European Laboratories

CERMAV: working on the fascinating sides of carbohydrate science

If you are looking for a broad coverage of carbohydrate science, visit CERMAV in Grenoble, France. The institute began as a paper research organization 33 years ago, but material science, glycobiology, rheology and molecular modelling have all established a firm position since. Director Serge Pérez tries to keep the scientists happy and communicating.

Serge Pérez must have a bad mood like the rest of us once in a while. It is difficult to imagine him at such moments however because he normally carries a broad smile, boldly underlined by one of his colorful ties. Perhaps he is the prototype of his own philosophy of how research should be done: with happy scientists!

His office, though, makes a less ebullient impression with its atmosphere of modern efficiency. The semi-circular wooden desk is half covered by small tidy piles of papers with small memo's on top – probably today's 'to-do' items. An eye-catcher is the large flat computer screen. The efficient office reflects another one of Pérez' philosophies: professionalism on the job. "I'm continuously asking myself questions about how to do this job the best I can. I do not believe in a technical approach to management. Maybe you can learn some tricks, but there are no real recipes to become a research manager."

"When examining experimental data we should be as sceptical as we are about computational data!"

And managing is what Pérez does most of his day at work. He runs CERMAV, one of the research institutes of France's primary research organization CNRS (see insert 'Hot debate on French research system' on page 14). The more than sixty staff members of CERMAV are involved in almost all aspects of carbohydrate science - from molecular biology to material science. Pérez accepted the position as director in 1996. "I had worked at CERMAV before, from 1984-1993, but then moved to Nantes where I worked as a research director of a small group of computational chemists for four years. I was then offered the position of director at CERMAV."

The scientist is happy in his current job. "You always have to fight to defend carbohydrate science against protein and nucleic acid science. This job gives me a strong position from which to promote the discipline." Another aspect of the job that appeals to him is "the transfer type of thing": the exchange and transfer of knowledge and experience to colleague scientists in or outside the institute, to his students, and also the communication with companies and the CNRS central management. Pérez: "I like meeting people, all sorts of people." The only shortcoming of the job as he sees it is that he cannot allocate his time as efficiently as he would like. "This office is sometimes like a church where people come in to confess, and I have to listen to everything!"

Be sceptical

Within the carbohydrate community, Serge Pérez is probably best known for his molecular modelling work. His interests in modelling were first aroused during a postdoctoral stay with Professor Bob Marchessault at the University of Montreal (1975-1977). Pérez: "I had a background in crystallography and had done some structural biology work during a one year postdoctoral stay in Oregon, USA. In Canada I was then introduced to the field of polysaccharides. I found these

CERMAV

Research Institute
Centre de Recherches sur les Macromolécules Végétales (CERMAV) (Associated with Université Joseph Fourier)

Director
Dr. Serge Pérez

Research Groups
- Structure and properties of glycomaterials, headed by Dr. Henri Chanzy
- Chemistry and physical chemistry of polysaccharides, headed by Prof.Dr. Marguerite Rinaudo
- Structure, interaction and dynamics of oligo- and polysaccharides, headed by Dr. Catherine Hervé du Penhoat
- Glycochemistry and molecular enzymology, headed by Dr. Hugues Driguez
- Molecular glycobiology, headed by Dr. Roberto Geremia
- Biochemistry of plant cell walls, headed by Prof.Dr. Jean-Paul Joseleau

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Number of employees
33 scientific staff
30 technical staff
20 Ph.D. students
27 postdocs
10 students

Research themes/key words
Cell wall, physicochemical properties, structure-function relationships, molecular modelling, material science, enzymology, carbohydrate engineering, bioinformatics.

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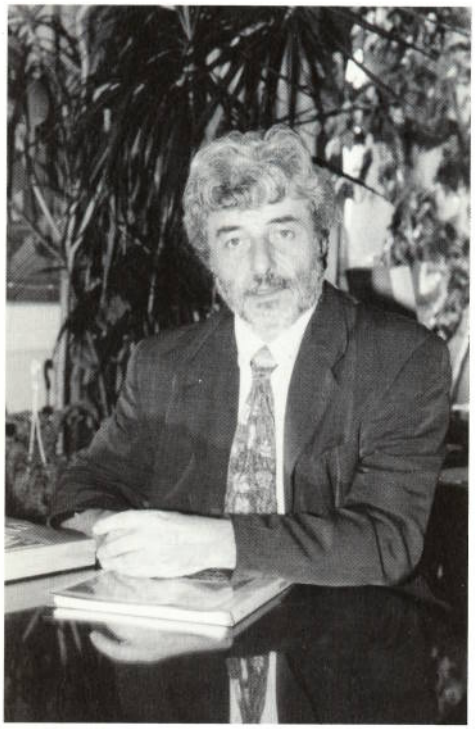


Serge Pérez (1947) was born as the grandson of Spanish immigrants in the Perigeux, an area in Southern France known for its truffles and fine wines. Pérez embarked on studies in physics at the University of Bordeaux in 1966 with the intention of becoming a geophysicist. Once enrolled in these studies, however, he found himself more interested in biophysics than in geophysics. In 1973, he received a Ph.D. degree in crystallography. After his Ph.D. studies he went abroad. He spent one year at the Institute of Molecular Biology of the University of Oregon in the USA, and, subsequently, three years at the Department of Chemistry at the University of Montreal in Canada as a research associate in the group headed by Professor Bob Marchessault. It was he who introduced Pérez to the field of carbohydrates. In 1977, he returned to France upon accepting a permanent position as a junior scientist of CNRS (Centre National de la Recherche Scientifique, see box) at CERMAV (Centre de Recherches sur les Macromolécules Végétales) in Grenoble. In 1984, he was appointed a Research Scientist at CNRS. After working in Grenoble for ten years, he moved to Nantes, to the Institut de la Recherche Agronomique, where he was awarded a position as Senior Research Scientist. Three years ago he moved back to Grenoble to become the chairman of the CERMAV. Pérez is married to Anne Imberty and has four daughters.

"Management of creativity, that is the most important part."

molecules fascinating because their diffraction spectra were so difficult to solve. The data from X-ray studies of an oligosaccharide or polysaccharide were not sufficient to solve the structure. To do this you need simulation methods. The beauty of molecular modelling is that it can explain very fragmented pieces of experimental evidence in a consistent way. And there is always the beauty of the images you can generate on the screen. But modelling is much more than that."

He considers the development of molecular modelling methods his largest contribution to glycoscience, but he seems very aware of the limitations of his favorite discipline. "You cannot use molecular modelling as a magic black box. If you use a force field for a particular problem, you have to know exactly how this force field has been parameterized to know its accuracy and limitations." He definitely does not believe a sales man that claims that his software is fit for all types of carbohydrates. At the same time, however, he defends his discipline: "Scientists rely so much on experimental data yet very often these cannot be reproduced. When examining experimental data we should be as sceptical as we are about computational data!"

"Invite scientists to cross conventional boarders."

The future belongs to biology

Although a central topic, molecular modelling of carbohydrates is only one of the many topics at CERMAV (see also the second part of the article: 'Current research topics'). A visit to this institute convinces one again of the high multidisciplinary character of carbohydrate science, with topics as diverse as 'physical properties of polysaccharide gels', 'genetically modified *E. coli* producing large amounts of oligosaccharides', 'the role of polysaccharides in cell wall expansion' and 'synthetic polymers reinforced with cellulose microfibers'. Pérez: "Carbohydrates represent the main compounds in biomass and are essential components of living organisms. We want to maintain the widest possible expertise in the field; having available all the tools that chemistry and physics can offer for unraveling the biological role of carbohydrates for which in many cases only speculations exist. We are now covering most of what we would like to cover at CERMAV, but are still missing a little bit of cell biology."

Pérez feels that the institute's broad approach has significant advantages in that it is possible to collaborate with many different laboratories. It also invites scientists to cross conventional borders. The institute is organized into six topical

Grenoble, a strong science cluster outside Paris

Although France is a highly centralized country also in terms of science, Paris is not the sole scientific center. The area Rhones-Alpes that includes the major cities Lyon and Grenoble has always played an important role in France's economy and has also an established science tradition. With a total population of 500,000, Grenoble holds over 50,000 students enrolled in studies at its three universities and polytechnics. All together the universities and scientific institutes in the Grenoble area employ over 12,000 people.

The presence of so many scientists has enabled the town of Grenoble to establish some highly advanced equipment by concerned action. Well-known examples are the synchrotron facilities (ESRF) and neutron reactor (ILL), but the scientists of Grenoble have also access to high-resolution NMR apparatus (750 MHz). Grenoble is also the main micro-electronics center in France and ranks as one of the top European centers for information science and technology.

groups, but there are also projects running across the disciplines. "These projects are not only interesting because of the science, but also for identifying the future managers. They are often run by young scientists." Pérez is not afraid that the 'critical mass' in some disciplines is too small. "Our groups may be small in comparison with those active in organic synthesis but the number of hands is not the most critical factor in our fields. Moreover, each of the groups is self-sufficient in terms of project budget and therefore large enough."

"The beauty of molecular modelling is that it can explain very fragmented pieces of experimental evidence in a consistent way."

Happy people

CERMAV's director has an interesting philosophy of how to perform the best work: "people have to be happy". He is not sure he can arrange that much, but he wants to create the optimum conditions for it. He feels that one contributory factor is the lifelong positions that CNRS employees get upon appointment. "People working here have no real stress about their future, but a stable view in life. Studies have indicated that less than 5% of the people at CNRS are not doing a good job, but this is the percentage that you could expect from any kind of organization." Other conditions he tries to optimize are access to facilities and funds and the emergency of new ideas: 'management of creativity'. Pérez believes that although realizing the first condition requires a lot of hard work, the second is the most difficult condition to accomplish. "After working in a laboratory for several years, people have a tendency to protect their own ideas and this sometimes prevents new views to flourish." One method that the director of CERMAV uses to establish an atmosphere of creativity is opening the workplace. "We have many people coming from all over the world at the institute. These people bring along their dreams and competence. I cannot force the people here to make use of this fresh air, but at least it comes in."

European theses

When it comes to major challenges in carbohydrate science, Pérez points to the production of large quantities of biological active oligosaccharides and polysaccharide engineering. Realization of the first objective will enable other scientists to study the function of carbohydrates, and, in the long term, make use of their characteristics. The growing understanding of structure-function properties of polysaccharides will eventually result in the synthesis of modified polysaccharides with a well-defined structure and function.

Pérez has European ambitions. He wants to promote networking, making sure that the relationship with fellow-scientists is growing steady with avoiding a lot of competition. One way of achieving this he believes, are so-called 'sandwich theses'. Ph.D.-students that divide their time between two European laboratories, taking advantage of the best of both. At the end of their studies, the students will receive a Ph.D.-title from both universities. After witnessing some successful examples with Brazilian-French Ph.D. theses, Pérez hopes to introduce the idea within the European network, creating a kind of European doctor title. "It will be quite complicated though. It requires bilateral agreements between a number of laboratories, a good organization and strong input from the supervisors, and of course good students that can cope with two supervisors and working benches".

Pérez hopes to stay on as director of CERMAV for at least another four-year term. "This is a big ship. Whenever you make a decision it takes some time before you know that you are on course, especially in research."

Hot debate on French research system

Centre National de la Recherches Scientifique (CNRS) is the largest of France's research organizations funded by the government. Its task is to produce fundamental knowledge and technology and to transfer this knowledge to society. CNRS employs a total of 11,500 scientists and 14,000 technicians and administrative staff, working within seven departments.

The organization has a strong democratic character. Scientists employed at CNRS have a lifelong appointment that is not restricted to one particular institute. Positions are established by the division directors of the national CNRS management, but granted to one of the job applicants by an independent committee elected every four years by the core of all French scientists.

The salaries of permanent employees and basic facilities are provided for by the French government. However, today only part of the research projects are financed directly by CNRS. At CERMAV, Grenoble, direct financing accounts for only 21% of the research projects, nearly one third of the projects is paid by the industry, 19% by various Ministries and 22% by EU-funds.

Recently, a hot debate is taking place in French about the country's research system. The discussion started when, in the start of 1999, the French science minister Claude Allègre announced a program of reforms aimed at the integration of universities and the CNRS, which should improve the researchers' mobility and facilitate the set-up of independent laboratories by young researchers.

As for CERMAV, this CNRS institute is associated with the Joseph Fourier University of Grenoble. Several of the scientists are in fact university professors. As the director of CERMAV, Pérez believes that strength resides in the combination of the two. "People who teach have a wider view about their discipline than full-time researchers, who focus more on details. This provides a nice balance."

Current research topics

Electron microscopy: focus quickly

In the glycomaterials department, headed by Dr. Henri Chanzy, the natural polymers cellulose, starch and chitin, and materials made from them are the subject of research. The group has many industrial contacts. In fact the majority of their projects is sponsored by the industry and several of those are marked as confidential.

The more fundamental studies concern the native structure of carbohydrates at the various levels of organization. The group has ample facilities available to do this including a transmission electron microscope complete with cryostat that allows the direct visualization of submicron objects in their hydrated or solvated environment. Recent projects include structural studies on model cellulose where microfibrils can be observed at the lattice resolution. The activity of cellulases at the surface of cellulose microfibrils can also be visualized.

The electron microscope is also used to study polysaccharide crystals such as inulin, xylan, mannan, chitosan and amylose by electron diffraction. Evidence of the usefulness of electron diffraction is best demonstrated by the appealing recent study of the composition and structure of filaments ejected by *Phaeocytis globosa*, a marine alga notorious for its clogging of fish nets (Figure 1).

Electron crystallography is a truly delicate job however since the polysaccharides are particularly sensitive to conditions in the electron microscope: the electron beam decrystallizes the polysaccharides and the vacuum can destroy their structures. Often there is ample time to focus a sample for an optimal image. Experience and intuition then steers the experiment to the very limits of the apparatus' capability. Electron crystallography is often complemented by X-ray diffraction experiments at the nearby European Synchrotron Radiation Facilities and neutron diffraction at the Institute Laue Langevin.

Useful cellulose microfibrils

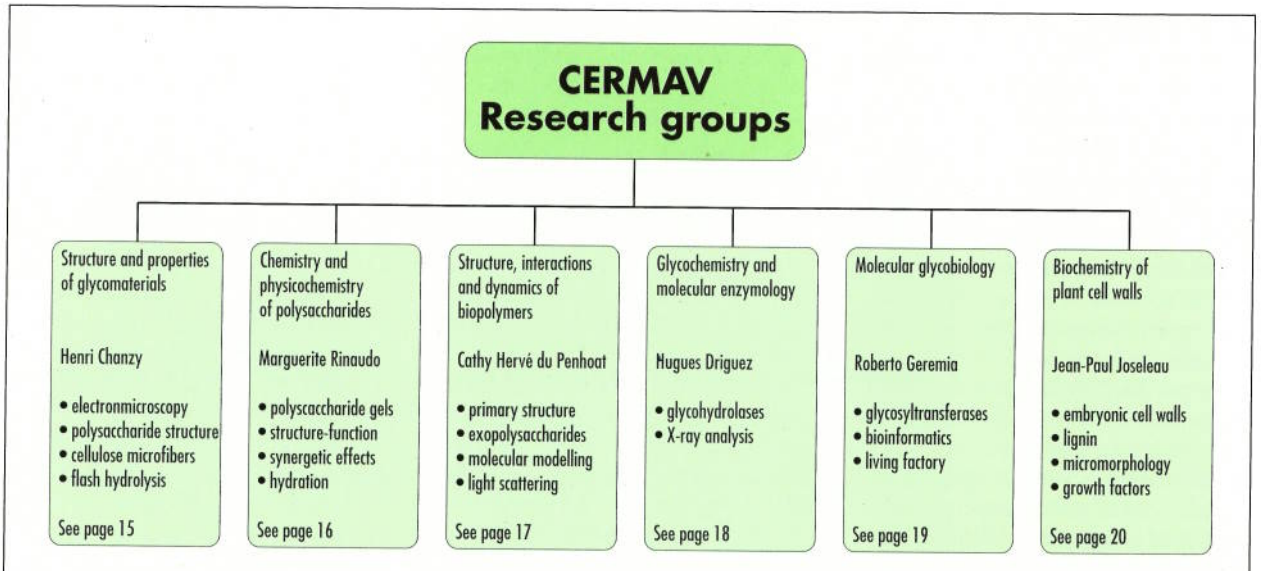
A hot topic in the glycomaterials group is the study of cellulose microfibrils from primary cell walls, commonly known as parenchyma cell cellulose (see also 'Carbohydrates of the cell wall' on page 20). Initiated in Grenoble six years ago this project has both fundamental and application-oriented aspects. Cellulose microfibrils are a main component (up to one third of dry weight) of all kinds of agricultural byproducts such as wheat bran, sugar beet, citrus and potato pulp. These products usually end up as cattle feed. CERMAV scientists believe that the biodegradable cellulose fibrils have strong potential as thickening or suspending agents (see also the article on ARD/Soliance in this issue).

An isolation method has been developed for the fibrils and today's studies include the analysis and optimization of mechanical properties of microfibrils from various sources (e.g. wheat bran, hemp, wood pulp, sugar beet pulp, cotton etc.). For example, it emerges that small amounts of pectins present as impurities in the fiber product play an important role during their preparation. The pectins prevent flocculation or sedimentation of aqueous dispersions of the microfibrils. The pectin also acts as a moisture-sensitive binder in films cast from the cellulose microfibrils. In a dry atmosphere, the pectins improve the strength of the film, whereas the Young's modulus decreases with pectin content in a humid atmosphere. Adding pectin to pectin-free cellulose films does not have the same effect, indicating that some critical native interaction is involved.

Blends of natural and synthetic polymers

Another application of cellulose microfibrils is as a filler and/or reinforcing agent in thermoplastics (see box 'Polypropylene reinforced with cellulose whiskers'). The glycomaterials group includes several polymer chemists, who design, study and develop polysaccharide-containing blends and composites. The polysaccharide is often more than just a cheap, abundant filler. Remarkable reinforcing effects of polysaccharide (micro)fibers have been observed even at concentrations of a few percents.

The hydrophilic nature of abundant, cheap polysaccharides that



The range of topics addressed at CERMAV in Grenoble, France, is almost as wide as carbohydrate science itself. The research is organized into six groups but scientists from various groups participate in joint projects. This article gives an overview of the ongoing studies.

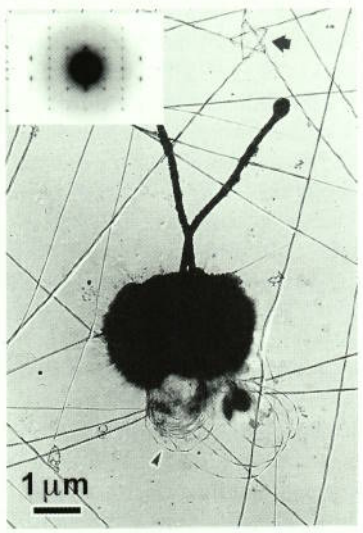


Figure 1. Electron microscopy image of the marine alga Phaeocytis globosa *ejecting a partly coiled set of filaments (small arrows). Around the microorganism some extended filaments and a star-like structure (large arrow) can be seen. CERMAV electron microscopy studies confirmed the assumption that the filaments consist of whisker-like α-chitin crystals, and provide clues to how the algae produce and eject the filaments. Inset: a representative electron diffraction pattern of a filament.*

often hampers their application as biodegradable material can be significantly improved in blends. Polyamide, for example, provides favorable intermolecular hydrogen bonds with polysaccharides, which can result in partial miscibility of the two components and a significant reduction in moisture sensitivity.

Gels are made by molecules

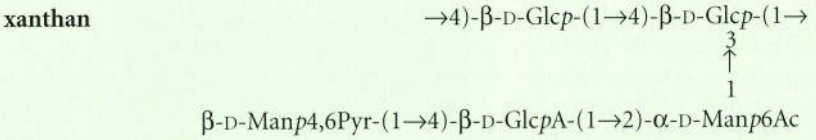
Before Dr. Serge Peréz became the director of CERMAV, Professor Marguerite Rinaudo led the institute for twelve years - the maximum term for a CNRS director. Rinaudo is one of the pioneers in the study and description of the complex behavior of water-soluble polysaccharides. After thirty years she finds her work more and more interesting because nowadays relationships can be formed between molecular structure and physicochemical properties.

Widely used and commercially important types of polysaccharides such as methylcelluloses, starch and chitin derivatives have often been the subject of her studies. A recent example is the study of the role of substituent distribution on the mechanism of gelation of methylcelluloses. Commercial types of methylcelluloses are highly heterogeneously substituted. At CERMAV, homogeneous types with different degrees of substitution were prepared and their gelling characteristics examined using microcalorimetry and rheology tests. The studies provided evidence for a strong contribution of highly-substituted units to gel formation. They also proved that a minimum degree of substitution of 1.3 is required for a phase transition. Similar studies have been carried out with alkyl-substituted chitosans, proving analogous interaction mechanism in gelation of this polymer.

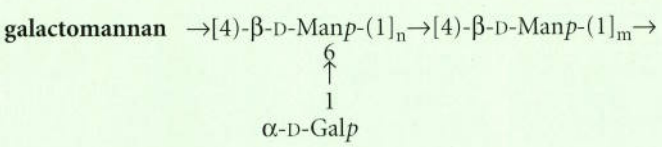
Polyelectrolytes are also among Rinaudo's favorite topics. In recent years, ionic bacterial polysaccharides have been studied. Because of their high regularity and homogeneity compared with plant polysaccharides, these bacterial carbohydrates turn out to be very suitable models for gaining insight into the relationship between structure, composition and physicochemical properties. A good example of this latter trend is the study of succinoglycan, a polysaccharide produced by a variety of bacterial strains (Figure 2). Depending on the source this polymer differs in the nature and content of succinate, acetate and pyruvate substituents, and also in the location of the succinate. Upon heating in solution, this polysaccharide undergoes a conformational transition between a helical ordered and disordered form. The transition also depends on the precise chemical structure of the polymer and the ionic concentration of the solution. Rinaudo and co-workers have succeeded in isolating three succinoglycan samples differing only in degree of substitution of succinate. Using differential scanning microcalorimetry, the behavior of the various polysaccharides in solution during several heating cycles was examined. The results indicate that both the content and the distribution of succinate groups have a strong influence on the conformational transition. On this basis, the scientists hypothesize that the conformation of the polysaccharide is directly related to the stiffness of the chain, and that the decrease in viscosity is not caused by a loss of aggregation or dimerization of helices, but is associated with a loss of interaction between the side chains and the main chain.

Synergy between polysaccharides in gel formation is another topic. While xanthan and galactomannans (Figure 2) do not form gels individually, mixtures of the two do. Various mixtures were characterized at different temperatures and ionic strengths using circular dichroism (CD), differential scanning calorimetry (DSC) and viscoelastic studies. Galactomannan was found to induce order in xanthan under circumstances in which it is usually disordered. However, galactomannan's interaction is more effective with disordered xanthan segments in terms of gel formation. The type of

xanthan



galactomannan



succinoglycan

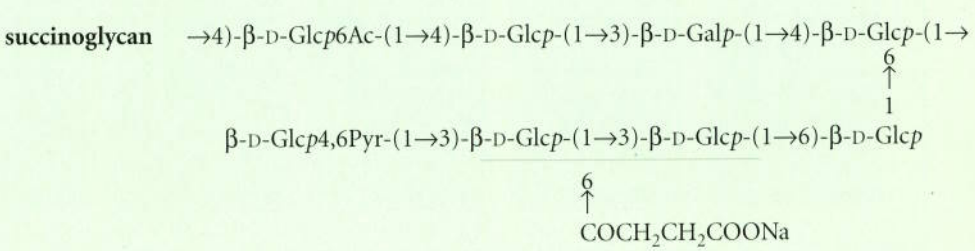


Figure 2: Primary structure of xanthan, galactomannan and succinoglycan.

Polypropylene reinforced with cellulose whiskers

Céline Bonini

Biosynthesis of cellulose provides crystallites in the form of slender rods called microfibrils, or whiskers when they are highly crystalline. The microfibrils reinforce cell walls. Cellulose whiskers can be obtained as aqueous dispersions by acid hydrolysis. The resulting whiskers are biodegradable, and have some remarkable properties: a high aspect ratio (ratio of length over diameter), a large surface area and high strength along length axis. These characteristics make the fibers preferable for reinforcing plastics in comparison with other fillers such as silica or carbon black.

We have developed new nanocomposites of cellulose whiskers and a synthetic polymer latex with improved mechanical stiffness (*1*). The reinforcement is attributed to the formation of network of whiskers linked by hydrogen bonds (*2*).

We are now studying whisker-reinforced polypropylene, a widely used thermoplastic. Because polypropylene is non-polar, it shows very little interaction with the cellulose whiskers. We have therefore modified the cellulose surface to obtain various types of interfaces and mixed the modified whiskers with atactic and isotactic polypropylene, providing a set of composites with varying morphologies and properties. Study of these model systems will help us to understand the mechanical properties of these new materials in terms of dispersion and interface, and may lead to more biodegradable reinforced thermoplastics.

1. Favier, V.; Cavaillé, J. Y.; Chanzy, H.; Ernst, B. Polymers reinforced with cellulose microfibrils. WO 9523824, Elf Atochem France, 1995.
2. Favier, V.; Chanzy, H.; Cavaillé, J. Y.; Polymer nanocomposites reinforced with cellulose whiskers. *Macromolecules* **1995**, *28*, 6365-6367.

ordering of xanthan in the complex seems to differ from that of ordered xanthan alone. Further investigations should reveal more details on the complex interaction, and the role of the mannose:galactose ratio of the galactomannan in gel formation.

Primary structure first

The largest group within CERMAV is devoted to structural and dynamical features of carbohydrates. The group has an enthusiastic leader in the person of Dr. Catherine Hervé du Penhoat. Scientists in her group often cooperate with other groups. They are determining the hydrodynamic volume of cellulose whiskers applied in the glycomaterials group, modeling the gel-forming polysaccharides studied in the group headed by Rinaudo, and analyzing primary structures of cell wall polysaccharides that are also of interest to the cell wall team.

Determination of the primary structure of carbohydrates is always a prerequisite for obtaining structural information and the group possesses all the necessary expertise and tools to do this, such as methylation analysis, HPLC, mass spectrometry and NMR spectroscopy. In fact, the institute owns an interesting library of over two hundred samples of all kinds of poly- and oligosaccharides, purified and identified over a period of thirty years. At the moment, the process of determining primary structures is focused on bacterial extracellular polysaccharides, e.g. the extracellular polysaccharides of lactic acid bacteria that play a key role in the rheological behavior and texture of fermented food and extracellular polysaccharides of rhizobacteria (see box 'The origin of good, sticky soil').

Another project concerned with the primary structure is the analysis of various pectins subjected to one or more enzymes in various stages of degradation, in order to understand the action patterns of pectinmethylesterases. Demethylation of pectin occurs during maturation of the cell wall and results in its stiffening (see also 'Carbohydrates of the cell wall' on page 20).

Molecular modelling

Most scientists in Hervé du Penhoat's group are studying the three-dimensional structure of carbohydrates by means of molecular modelling. In some cases, emphasis is primarily on obtaining structural information unavailable using other methods such as crystallography or NMR spectroscopy. Recent examples include the calculation of low energy conformations of agarose and the cyclodextrin analogue cyclotrikis-(1→6)-[α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]. In the latter case, the Grenoble scientists used a self-written modelling program: METROCYCLIX. This frequently happens because standard software is often unsuitable for carbohydrates.

The Grenoble scientists do like a challenge in this field. Last year they calculated the preferred conformations of the four oligomeric fragments of rhamnogalacturonan II (RG-II), a highly complex pectic mega-oligosaccharide (28 moieties) containing a number of rare monosaccharides. The mega-oligosaccharide has a backbone of at least seven (1→4)-linked α-D-galacturonic acid (GalA) residues to which four structurally well-identified side chains are attached in a fashion as yet unknown. RG-II is released from the primary cell wall of higher plants, and has an unusual highly-conserved structure. It is believed to complex borate, an essential micronutrient.

Every disaccharide segment present in the fragment has been modelled and its potential energy surface described. Using this information the complete fragments of RG-II were built and possible structures submitted to energy minimization. However, this produced a large number of viable conformers for each chain. The complete molecule is currently being constructed by comparing possible conformers from this database with the results of recent NMR work on RG-II and related oligomers.

Another research example is the

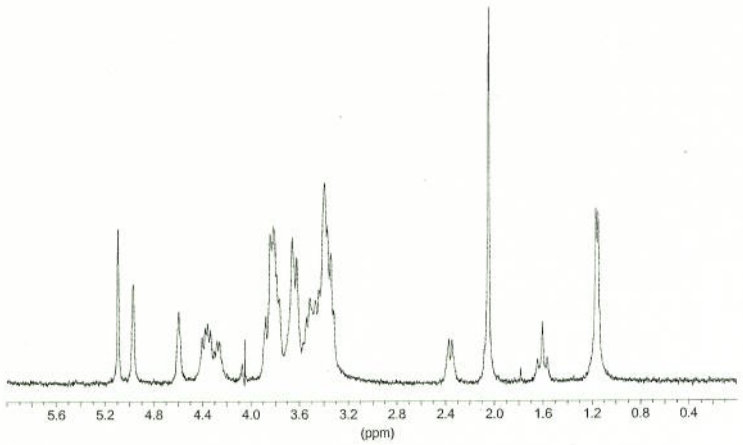
The origin of good, sticky soil

Cecile Vanhaverbeke

When a gardener uproots a plant, a clump of soil is stuck to the roots that is more or less voluminous depending on the vegetable species and on the type and the moisture level of the soil. The plants actively form this clump called the rhizosphere by controlled delivery of organic carbon to the soil via root exudates. Bacteria present in the rhizosphere live on this carbon source. Some of the rhizobacteria then synthesize exopolysaccharides (EPS) that cause the soil to stick around the roots. In this way the bacteria control the soil macroporosity which is important for a good uptake of water by plants.

The subject of my Ph.D. thesis is the study of EPS of rhizobacteria (*Rhizobium* and *Burkholderia*). This includes the characterization of the physicochemical properties as well as the determination of the primary structure using various methods such as HPLC, mass spectrometry, NMR spectroscopy (COSY, TOCSY, HMQC, HMBC, etc.). The NMR data (heteronuclear coupling constants, nuclear Overhauser effects) will be used in molecular modelling. Programs implemented in our laboratory for studying carbohydrates (METROPOL, POLYS, CHA-CHA) will be used to do this.

While we expect to gain a better fundamental understanding of the role of EPS of rhizobacteria in improvement of soil structure, the use of EPS in an industrial context is also interesting.



NMR spectrum of the exopolysaccharide of rhizobateria.

study of the widely-used polyelectrolytes pectin, alginate and glucuronan that form gels in the presence of divalent cations. The gelation mechanism of these acidic polysaccharides is a much discussed topic in carbohydrate science. Pérez and co-workers modelled four regular homopolymers of the acidic moieties of these polysaccharides to study their chelating properties. Both (1→4)-α-D-galacturonan and (1→4)-α-L-guluronan were found to have a high stereospecificity for calcium binding, and well-defined chelation sites. In contrast, (1→4)-β-D-mannuronan and (1→4)-β-D-glucuronan do not display any specificity. These findings add to the understanding of the physicochemical properties of the natural polysaccharides.

How glycohydrolases hydrolyze

Once you know that the threads in the two glycobiology groups at CERMAV are enzymes acting on glucans, the pieces all fall into place. Without this important piece of information a visitor is lost however. Research projects here range from highly fundamental studies to the structure and mechanism of α-amylase, via the production of oligosaccharide mimics of pneumococcal polysaccharides and the classification of glycosyltransferases, to application-directed projects such as the large scale production of chitooligosaccharides using genetically modified bacteria.

The group supervised by Dr. Hugues Driguez focuses on glycohydrolases. A main topic is the mechanism of enzymes acting on plant polysaccharides such as the endo-acting α-amylase and cellulases. Their activities have been monitored using bifluorescent substrate analogues. The results suggest that cellulases have tunnel-like active sites that are occasionally opened by 'the lid', a peptide loop. The conformational change has been confirmed by the X-ray structure of this enzyme in complex with a cellotetraose as determined at Prof.Dr. Alwyn Jones' laboratory in Uppsala, Sweden.

In the framework of a EU-project on starch degrading enzymes, the motion in glucoamylase G1 of *Aspergillus niger* has been studied using quasi-elastic light scattering. Glucoamylases catalyze the hydrolytic release of β-D-glucose from the non-reducing ends of starch. They possess a starch-binding domain or module (SBM) and a catalytic module (CM) separated by a flexible highly glycosylated peptide linker. A popular hypothesis asserts that the SBM attaches to the starch granule and upon binding with it pulls the catalytic module into an appropriate position to cut the glucose moiety on the non-reducing end. The SBM then slips over the starch chain allowing the CM to cut off the next unit. The hypothesis has not yet been confirmed because crystallization of the enzyme has failed, probably because of the flexible linker region. Driguez and co-workers synthesized substrate mimics that contain both acarbose, an inhibitor that binds to the CM and β-cyclodextrin which interacts only with SBM. The hydrodynamic dimensions of the enzyme were determined with and without the ligand using light scattering experiments. The results indicate that the ligand stabilizes a more compact enzyme conformation, thus confirming the 'pull' theory. However, the results also suggest that the enzyme may cut off several glucose moieties at the non-reducing end once bound to the starch. This new concept is now under further investigation at Carlsberg Laboratory, Copenhagen, Denmark (Dr. Birte Svensson) using glyco-

amylose mutants with linkers of various lengths.

The roots of all carbohydrates

Dr. Roberto Geremia heads the other glycobiology group. The central theme here is glycosyltransferases, the enzymes that play a key role in the biosynthesis of polysaccharides, glycoproteins and glycolipids. The study of this class of enzymes is also severely hampered by a lack of available crystal structures (see also CarboHighlights page 7 of this issue). The group is therefore highly creative in using other means to collect structural information, for example by using bioinformatics and molecular modelling. By computer-assisted comparison of all known amino acid sequences of galactosyltransferases present in protein databases, five families could be discerned and described in terms of length and locations of conserved regions. More importantly, the CERMAV bioinformaticians found a possible 'signature' for glycosyltransferases. Almost all enzymes possess an acidic motif (Asp-x-Asp). As this motif is widespread among glycosyltransferases it is also likely to be involved in substrate binding.

The group also makes extensive use of fold recognition methods to propose possible conformations for glycosyltransferases. A recent achievement in this field is a three-dimensional model of the nucleotide binding domain of pig α-1,3-galactosyltransferase. This enzyme is responsible for the biosynthesis of the Gal(α1-3)Gal epitope that causes hyperacute rejection of pig organs in humans upon xenotransplantation (see also box 'The living factory').

Recently, the glycobiologists have developed strategies for cloning glycosyltransferases in order to obtain larger quantities of the enzymes for studies. Glycosyltransferases of plant origin are cloned because of their great biotechnological potential in using plants as expression vectors for recombinant glycoproteins. Glycosyltransferases involved in the biosynthesis of bacterial exopolysaccharides are also under investigation. Examples include the enzymes involved in the synthesis of succinoglycan in *S. meliloti*, acetan in *A. xylinum* and xanthan gum in *X. campestris*. A mechanistic understanding of these glycosyltransferases is important for understanding the biosynthesis of bacterial cell wall components, and may also lead to the engineering of novel polysaccharides of industrial interest.

Let bacteria do the hard work

The close cooperation between the glycobiology groups is exemplified in the 'living factory' project. The glycobiologists realized that the utilization of glycosyltransferases for synthetic purposes, is severely hampered by the high costs of the required carbohydrate donors: the sugar nucleotides. Their idea was to use microorganisms and their intracellular pool of sugar nucleotide for the *in vivo* synthesis of 'recombinant oligosaccharides' by adding the genes encoding for the glycosyltransferases to produce them to the genome of a bacterium.

The idea seems simple, but is not as straightforward as producing a recombinant protein. Not only must the bacteria express the enzyme, it must also 'allow' it to function and accumulate its products. Nevertheless, CERMAV decided to try to produce recombinant chitooligosaccharides in *Escherichia coli* by introducing the *NodC* and *NodB* gene, encoding respectively for a chitooligosaccharide synthase and chitooligosaccharide *N*-deacetylase. Chitooligosaccharides were chosen because they are plant growth regulators, they are very difficult to isolate or to synthesize in larger quantities, and their biosynthetic pathway is known.

Dr. Eric Samain, a biotechnology expert, has succeeded in feeding and growing the genetically modified bacteria in such a way that they yield up to 2.5 g/L penta-*N*-acetyl-chito-

Serge Pérez and co-workers in front of the CERMAV building.

pentaose and its deacetylated derivative tetra-*N*-acetyl-chitopentaose. The bacterium expresses both of the enzymes at low but sufficient levels to allow production of the oligosaccharides without disturbing the bacteria's metabolism. Purification of the products is straightforward. Charcoal adsorption and ion-exchange chromatography do the trick.

The success inspired the CERMAV scientists to study the potential of recombinant bacteria to produce other and more complex carbohydrates. Mono-6-*O*-acetylated chitooligosaccharides were obtained by introducing the *nodL* gene and sulfated analogues by introducing *nodH*. It was possible to isolate the major compounds in reasonable yields although one problem encountered in the growing number of enzymes introduced was the difficulty in separating the various products.

Carbohydrates of the cell wall

In 1966, when CERMAV was established to conduct fundamental research into cellulose and lignin, thereby complementing the applied research of the Centre Technique du Papier and the l'Ecole Francaise de Papeterie in Grenoble, immediately a group devoted to the study of the cell wall was set up. Today, the group is supervised by Professor Jean-Paul Joseleau, who also lectures biochemistry at the affiliated Université Joseph Fourier. Since the establishment of the research group, over thirty years ago, the aim has shifted from identifying cell wall components and structures towards the biochemical and physiological processes by which plant cell walls develop.

The plant cell wall is a highly complex and dynamic composite. The walls of growing plant cells (primary cell walls) are relatively thin and flexible, allowing the cell to expand in size. They consist of cellulose microfibrils embedded in a matrix of proteins, hemicelluloses and pectins. Once cells have ceased growth, they frequently lay down a secondary cell wall between the plasma membrane and the original primary cell wall. Secondary walls are thicker and more rigid than primary ones, providing mechanical strength to the plant. They lack pectin, and are strengthened by lignin, a complex polymer of phenolic residues. The orientation of cellulose microfibrils also differs in primary and secondary cell walls. In the primary walls they are more or less randomly orientated, whereas in secondary cell walls they are highly ordered. Secondary walls frequently contain several layers of cellulose fibers each with its own orientation, forming a strong laminated structure.

The dynamics of the primary cell walls during growth are a main topic in Grenoble. The precise nature of the modifications taking place and the enzymes involved in them are being studied using cell cultures in suspension as a model system. The major soluble carbohydrates present in the cell walls and the cell culture are being analyzed (using methylation analysis and NMR spectroscopy) during various stages of development. Recent examples include the study of an embryonic cell line of *Pinus caribaea*,

The living factory

Large-scale production of a trisaccharide responsible for the rejection of xenografts

Emmanuel Bettler

While scientific and commercial interest in oligosaccharides is increasing, their availability is limited as production relies on chemical and chemo-enzymatic synthesis. In the framework of an EU-sponsored project on xenotransplantation (B104CT972242) and in the endeavour to find a more economical method for synthesizing larger quantities of biologically-interesting oligosaccharides, we investigated the possibility of producing oligosaccharides in genetically modified *E. coli* that express the appropriate glycosyltransferases.

More precisely, we attempted to produce larger amounts of the trisaccharide Gal(α1-3)Gal(β1-4) GlcNAc. This is a major target of natural human antibodies after a xenotransplantation, and is responsible for the hyperacute rejection. The production of this oligosaccharide could open new doors for the treatment of the hyperacute rejection, e.g. by removing circulating antibodies from the blood stream by absorbing them on a column coated with the trisaccharide.

In a first step towards our goal, the *Azorhizobium chitin* pentaose synthase NodC (a β-1,4-GlcNAc transferase) and the *Neisseria* β-1,4-galactosyltransferase LgtB were co-expressed in *E. coli*. The major oligosaccharide isolated from the recombinant strain was subjected to LC-MS, FAB-MS and NMR analysis and identified as Gal(β1-4)[GlcNAc(β1-4)]$_4$ GlcNAc (*1*). Yields were over 1.0 g/L.

The hexasaccharide turns out to be an acceptor *in vitro* for pig α-1,3-galactosyltransferase, which suggests that additional expression of this enzyme in *E. coli* will allow the production of the heptasaccharide Gal(α1-3)Gal(β1-4)[GlcNAc(β1-4)]$_4$ GlcNAc. The trisaccharide responsible for rejection of xenografts can be obtained from this oligosaccharide by the action of a chitinase.

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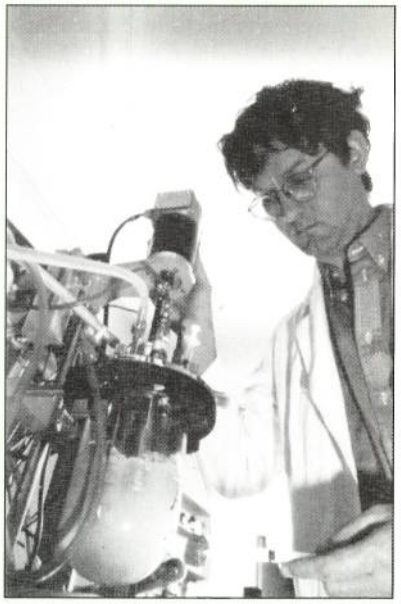

Eric Samain at the bioreactor.

a fast growing and high yielding coniferous species for timber and pulp production, and *Rubus fruticosus* cells grown in suspension. These cell-growing techniques are important in clonal propagation of genetically transformed plants.

The interaction between cellulose and hemicelluloses of the growing plant cell is also being examined by *in vitro* measuring the affinity between the two polymers, by modelling the complex, and by *in vivo* visualizing the distribution of the hemicelluloses along cellulose microfibrils using electron microscopy. Chemical, enzymatic and immunological markers are developed in this group to identify wall polymers *in muro* (within the cell walls) and to visualize their distribution at the ultrastructural level. Novel antibodies directed against various lignin substructures have recently been obtained. Thanks to the unique collection of immunological probes, the CERMAV cell wall group has the opportunity to study the fine topological organization of the lignified walls. The markers are used to study lignification in developing tissues, to characterize lignins in transgenic plants in which the lignin biosynthetic pathway has been modified and to unravel the mechanisms by which filamentous fungi attack and degrade lignified walls of woody plants.

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