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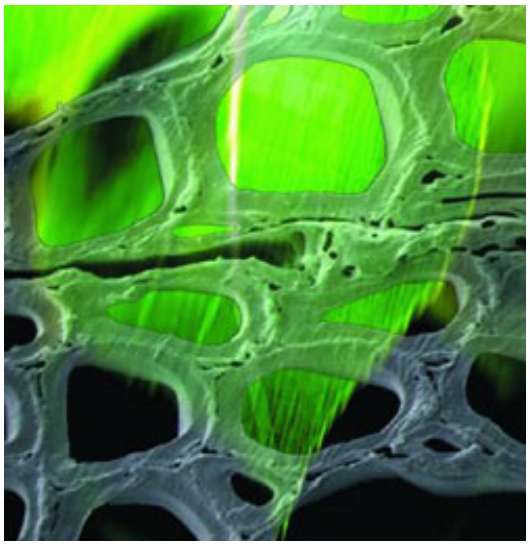
ONLY FACTS WILL END LIGNIN WAR

Data, not verbiage, will determine if new lignin biosynthesis model will prevail

A. MAUREEN ROUHI, C&EN WASHINGTON

It's topsy-turvy in the world of lignin biosynthesis research. The field is embroiled in a controversy that has researchers broiling: One camp warns of dinosaurs clinging to old notions; the other protests that allegedly unsubstantiated claims are being promoted as facts. At issue is whether the polymerization that forms the biopolymers called lignins is under the strict control of proteins.

The opposing views are pushed by researchers who are passionate about their work and are competing for funds. Reviews of grant proposals become skirmishes in the bigger war, and the controversy is driving away resources from a field that does not attract a lot of money to begin with, sources say.



RIGID WALLS **When lignification is complete, the only traces of previously living cells are thick walls made rigid with lignin and cellulose.**

Lignins are phenolic polymers that form in cell walls of terrestrial plants. After cellulose, they are the second most abundant biopolymers in nature, providing strength and facilitating water and nutrient transport in land plants. They also are one of the most difficult materials to study. Their structures are complex, their isolation is difficult, and their characterization requires a compromise between purity and structural integrity. One researcher says he left the field because lignins are just too messy. Progress is hard.

Except as part of wood and timber, lignins generally are a nuisance to humans

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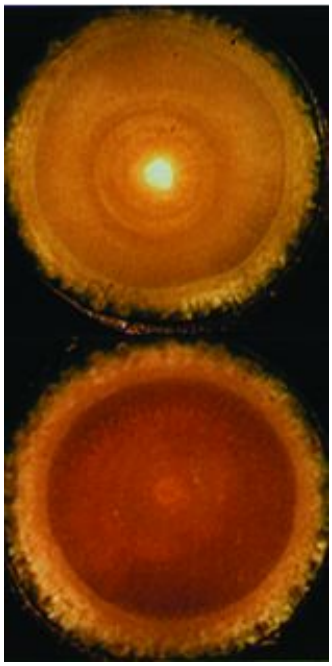
at present. The pulp and paper industry wants to reduce the amount of lignins in trees, because they are very difficult to remove to free the cellulosic fibers. Lignins could be burned to produce energy, as happens in the paper and pulp industry. Some researchers are studying how they may be converted to things like plastic, adhesives, or gasoline. The more that is known about lignins, the more likely they can be used.

SINCE THE 1950S, the accepted view has been that lignin forms by random coupling of phenolic monomer radicals. About three years ago, however, a group of researchers proposed that the process is fully controlled by proteins. The manner in which this new model is being advanced has riled many in the lignin research community. A story in C&EN (Nov. 13, 2000, page 29) fueled the controversy.

According to the old model, the radical couplings that produce lignin proceed under simple chemical control. The plant regulates the amount and type of monomers that are available at the lignification site. Oxidizing enzymes produce the radicals, but nothing except chemical control dictates coupling and polymer structure. Decades of research are consistent with this model, says John Ralph, a forestry professor at the University of Wisconsin, Madison, and a researcher at the U.S. Dairy Forage Research Center (USDFRC) in Madison.



BLUSH ON WOOD

Genetic engineering to alter the lignin in aspen has produced trees with rosier wood (bottom) than that in unaltered trees (top).

Norman G. Lewis, a professor of plant biochemistry at Washington State University, Pullman, and others disagree. They say the polymerization that forms lignins must be controlled by proteins and there is more than a preponderance of evidence to that effect.

In 1997, Lewis, Washington State senior investigator Laurence B. Davin, and Simo Sarkanen, a bioorganic chemist and a professor of wood and paper science at the University of Minnesota, St. Paul, discovered "dirigent" proteins. The name comes from the Latin dirigere, which means to guide. The proteins have no catalytic activity, serving only to orient the coupling of monomer radicals during biosynthesis of lignans--natural products ranging from dimers to oligomers that share with lignins three precursors called monolignols: p-coumaryl, coniferyl, and sinapyl alcohol. During dimer formation, dirigent proteins recognize specific substrates and control the regiochemistry and stereochemistry of coupling, resulting in optically active products.

That discovery led Lewis, Davin, and Sarkanen to propose that similar control must exist for lignin assembly from monolignols. Nature cannot be so careless, Lewis says, as to allow its second most abundant polymer to be made haphazardly, as the old model would have it.

Arguments and counterarguments have been flying since the new model surfaced. The dispute has become so acrimonious and personal that one researcher tells C&EN that he would never have entered the field if he'd foreseen that science would become a substitute for going to war.

Just show us the data, sources say. People have come away from hearing Lewis talk or from reading his papers thinking that he has the proteins and the genes for lignification. Therefore, they say, the issue can be settled easily. If these

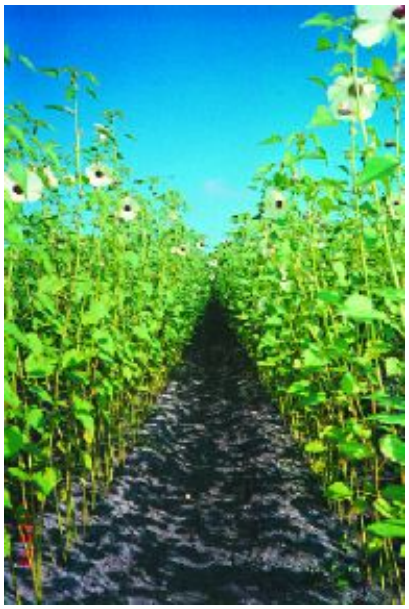
proteins control lignification, then knocking out their genes should shut down lignin formation.

But Lewis says, "I have never stated that we have the genes that control the system that's involved in lignification."

In that statement lies one source of exasperation, not only for critics of the new model, but also for observers who simply want to know what's going on. Just what Lewis is claiming appears to shift over time.

Lewis knows lignins and lignans are products of distinct metabolic pathways. However, the biochemical underpinnings must be the same, he argues, because for other processes where identical monomers have multiple outcomes--for example, glucose forming either cellulose or cellobiose--the same principles of protein chemistry operate.

TO DATE, evidence that proteins which control lignin biosynthesis actually exist is limited to the detection of dirigent-protein–like epitopes coinciding with lignification in developing tissues. But in the cell wall there are hundreds of proteins with no known function, says Ronald R. Sederoff, a forestry professor at North Carolina State University, Raleigh. To prove protein involvement, one must show "that the protein is necessary and sufficient for a specific step in lignification."



CRAFTY PLANT **If only monolignols make up lignins, then kenaf plants--which incorporate in their lignin the ester of a monolignol--are standing tall with little support from lignin.**

Lewis, however, says, "It would be naive if we didn't take into consideration" that proteins are associated with lignin initiation sites.

"That's fine," Ralph replies. But taking proteins into consideration is a "long way from a protein that is supposedly dictating absolute chemical structure."

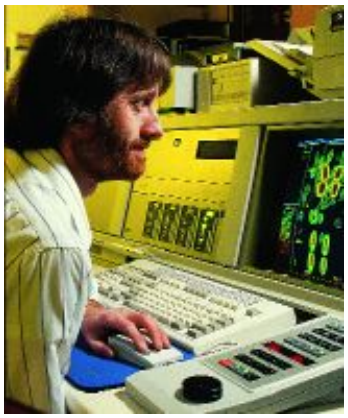
Despite having only circumstantial evidence, Lewis, Davin, and Sarkanen ardently promote their model. Their talks and papers project an inevitability that their model will be proven true--all that's needed is to fill in details. The new model already has appeared in a textbook.

"People are being misled," says Ronald D. Hatfield, a research plant physiologist at the USDFRC. "People who are not well versed in cell-wall chemistry and biochemistry are allowing this theory in textbooks, where it is presented not side by side with the other theory, but rather as the new accepted theory," Hatfield comments.

Other scientists object that nothing now known about lignin requires invoking the new model. According to Knut Lundquist, an emeritus professor of wood chemistry at Chalmers University of Technology, Göteborg, Sweden, "You must have some structural feature that needs to be explained by the existence of dirigent proteins."

Lewis says the dominance in lignins of a specific linkage between two monomers, the so-called 8-O-4 linkage, implies regioselectivity that cannot be explained by the old model. But others say the selectivity requires only the plant's careful control of the supply of monomers to the lignifying zone and/or the rate of radical generation. Last year, for example, Gösta Brunow, an

emeritus professor of organic chemistry at the University of Helsinki, in Finland, showed how the regioselectivity could be achieved in a chemical system. Yet Lewis says such experiments have "no bearing" on lignins.



DIAMETRICALLY OPPOSED **Ralph (left) and Lewis support what seem to be irreconcilable views.**

ANY MODEL of lignin biosynthesis must account for the biological facts, Lewis says. Prior to lignin formation, the cell deposits layers of cellulose, hemicellulose, and other components to define the boundaries of the cell wall that will be lignified. The monolignols diffuse through this matrix to the farthest end of the wall, where polymerization occurs. The growing polymer then works its way back toward the plasma membrane.

Key to Lewis' reasoning is the varying composition of lignin in different parts of the wall. In certain woods, for example, the lignin at the outermost wall is derived primarily from p-coumaryl alcohol, while that in an inner wall is derived mainly from coniferyl alcohol.

To explain that differential composition, Lewis believes that proteins direct monolignols to specific sites. But Hatfield says the difference can be explained by the plant shifting production from one monolignol to another. "By doing so, the plant directs what goes on in the wall, and there's no need to select anything," Hatfield says. "That makes much more metabolic sense." Selection by a protein must mean that the plant is producing multiple precursors all the time, which would be wasteful.

The new model includes three key elements to account for what's been observed. First, the model holds that lignification is controlled by arrays of dirigent sites--sites that bind monomer radicals--provided by proteins located at the farthest end of the wall. The sites dictate monomer composition, radical orientation prior to coupling, and the sequence of interunit linkages. Second, only radicals derived from the monolignols are targeted to these sites, where they polymerize to form a primary lignin chain. And third, copies of the primary lignin chain are formed by template polymerization, enabling the lignin to grow from the outside and back toward the plasma membrane.

THE PROBLEM, sources say, is that these elements don't jibe with other observations about lignin: its lack of optical activity, the incorporation of monomers other than the monolignols, and the lack of a repeating unit.

According to the first key element of the new model, proteins absolutely control monomer coupling, just as they do in lignan biosynthesis. But "if such a process were occurring, one should see optically active lignin," says Richard F. Helm, a professor of wood science and forest products at Virginia Polytechnic Institute & State University (VPI).

Sources perceive Lewis as having flip-flopped on the issue of optical activity. They believe Lewis once entertained the possibility that lignins should be optically active and then changed his mind when it was shown that they are not.

Lewis strenuously denies flip-flopping on this issue. In published work, however, he refers to the lack of optical activity as "reputed," "apparent," or

"presumed." These qualifiers suggest that the issue is still under discussion. He also usually explains how optical inactivity is possible, even when absolutely protein-controlled coupling likely would yield optically active products.

Talking to C&EN recently, Lewis says the protein only provides sites for the monomer radicals to bind, but that the coupling does not need to have the same stereochemical consequences as in lignan formation. In published work, however, Lewis and his collaborators state that the monomer-binding sites in lignification proteins should be of the same type as those in the dirigent proteins controlling lignan formation, which "juxtapose [the radicals] into the correct relative orientations for regio- and stereoselective coupling."

The second key element of the new model requires specific monomer radicals to bind to specific sites in the protein. If so, lignin production should shut down in mutants or transgenic plants lacking the correct monolignols. Research shows lignification does not stop in mutant or transgenic plants. Lewis, however, questions whether what such plants make really is lignin.

So what exactly is lignin According to Ralph, lignin is the polymer in plant cell walls synthesized from hydroxyphenylpropanoids by radical coupling in a near-random--rather than a statistically, completely random--fashion. The definition incorporates what he believes about lignin biosynthesis.

So does Lewis' definition, which is different. Lewis says lignin is a polymer of a requisite molecular weight (at least 100,000) that is derived from the three monolignols and that performs the structural functions of lignins in healthy, genetically unperturbed plants. Lewis further stipulates that claims about lignin should be based on pure samples.

Molecular weight is essential, Lewis says, because his own work shows that some of the materials claimed as lignins are really high-molecular-weight lignans. But according to Ralph, the issue should not be molecular weight but the type of polymerization. The polymerizations that produce high-molecular-weight lignans and those that produce lignins, he says, "can usually be distinguished."

Lewis' requirement for purity takes aim at structural work by Ralph on lignins associated with carbohydrates. But other scientists say there's no such thing as a pure lignin. Lignins are isolated from their associated carbohydrates only with great difficulty, so by the time all the carbohydrates are gone, it's not the original material anymore.

And then, Lewis continues, researchers must compare the material that mutant and transgenic plants are forming with what the normal plant makes. Some mutant or transgenic plants look sickly, so the material is not performing its function, Lewis argues, and therefore what the plants are producing could not be lignin.

Lewis "does not consider the large and growing body of evidence that lignin is readily modified" by changes in the level and composition of monomer precursors, Sederoff says. That evidence comes mainly from mutant and transgenic plants.

"Plants lacking enzymes in their monolignol biosynthetic pathways often incorporate phenolics other than the traditional monolignols," Ralph says. Recently, for example, he and others showed that mutant poplars that can't efficiently make sinapyl alcohol incorporate 5-hydroxyconiferyl alcohol into the lignin instead [*J. Agric. Food Chem.*, 1, 48 (2001)].

"These plants have no problem incorporating something they are unlikely to have dirigent proteins for," Ralph says. "Obviously, the resultant lignins have different properties from their natural counterparts, since the structure and

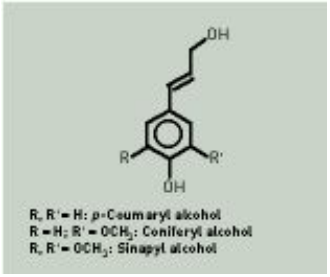
composition are different. I think the plant would call it a lignin if it could talk."

NORMAL PLANTS also incorporate monomers other than the monolignols, Ralph notes. For example, kenaf, a plant grown for its fiber, incorporates sinapyl acetate monomers. He adds that all grasses incorporate sinapyl and coniferyl p-coumarates; willow, aspen, poplar, and various palms appear to incorporate coniferyl and sinapyl p-hydroxybenzoates.

Lewis would ask to see molecular weight ranges before considering any results implying monomer substitution. But his collaborator Sarkanen is more flexible. He says 5-hydroxyconiferyl alcohol and those esters mentioned by Ralph could be accommodated by the new model. "The key point is the orbital structure of these molecules," Sarkanen says, because the monomer-binding sites of the protein would bind substrates through nonbonding orbital interactions. "At a first level of approximation, there's no difference" between these monomers and the monolignols.

But monomers significantly different from the monolignols are another matter. Lewis and Sarkanen both decry so-called metabolic plasticity, an idea promoted by Ralph and others. According to this idea, plants are enormously flexible in incorporating monomers into lignin without disrupting lignin function. Ralph has gone so far as to suggest that neither lignin composition nor lignin structure is important, so long as the plant has a polymer with the required properties.

THE IDEA of metabolic plasticity gained visibility with a paper about abnormal lignin in a mutant loblolly pine [*Science*, **277**, 235 (1997)]. Ralph and coworkers reported incorporation of dihydroconiferyl alcohol and a 2-methoxybenzaldehyde in the lignin of this pine. Ralph erred in a structural assignment but corrected the error later [*Proc. Natl. Acad. Sci. USA*, **95**, 12803 (1998)]. What he had identified as a 2-methoxybenzaldehyde turned out to be a coniferyl aldehyde.



The misassignment "makes no difference to the arguments," Ralph says. "It still represented new structures in the polymer that were not in the normal plant," he explains.

Lewis calls Ralph's correction a retraction. He seems to hint that because of this error, everything Ralph reports must be suspect.

One erroneous assignment, Ralph says, does not nullify the body of evidence that firmly establishes monomer substitution in lignification. Lignification is not unique in nature in this regard, he points out, noting as an example the discovery that beetles indiscriminately assemble macrocycles from monomeric units. A "chaotic assembly," he adds, benefits plants by foiling the ability of natural foes to develop ways to attack the material, which is notoriously hard to degrade.

The third key element of the new model is replication of the primary lignin chain by template polymerization. This implies that lignins must have a regular repeating structure. However, no order or periodicity of units has been found in lignins. In fact, Ralph maintains that it is "astronomically improbable" to find chemically ordered regions in lignin and that "the search for regularity is futile."

The work of Sarkanen is key in developing this aspect of the new model. Using methods to prepare synthetic lignins, he has shown that in the presence of a template--a soluble, high-molecular-weight so-called kraft lignin sample--coniferyl alcohol forms high-molecular-weight products without going through low-molecular-weight intermediates. In the absence of the template, mostly low-molecular-weight products form. Lewis and Sarkanen consider

these results as key evidence for template polymerization in lignification.

Other scientists, such as Lundquist, have expressed skepticism about Sarkanen's experiment. But regardless of its validity, the experiment tells nothing about how the products are related, if at all, to the template. They don't establish that template polymerization occurs in lignification. Sarkanen is seeking funding to explore this matter further.

Another complaint about the new model is that its proponents have not laid out clearly how the template works. Sarkanen and Lewis say they expect the template to dictate interunit linkages and constituent monolignol residues. Both say they wouldn't be surprised to find some repeating unit, such as an 18-mer.

Their idea is reminiscent of one proposed in the 1950s by a researcher named Kaj Forss. He proposed the existence of an 18-mer unit in lignin. But his evidence was insufficient, and the idea could not be taken seriously, according to Wolfgang G. Glasser, a professor of wood science and forest products at VPI.

Sarkanen, however, says, "We and others have separate, quite different data that also argue for an 18-mer-type repeating unit."

Assume for the sake of argument that template polymerization occurs. Does the template have to consist of proteins

"Isn't the cell wall itself a template" Helm notes. "To me, a template is just a surface from which a reaction can occur. The template is already there. It happens to be pectin, hemicellulose, and cellulose. I don't need dirigent proteins to make it work."

"It does not have to be a protein," Glasser agrees. "It could be another metabolite created by the cell that becomes immobilized somewhere at the cell wall."

Even Sarkanen says the template does not have to be a protein a priori. But, he says, the preponderance of evidence argues that it must be a protein.

BY EVIDENCE, Sarkanen means the detection of dirigent sites and proline-rich proteins in the region where lignification is just starting. "That alone, of course, doesn't prove anything," he says. At the same time, he says, proline-rich proteins have been shown to associate strongly with phenolic components. "That alone still doesn't prove anything either. But whatever information is available at this time supports the working hypothesis of template polymerization."

Ralph argues that the proponents of the new model have not thoroughly tested their ideas. "The scientific method requires of someone who proposes a theory to come up with every experiment to discredit the theory," he says. "The theory becomes stronger by not being able to find proof against it."

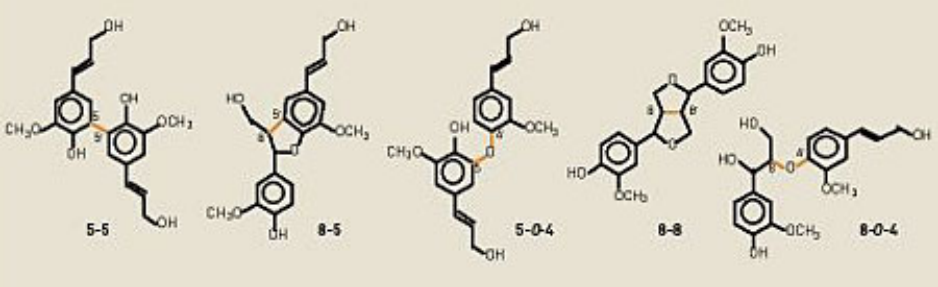
Lewis sees it differently. In hypothesis-driven research, the way to move forward is "not to look for evidence that doesn't support it," Lewis replies to C&EN asking what experiments he plans along such lines. "You look for evidence that will support your hypothesis, and you do controls."

Lewis and his collaborators have a lot to prove. They need to produce direct proof of a template. They must isolate the proteins, conduct experiments in vitro, and show that results with proteins are different from those in the absence of proteins and that the difference is due to a directing effect. "Many other things are in the cell wall that could be dirigent. They could be macromolecules or small molecules," Glasser says.

Ultimately, reasoning--no matter how compelling--cannot replace data. "It is always stimulating to discuss new ideas," Brunow says. "But in experimental

science, it is customary to present experimental evidence when debunking old notions and setting winds of change ablowing."

"With real scientific controversies," Glasser notes, "the truth often lies in between the conflicting viewpoints. I would not be surprised if that were the case here."



PICK A LINK **Part of lignin's complexity is the numerous coupling modes possible--such as these for two coniferyl alcohol monomers--of which the 8-O-4 link is the most predominant.**

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