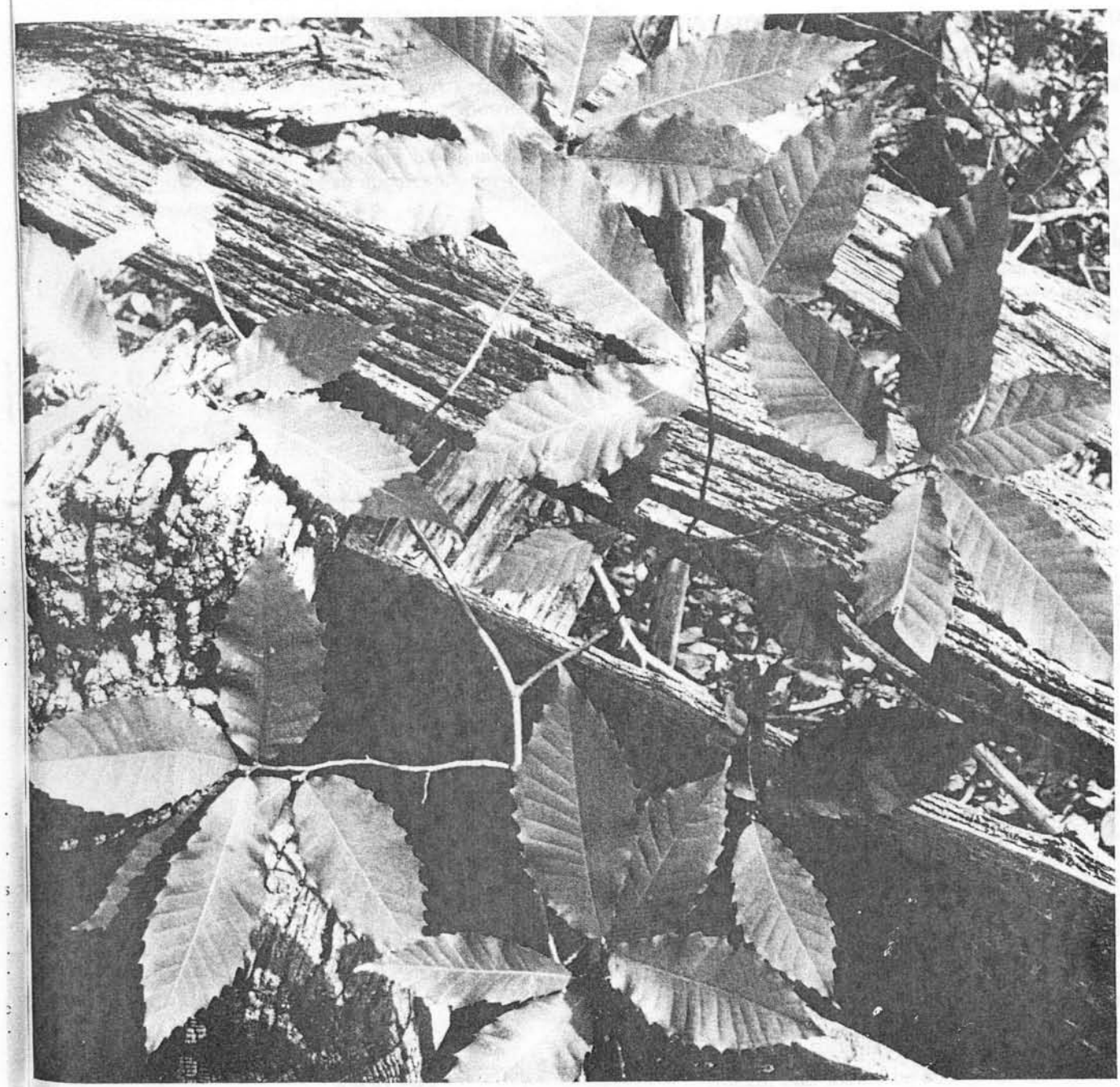
FRONTIERS
of PLANT SCIENCE

FALL 1981



Chestnut Sprouting—See page 6

THE CONNECTICUT AGRICULTURAL EXPERIMENT STATION NEW HAVEN

Searching for better plants by altering enzyme reactions

By Kenneth R. Hanson

Better crop plants have been sought since man first turned from hunting and gathering to growing food. Science has contributed to this search in many ways. Genetics has provided methods for introducing such desirable features as disease resistance or larger grain size into cultivated plants. Physiologists have shown why certain characteristics can lead to better yields and thus set goals for plant breeders. Advancing biochemical knowledge now offers biochemists an opportunity for making major improvements in agriculture.

A series of small steps has produced knowledge about a means of controlling the formation of some plant products.

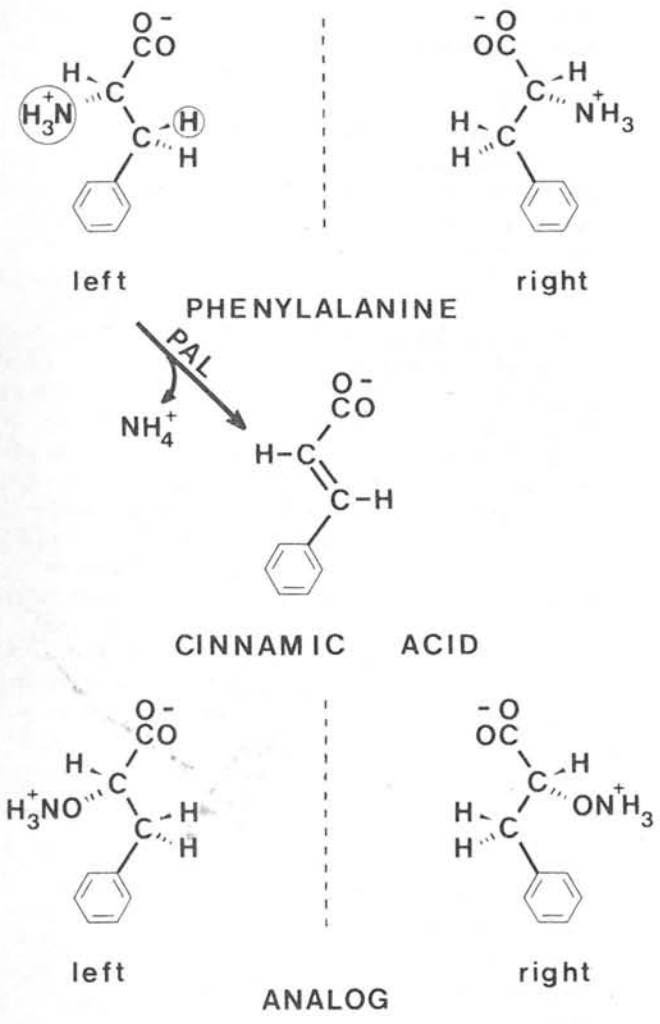


Fig. 1. Phenylalanine and its analog exist in forms which are mirror images of each other. The "natural" form of phenylalanine found in plants (and other living organisms) is indicated as "left". When the enzyme PAL acts on this form the circled hydrogen is lost along with ammonia.

Plants produce food by photosynthesis, which employs the energy of the sun to convert carbon dioxide and water into sugars. These sugars in turn are used to produce a vast array of chemical compounds. Biochemists have identified sequences of chemical changes and have examined many of the enzymes that determine which reactions occur, where in the cell, and when they take place. Enzymes exercise this control because as catalysts they participate in specific chemical reactions in a cyclic manner (they are not used up) and thereby cause particular reactions to take place at a rate fast enough for plants to grow.

An important goal of biochemical research is to understand how enzymes work. Much research on therapeutic drugs now begins by studying the properties of an enzyme and devising ways to block its action. Topics explored include the sequence of chemical changes during catalysis and the three dimensional interactions between the enzyme and the compound it acts upon. Such an approach should also be usable in agriculture, but this area is a largely unknown territory to be explored. The trail from learning about enzymes to producing a better crop plant is unmarked. Experience has shown scientists how best to explore such an unknown territory: they must be alert for landmarks, take determined steps, and map carefully as they go.

The exploration I shall describe concerns an enzyme, phenylalanine ammonia-lyase, usually called PAL. It controls the formation of the plant structural material lignin, many flower pigments, and compounds that help to protect plants from infection and insect attack. Phenylalanine is an amino acid which, together with other amino acids, is used to form proteins. PAL converts phenylalanine to ammonia, which is reused to form amino acids, and cinnamic acid. Cinnamic acid by a sequence of changes then produces lignin or other substances. PAL, therefore, acts as a spigot to divert phenylalanine from protein formation to entirely different types of plant constituents. If one can determine how PAL works and how its activity is controlled, one should be able to discover how to turn the spigot on and off in plants.

Dr. Evelyn Havir and I have studied PAL extensively. We have purified the enzyme from potato slices and corn shoots. We have also purified PAL from soybean cells grown in culture. We found that PAL is composed of four subunits, each of which is about 83,000 times the weight of a hydrogen atom. (For comparison: on the same scale

phenylalanine weighs 165, water 18, and human hemoglobin, the red blood protein, has two subunits weighing 15,000 and two weighing 16,000.) The catalytic reaction takes place at only two locations on the protein of PAL. We have found that if phenylalanine is bound to the first site, it is harder to bind phenylalanine to the second. We have also tried to picture the precise chemistry of the enzymatic reaction.

An early stage in studying the reaction was to determine which of the two hydrogens attached to the same carbon atom of phenylalanine was lost when cinnamic acid is formed (Fig. 1). We also found that an unusual chemical group on the enzyme combines with phenylalanine to help remove ammonia. Chemical electronic theory helped us picture the reaction as the collapse of a staggered phenylalanine molecule with portions lying in two parallel planes to a cinnamic acid molecule lying entirely in one plane. The molecule of phenylalanine collapses as hydrogen and ammonia are removed. This, however, was theory. To support the hypothesis other arguments were needed.

Professor Amrhein and associates in Germany found that a close chemical analog of phenylalanine in low concentrations blocks the action of the enzyme. Equal binding to the enzyme occurs when the concentration of the analog is 10,000 times lower than that of phenylalanine. Supplied to a cutting of periwinkle (myrtle), the compound was bound so effectively that the plant produced a white flower. The purple pigment in the normal flower is formed from the cinnamic acid produced by the action of PAL. The analog had turned off the spigot without damaging other enzymes in the plant.

The only difference between the analog and phenylalanine is that there is an oxygen atom between the nitrogen atom and a carbon atom (Fig. 1). The explanation of why this small change leads to a stronger binding could be a clue as to how the enzyme acts as a catalyst. Energy is required to break chemical bonds. This energy comes directly or indirectly from the water in which the enzyme is dissolved. Molecules of water share their energy of motion when they crash into the protein. The greater the energy required to reach the breaking point of the bonds, the fewer water molecules that have sufficient energy to break the bonds, and therefore, the slower the reaction. Let us suppose that when phenylalanine binds to the enzyme two opposite processes lead to a net attraction. The protein molecule is distorted as if it were a spring being squeezed, but the compound is attracted to the surface of the enzyme by stronger forces which include those between positive and negative charges. Let us further suppose that as the chemical bonds are breaking the direct attraction is similar but the tension of the "spring" is released because the shape of the reacting molecule changes. Because part of the energy required to tear the chemical bonds apart is supplied by the protein spring, less energy is required from the water. The energy mountain to be scaled is lower, therefore the reaction is faster (Fig. 2). According to this hypothesis, much of the effectiveness of the enzyme as a catalyst can be attributed to this "spring" effect. It follows that a compound that fits

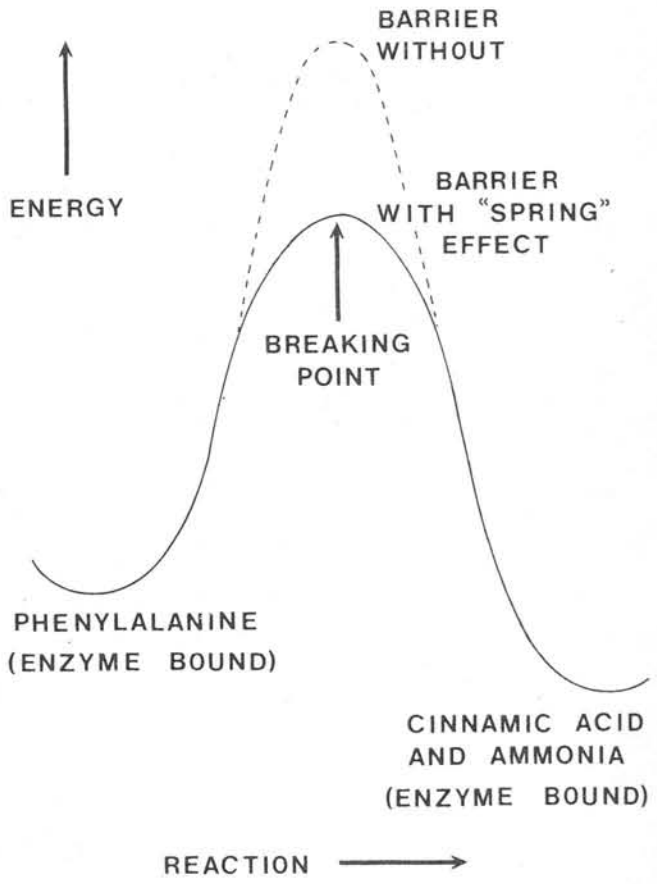


Fig. 2. An energy diagram to explain catalysis by PAL. The lower the energy barrier, the faster the reaction.

the catalytic site of the enzyme in the "relaxed-spring" position and is directly attracted to the same extent as phenylalanine will be bound very tightly. The analog could be strongly bound for this reason.

By comparing the binding of different compounds I concluded that there must be a balance of distortion and attraction when phenylalanine binds. I eliminated the possibility that stronger chemical bonding caused the tighter binding of the analog. Phenylalanine exists in two forms, which are related as the left hand is to the right (Fig. 1). Both forms bind to the enzyme to a similar degree, but only the "left-handed" is attacked by the enzyme at a significant rate. The analog also exists in left- and right-handed forms. When I found that the effects of both forms were similar, I was faced with a real conundrum: how could both resemble the breaking point in a chemical reaction which can only take place with the left-handed phenylalanine molecule?

As often happens in science, the trail seemed to have come to an abrupt end. No answer to this question occurred to me for about a year. Eventually, I used wire molecular models, which show the positions of atoms and the bonds joining them, to suggest an answer.

First, I arranged the left-handed phenylalanine model in the shape I had reasoned was a plausible starting point for the reaction. When I placed the right-handed model over this, the only important difference was in the twisted

center of the molecule. Under these conditions, the binding to the surface of the enzyme should be the same. (For comparison place your hands together with the palms upwards and the thumbs folded into the palm. Fingers 2–5 on the left hand resemble 5–2 on the right, hence either hand could fit into the same glove. The backs of the hands are similar: the only marked difference is the direction of the folded thumb.) Although the binding should be the same the catalytic interactions should differ. The hydrogen lost in the reaction is associated with the twisted center of the molecule. If removal of hydrogen is assisted by a group on the enzyme, removal will occur only with one form. The group will be in the wrong place to assist when the other form binds with a mirror-image twist.

The next step was to fit the models for each form of the analog into the space presumed to fit the two forms of phenylalanine. This was again achieved with the molecules having mirror-image twists. The important difference was that a portion of the analogs no longer occupied the position assigned for phenylalanine. Instead, it was at a point that would be appropriate if the left-handed phenylalanine had reached the breaking point in the reaction. If the enzyme reaches a relaxed-spring state at the breaking point, then the analogs should bind to the enzyme in this state, and its strength of binding is explained.

The model building had fused two hypotheses: one about the three-dimensional course of the reaction, the other about energy changes during catalysis. Because of this fusion, both seem more plausible. We now know more about the way in which the enzyme works.

Much has been learned about a key enzyme. A series of small steps has produced knowledge about a means of controlling the formation of a group of plant products. We know one way to turn the spigot off. Armed with such knowledge it should be possible to learn more about PAL regulation and produce plants in which the regulation is altered.

Because the products of PAL action relate to structural strength, disease resistance, and other characteristics, some plants with changed PAL regulation should be better crop plants. The goal of producing such plants is now closer than when we started our journey into the unknown territory.

Studies of ticks and animals assess risk of spotted fever

By Louis A. Magnarelli and John F. Anderson

During 1965-1980, 19 persons may have contracted Rocky Mountain spotted fever (RMSF) at widely separated sites (Fig. 1) in Connecticut. Fortunately, because of early diagnosis and proper antibiotic treatment, each of these people recovered. We are studying the ticks that may transmit the causative agent, *Rickettsia rickettsii*, to wild animals and humans.

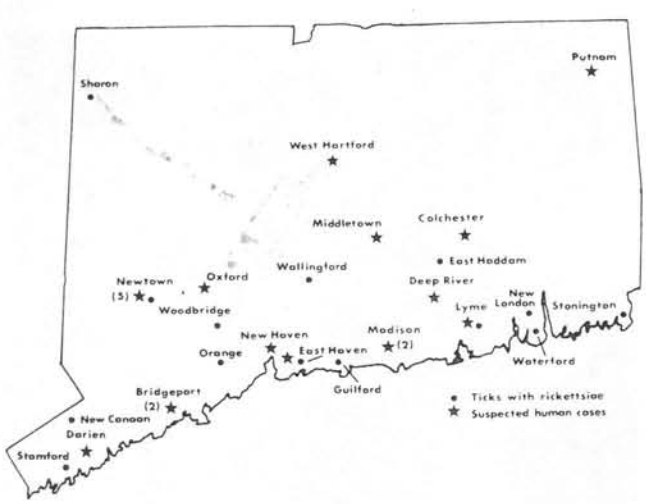


Fig. 1. Geographic distribution of human cases of RMSF and rickettsiae-infected ticks in Connecticut.

Our findings indicate widespread distribution of rickettsiae in Connecticut.

Realizing that RMSF occurs in Long Island (New York), Rhode Island, and in the coastal areas of Massachusetts, we began research in 1976 in collaboration with the U.S. Public Health Service in Hamilton, Montana to assess the risk of human infection in Connecticut. It is important for us to determine the prevalence of infected ticks and to determine the extent of spotted fever infections in wildlife so that we can better understand how ticks relate to the incidence of this disease in Connecticut.

Because the American dog tick, *Dermacentor variabilis*, is believed to be a chief vector of *R. rickettsii*, we studied areas with heavy infestations of this tick and localities such as Newtown where suspected human cases of RMSF may have originated. Ticks and animal blood samples were also provided by veterinarians, staff of the Department of Environmental Protection, town and state health officers, and the public.

During 1976-1980, 6,201 ticks were processed and examined for rickettsia-like organisms by tests described in *Frontiers of Plant Science* (1978, Vol. 30(2):4-5). Of the 189 (3.1%) ticks positive for rickettsia-like organisms in initial tests (Table 1), only 57 (0.9%) reacted with the

Table 1. Tick species and number of specimens carrying rickettsia-like organisms in Connecticut, 1976–1980.

Tick Species	Total examined	No. (%) with rickettsia-like organisms	No. (%) with rickettsiae of the spotted-fever group
Dermacentor variabilis	3,993	157 (3.9)	46 (1.2)
Ixodes cookei	71	0	0
Ixodes dammini	2,004	32 (1.6)	11 (0.6)
Ixodes dentatus	9	0	0
Ixodes marxi	1	0	0
Ixodes texanus	90	0	0
Rhipicephalus sanguineus	33	0	0
Totals	6,201	189 (3.1)	57 (0.9)

antibody reagent (Fig. 2). Both male and female American dog ticks and those of another tick, *Ixodes dammini*, were found harboring spotted fever-group (SFG) rickettsiae. About 13 other tick species inhabit Connecticut, but none of these has been found infected with SFG rickettsiae in the state.

Infected ticks were ground and inoculated into laboratory-bred meadow voles or were placed into flasks containing animal tissues to identify the particular rickettsia. *Rickettsia montana* was isolated from four American dog ticks collected in Woodbridge and Branford. This organism is closely related to *R. rickettsii*, the agent that causes RMSF in man, but there is no evidence that *R. montana* causes disease in humans or laboratory animals. We know that *R. rickettsii* is present in Connecticut because we isolated it from the blood of a person from West Hartford who had the typical clinical signs and symptoms of RMSF. This person recalled being bitten by a tick and had not traveled outside the state for several weeks before becoming ill.

Domestic and wild animals are also bitten by ticks and are exposed to rickettsial organisms. Most dog owners are familiar with these external parasites and often remove them from their pets. At times, ticks can be very abundant. For example, we removed over 100 adult American dog ticks from one opossum. Blood tests detected antibodies to SFG organisms in samples from 13 of the 19 mammalian species we have studied in Connecticut (Table 2). Particularly high rates were noted for 79 gray squirrels (38% positive) and 282 raccoons (23.8% positive). Blood tests of squirrels and raccoons captured at Newtown (an area with five known human cases of RMSF) identified antibodies specific to the causative agent of RMSF.

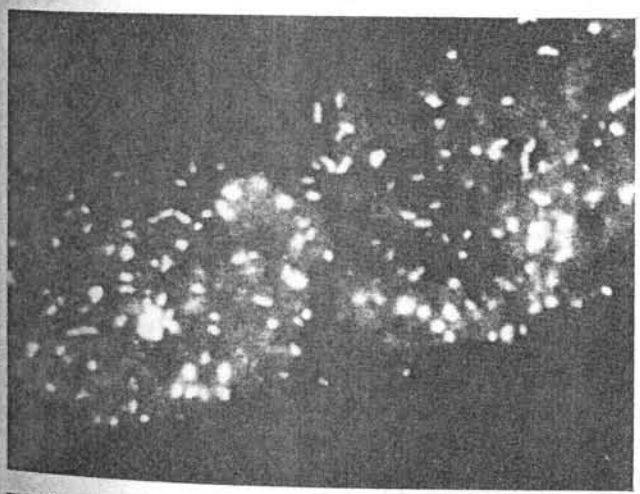


Fig. 2. Microscopic view of spotted-fever group rickettsiae in tick tissues fluorescing in antibody test.

American dog ticks are abundant from April through mid-July in grasslands at the forest edge, particularly along trails used by medium-sized mammals. Larval and nymphal dog ticks ingest blood from small rodents such as field mice and meadow voles. If they feed on an animal infected with rickettsiae, the ticks retain the microorganisms as they develop to adults. These adults—both male and female—may then transmit the disease agents to people, dogs, raccoons, opossums, or other medium- to large-sized mammals they may feed on. If an infected female lays eggs, the disease organisms may be passed to the offspring in the next generation.

Our findings indicate widespread distribution of rickettsiae in Connecticut. Although we have encountered few infected ticks, our blood analyses of wild and domestic animals suggest that exposure to *R. rickettsii* may occur more often than previously thought. Therefore, the relatively low number of human cases reported since 1965 should be viewed as a conservative estimate of rickettsial activity in the state.

Table 2. Mammalian species and number of animals carrying antibodies against spotted fever-group rickettsiae in Connecticut, 1976–1980.

Animal	Total blood samples analyzed	No. (%) with antibodies to rickettsiae
Beaver	2	0
Chipmunk	38	7 (18.4)
Eastern woodrat	1	1 (100)
Gray squirrel	79	30 (38)
Meadow vole	17	1 (5.9)
Mink	1	0
Norway rat	5	2 (40)
Opossum	50	4 (8)
Rabbit	9	0
Raccoon	282	67 (23.8)
Red fox	1	0
Red squirrel	2	0
Redback vole	6	0
Short-tailed shrew	10	1 (10)
Striped skunk	11	2 (18.2)
White-footed mouse	826	41 (5)
White-tailed deer	549	14 (2.6)
Woodchuck	16	3 (18.8)
Woodland jumping mouse	3	2 (66.7)

Because we have found the American dog tick throughout Connecticut, there is potential for RMSF at any site where this tick thrives. Little is known, however, about how rickettsiae become established in tick populations, how they circulate in nature, or about how environmental factors such as temperature and relative humidity influence the survival of ticks and rickettsiae. To learn about these and other factors, we are investigating annual changes in patterns of tick infectivity and in antibody production by wild animals.

Suggested Readings

1. Magnarelli, L.A., Anderson, J.F., and Burgdorfer, W. 1979. Rocky Mountain spotted fever in Connecticut: human cases, spotted-fever group rickettsiae in ticks, and antibodies in mammals. Am. J. Epidemiology 110(2):148–155.

2. Anderson, J.F. and Magnarelli, L.A. 1980. Vertebrate host relationships and distribution of ixodid ticks (Acari: Ixodidae) in Connecticut, U.S.A. J. Medical Entomology 17(4):314–323.

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4. Magnarelli, L.A., Anderson, J.F., and Burgdorfer, W. Geographic distribution of human cases of Rocky Mountain spotted fever, infected ticks, and seropositive mammals in Connecticut. *In* W. Burgdorfer and R.L. Anacker, eds., Rocky Mountain Laboratory Conference on Rickettsiae and Rickettsial Diseases. Academic Press, New York, NY (In press).

Knowledge of genetic barriers helps control chestnut blight

By Sandra L. Anagnostakis

For 77 years, the chestnut blight fungus, *Endothia parasitica*, has been relentlessly killing American chestnut trees in our New England forests. The chestnuts, however, continue to persist by sprouting from the living root systems of blight-killed trees. Chestnut research at the Experiment Station during the past decade has centered on attempts to introduce a natural biological control that has evolved in Italy. If the cure can be induced to spread naturally here, it may be possible to save the American chestnut before the declining root systems cease sprouting.

The cure was brought to the attention of Station scientists in reports that blight-caused cankers on European chestnut trees were healing spontaneously. Results were encouraging after the blight-curing factors were introduced into American strains of the chestnut blight fungus. Research has determined conditions that produce the highest rate of cure. The Station has since found naturally-occurring American blight-curing strains of the fungus in several states, but none so far in Connecticut.

We know little about the cure in Italy, but in every case examined the cure has been associated with abnormal strains of the blight fungus. Several kinds of these strains, which cause little harm to healthy trees, can usually be recovered from superficial cankers on chestnut trees all over Italy. Pairing of normal killing strains with the abnormal strains in the laboratory can cause the killing (virulent) strains to become abnormal. The abnormal strains (referred to as hypovirulent) can cure cankers caused by virulent strains if inoculated into or around virulent cankers. Thus, hypovirulent strains can be used as a treatment to control the blight.

Station scientists suspected that the abnormal strains contained viruses when Peter Day found that all hypovirulent strains tested contained double-stranded ribosenucleic acid (dsRNA) and none of the virulent strains tested contained dsRNA, the genetic material of fungal viruses. The theory that hypovirulence was associated with a fungal virus suffered a set-back when J. Allen Dodds could not isolate typical virus-like particles from dsRNA-containing strains. After isolating dsRNA from many hypovirulent strains, he finally recovered some amorphous mem-

It may be possible to save the American chestnut before the declining root systems cease sprouting.

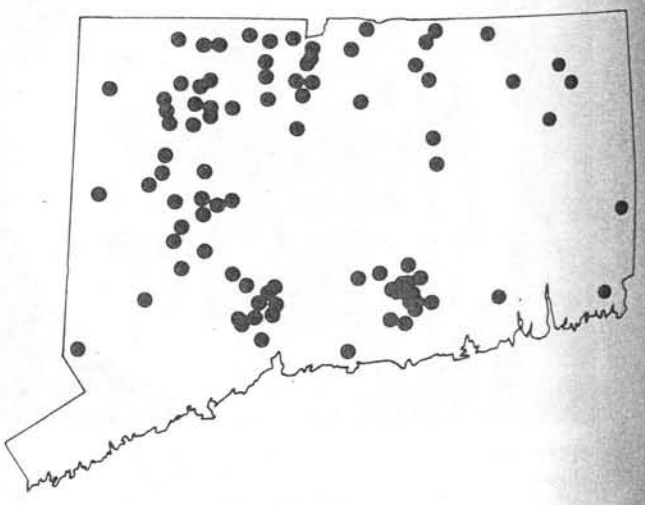

Fig. 1. Strains from these locations have been tested for vegetative compatibility.

brane-bound structures containing dsRNA. These can only be seen with an electron microscope and often resemble the Schmoos of Lil'Abner fame. The membrane-bound structures may be a new kind of virus or some as yet undescribed kind of agent.

While Dodds worked on the dsRNA problem, various hypovirulent strains were tested against blight in chestnut trees. We soon discovered that treatment with a single hypovirulent (H) strain would cure only one-fifth to one-half of the cankers in our Connecticut woods. We studied interactions between virulent strains in the laboratory for an explanation. We found that some virulent (V) strains paired on agar medium merged, while others formed a line, or barrage zone, where they met and never merged. By crossing strains and examining their offspring, we found that the formation of a barrage zone is controlled by several genes in the nucleus of the fungus. Strains with identical genes merge with each other and are said to be in the same vegetative compatibility (v-c) group. Strains that differ at even a single gene will form barrage zones and are therefore incompatible.

The dsRNA of hypovirulence was easily transferred between strains in the same v-c group, but often could not be transferred between strains in different v-c groups. We knew that dsRNA must be transferred to convert a virulent strain to hypovirulent to cure a canker; therefore, vegetative compatibility was a real barrier to successful treatment of the disease.

Most, but not all incompatible pairings of H and V failed to cure. We clearly demonstrated the effects of this vegetative isolation system in a field test of pairs of strains different from each other at 0, 1, 2, or at least 5 v-c genes. Several V strains were inoculated first, then the incipient cankers were treated 2 weeks later with either V or H strains. Treatment with V strains had little effect. Effectiveness of the treatment with H strains varied depending on the number of v-c genes that differed between the strains.

So far, analyses of 179 strains of *E. parasitica* isolated from Connecticut cankers have divided the strains into 67 v-c groups. In a similar survey in Europe, J. Grente found 22 v-c groups among 141 strains.

C. Caten has suggested that vegetative incompatibility in fungi can be viewed as a cellular defense mechanism. Since we wanted to encourage the dsRNA factors in hypovirulence to spread from H to V strains and therefore cure chestnut blight in our forests, we started looking for ways to foil this defense system erected by the blight fungus.

We planned field tests that included several kinds of H strains to find the most efficient way to use H strains on cankers caused by V strains in diverse v-c groups. The strains tested were in several v-c groups and had different abilities to grow in chestnut trees. Fifteen forest plots, each with 24 sprout clumps of American chestnut, were used. Uniform infections with virulent *E. parasitica* were introduced into the trees, using bark pieces from a natural infection in each area. The treatments were begun 5 weeks later. A group of eight H strains was used in four different ways:

1. Spores from eight H strains were mixed in water and sprayed on the cankers.
2. Four pieces of a culture of an H strain were put into holes around each canker.
3. One piece of a culture of each H strain was put into eight holes around each canker.
4. A mixture of all eight H strains (a slurry with agar) was put into four holes around each canker.

All treatments limited the size of the cankers as compared to the untreated controls, but treatments 3 and 4 were the most effective. The cankers treated were caused by *E. parasitica* strains in 25 v-c groups. Five strains were the same as v-c types among the H strains used in the experiment. In the test plots where only one H strain was used per canker it was possible to see which v-c groups were controlled by which H strains. It seems clear that we must deploy H strains in several v-c groups to be successful in our attempts to help a naturally-spreading cure of chestnut blight.

In Italy, dense forest stands of European chestnut trees are being used again as a timber source because the blight cankers rarely kill them. Although we can cure blight cankers on individual American chestnut trees, we have seen no evidence of natural spread of the biological control in our test plots.

There are many differences between our situation and that in France and Italy. The trees are a different species. Chestnut trees grow alone in dense plantings in Italy and in orchards in France, while in this country, chestnuts grow among other hardwood species in the forests. Although in some areas of Virginia there are dense stands of American chestnut sprouts comparable to the density in Italy, in this country the cure must generally move farther to get from tree to tree. It is also possible that some carrier, such as a bird, mammal, or insect not present here may be responsible for the spread of the H strains in France and Italy. We hope that between field and the laboratory studies we will find the clues that will help bring about a biological control of chestnut blight.

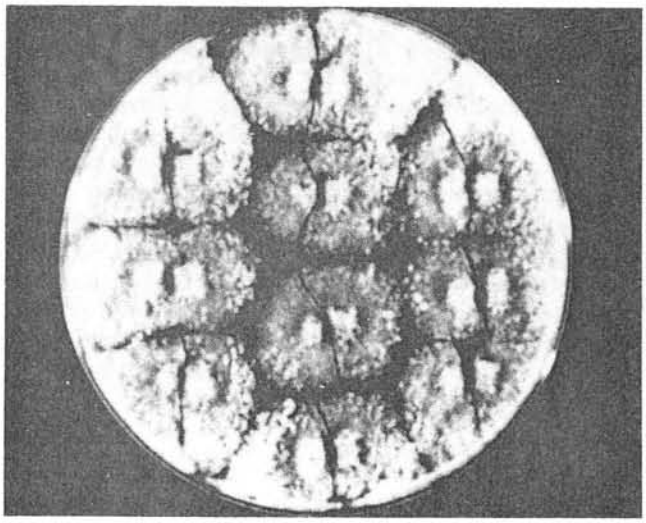

Fig. 2. A vegetative compatibility (v-c) test of one strain paired with 10 different v-c types. The strains in the middle of the first row are compatible; the line (barrage zone) between the rest of the pairs indicates incompatibility.

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THIRD CLASS

The gypsy moth in Connecticut during 1981

The gypsy moth attracted much attention during 1981—both from the public and the Experiment Station.

The annual defoliation survey revealed that 1,482,216 acres were defoliated by caterpillars, more than double the previous record 654,102 acres defoliated in 1971. Only three of the 169 cities and towns: New London, Sprague and Scotland, escaped defoliation. The heaviest defoliation was experienced in New Haven, Litchfield, Fairfield, Middlesex, and Hartford Counties. These counties are expected to experience less defoliation in 1982, while the counties to the east will experience more defoliation than in 1981.

A rash or *urticaria* affected many people for the first time in 1981. This condition, presumably caused by hairs of the small caterpillars, affected more than 1000 people in Bristol alone and was treated by over-the-counter drugs.

A large experiment in the aerial application of a biological spray, *Bacillus thuringiensis (Bt)*, was executed in Harwinton in collaboration with the U.S. Forest Service and the Dept. of Environmental Protection. Because the timing and the weather were perfect, the control was about the maximum that can be expected. Defoliation in untreated plots was 60 percent. One spray reduced defoliation to 35 percent, and two sprays reduced it to 15 percent. Investigations of caterpillars in the sprayed plots revealed that the decreased defoliation was caused indirectly by delayed development and increased attack by parasitic wasps as well as directly by the pesticide.

A parasite that the Experiment Station released in 1980 was found in a gypsy moth pupa during 1981.

The Experiment Station is continuing its studies of parasites of the pest and of defoliation on trees, especially of evergreens defoliated in 1981, and will survey for eggs of the gypsy moth as requested by towns during the winter.

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