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In-situ Raman microprobe studies of plant cell walls: Macromolecular organization and compositional variability in the secondary wall of *Picea mariana* (Mill.) B.S.P.

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Abstract. Native-state organization and distribution of cell-wall components in the secondary wall of woody tissue from *P. mariana* (Black Spruce) have been investigated using polarized Raman microspectroscopy. Evidence for orientation is detected through Raman intensity variations resulting from rotations of the exciting electric vector with respect to cell-wall geometry. Spectral features associated with cellulose and lignin were studied. The changes in cellulose hands indicate that the pyranose rings of the anhydroglucose repeat units are in planes perpendicular to the cross section, while methine C-H bonds are in planes parallel to the cross section. Changes in bands associated with lignin indicate that the aromatic rings of the phenyl-propane units are most often in the plane of the cell-wall surface. However, regions where lignin orientation departs from this pattern also occur. These results represent direct evidence of molecular organization with respect to cellular morphological features in woody tissue, and indicate that cell-wall components are more highly organized than had been recognized. Studies carried out in order to establish the usefulness and sensitivity of the Raman technique to differences of composition within the cell walls provide evidence of variations in the distribution of cellulose and lignin. Such compositional differences were more prominent between the walls of different cells than within a particular cell wall.

Key words: Cell wall structure – Cellulose – *Coniferae* – Lignin – *Picea* – Raman microprobe.

Introduction

Review of the two recent treatises on the architecture of plant cell walls (Preston 1974; Frey-Wyssling 1976) shows a gap in our knowledge of wall structure. Most information available falls into one or the other of two major categories. The first includes descriptions of morphological features as observed using optical and electron-microscopic procedures. The second category consists of information concerning the molecular structure of constituents as derived from physical and chemical analyses of isolated samples of individual components. Overlap between the two categories is constrained primarily because isolation of the different components in quantities sufficient for analytical purposes usually requires procedures such as those described, for example by Browning (1967), which are severely disruptive of the native structures. Limited chemical characterization of intact samples of plant tissue is possible with microscopic staining reactions. Such reactions, however, involve rather complex charge-transfer phenomena and are not easily interpreted in terms of molecular structures

Studies of the variation of chemical composition between different morphological regions in woody plants have been undertaken in the past (Meier 1961; Simon and Timell 1978; Hardell and Westermark 1981; Whiting and Goring 1983). These investigations have involved chemical analyses of separated tissue regions or the isolation of tissue fractions. These approaches require complete physical separation of the morphological regions of interest (Hardell and Westermark 1981) or isolation of the tissue fractions based on the physical properties of the components (Whiting and Goring 1983; Whiting et al. 1981). In this light, we considered it worthwhile to explore the potential of Raman spectroscopic studies on intact native tissue; such studies are now possible with Raman microprobe systems possessing resolution capabilities in the 1 μm range.

The Raman microprobe (Rosasco 1980) presents a promising new opportunity to fill a gap in

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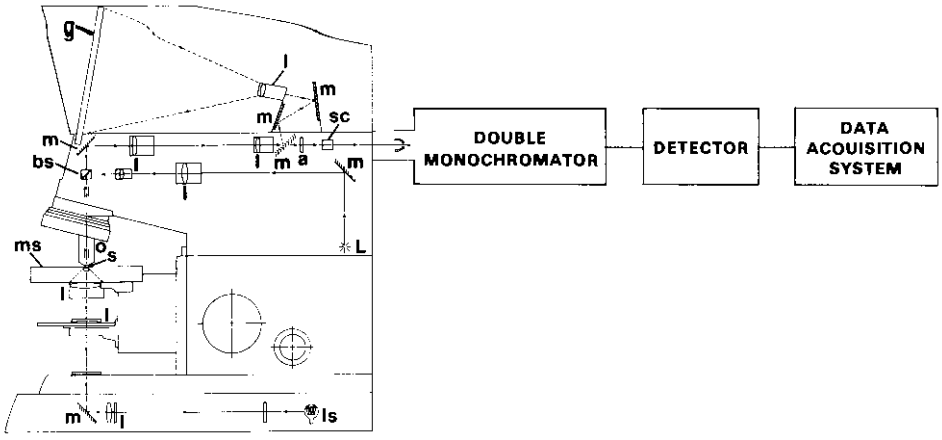

Fig. 1. Optical pathway in a Raman microprobe setup. Incident and scattered light directions are shown by *arrows. o,* Objective; *s,* sample; *ms,* microscope stage; *bs,* beam splitter; *m,* mirror; *a,* analyzer: *sc,* scrambler; *L,* laser; *g,* glass screen; *l,* lens assembly; *ls,* light source

our knowledge of the cell wall, for it provides for the first time the possibility of acquiring direct information concerning the molecular structure and composition of individual morphological features of undisrupted plant tissue. Because spectra can be recorded from domains as small as 1 μm in diameter, the problem of morphological heterogeneity is overcome to a substantial degree. Furthermore, the Raman spectra arc recorded in a backscattering mode so that optical heterogeneity or opacity of the sample is not a problem; the spectral information pertains to material within 1 μm of the surface of the sample under study.

In laser-excited Raman spectroscopy the sample under investigation is exposed to a focused laser beam and the scattered light is analyzed (Gilson and Hendra 1970). A small fraction of the scattered light is shifted in frequency. The shift in frequency is equivalent to the vibrational frequencies of the samples under investigation. This information is similar to the information in a typical infrared spectrum. Raman spectroscopy has some important advantages over infrared in the study of biological tissue, however. The primary one is that there is little or no interference from water; highly polar bond systems, which result in intense infrared bands, have relatively low polarizabilities and, hence, very weak Raman spectra. In addition, control of the plane of polarization of the incident laser light, coupled with analysis of the polarization of scattered light, can facilitate assignment of the spectral features and in many instances result in information concerning molecular orientation (Andersen and Muggli 1981 ; Harrand 1983).

The microprobe extension of the standard Raman technique involves utilization of an accessory consisting of a specially designed microscope system that serves two key purposes simultaneously. The first is to focus the laser beam on the region to be investigated down to a diameter of approx. 1 μm. The second function is to gather the scattered Raman light and transmit it into the entrance slit of the spectrometer. The microprobe thus permits microscopic examination of the tissue under study and identification of individual morphological features the Raman spectra from which are recorded. Its potential in studies of the structure of woody tissue has been illustrated in our preliminary reports (Atalla and Agarwal 1984, 1985). Here we provide a more comprehensive account of studies concerning orientation of cell wall components and their distribution in the secondary wall of *Picea mariana* tissue.

Material and methods

Plant specimens. Preliminary studies were carried out on sections from wood chips available at the Institute hut not identified beyond their origination from a log of spruce from a location in northern Wisconsin. Most of the results reported here were for a log collected specificially for this investigation. It was identified as *P. mariana* (Miller) B.S.P., 55–57 years old, grown in a northern Wisconsin location. The samples were taken from the main stem which measured 19 cm in diameter at a height of 1.4 m. Sections were prepared by microtome from segments between the 15th and 40th rings, and were approx. 30 μm thick. For both transverse and longitudinal sections, spectra were recorded only from the S_2 layer.

Sample preparation. The sections were repeatedly washed with distilled water, prior to mounting in the following manner. A flat-base Pyrex glass beaker was fixed to a slide in a manner that allowed transmitted light to pass through. On the inner side of the bottom yet another portion of a glass slide was attached. A wet sample was sandwiched between the slide portion and a cover slip through the center of which a small hole had been drilled; the cover slip held the sample immobile while D_2O was slowly added into the beaker until the sample was completely immersed. The beaker was then mounted on the stage of the microprobe, and the water immersion objective was lowered into the D_2O. In order to avoid evaporation or exchange with atmospheric moisture, a thin layer of mineral oil was placed on the surface of the D_2O after the objective was submerged in the medium. This sampling procedure has

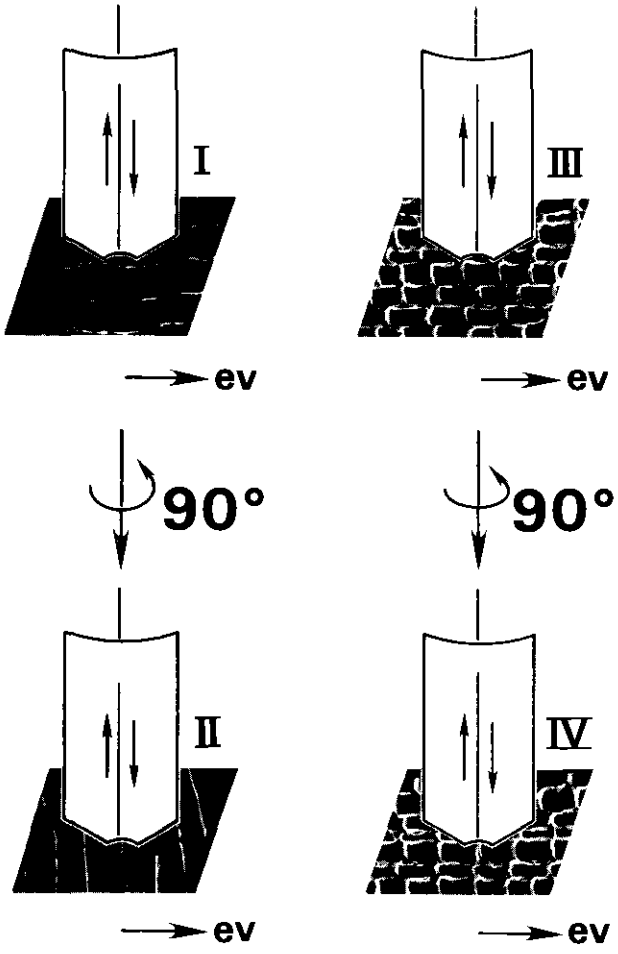

Fig. 2. Scattering geometries. *1* – electric vector (ev) direction parallel to the cell-wall surface in a longitudinal section; *II* – ev perpendicular to the cell-wall surface (longitudinal section): *III* – ev parallel to the cell-wall surface in a cross section, and *IV* – ev perpendicular of the cell-wall surface (cross section)

enabled us to acquire the best Raman spectra from native tissue, which otherwise is highly fluorescent. Details of the procedure are given elsewhere (Atalla and Agarwal 1984). Delignified tissue sections were prepared by the acid-chlorite method at 60° C (Browning 1967).

Roman microprobe. The system consisted of three major components: a Raman spectrometer, a modified optical microscope, and an automated system for data handling. The spectrometer used was a Jobin Yvon Ramanor HG2S System (obtained from Instruments SA, Metuchen, N.J., USA) using the 5145 A° line of a Coherent Radiation (Palo Alto, Cal., USA) 52A laser for excitation. A Tracor Northern (Madison, Wis., USA) TN 1500 data analyzer in the photon counting mode was used to accumulate spectral data. The System could be programmed for multiple scanning when desired. The reported spectra are averages of four to ten scans.

The microprobe consists of a Nachet microscope (from Instruments SA), especially modified to allow coupling of the laser into its optics so that the exciting radiation is focused on the specimen through the objective. The Raman-scattered light is collected into the same objective and focused on to the entrance slit of the Raman spectrometer. In addition, the sampled area and its location relative to other morphological features could be viewed and determined precisely. A schematic illustration of the microprobe is shown in Fig. 1

Spectra were recorded using a 100X objective with a numerical aperture of 1.18; it gave a focused spot of 1–2μm in diameter. Laser power at the sample was approx. 5 mW. The entrance slits of the double monochromator were set at 800 μm, corresponding to a resolution of approx. 8 cm^{-1}; this wide opening was necessary to allow sufficient energy into the monochromator. Spectra were scanned at a rate of 60 wavenumbers/min and the dwell time was 1 s. In most instances four scans were needed to obtain an acceptable signal-to-noise ratio, though the spectra of better quality were obtained using 10–12 scans. Multiple scans with a short dwell time per channel were preferred to a single scan at longer dwell time so that any long term drift in laser power would not distort the spectra. The spectral region between 250 cm^{-1} and 3,700 cm^{-1} was studied.

Most spectra were recorded with an analyzer in the scattered beam: but it was noticed, from the results on the longitudinal sections, that its presence did not result in any major changes. Therefore, in some cases spectra were also recorded without the analyzer because a much higher signal throughput could be achieved.

Pairs of spectra reported in parallel and perpendicular modes are from the same location in the cell wall. Because of dichroism in the optics of both the microscope and the spectrometer, rotation of the electric vector relative to morphological features was accomplished by rotating the sample on a precisely aligned rotating stage; the polarization of the incident beam and the orientation of the analyzer relative to the optical components of the microscope and the spectrometer were kept constant.

Scattering geometries. Four different scattering geometrics were employed; these arc shown in Fig. 2. In geometrics I and II, longitudinal radial sections were studied with the electric vector of the laser beam parallel and perpendicular, respectively, to the cell-wall surface. The transition from I to II was accomplished by rotating the stage 90° about the axis of the microscope objective. Cross sections were used in the other two geometries. As in the first instance, the cell-wall surface was parallel to the electric vector in one case (III) and perpendicular to it in the last arrangement (IV). Here again the transition from III to IV was accomplished by 90° rotation of the stage.

Laser-power effects. During operation the microprobe requires the laser to be focused to a tiny spot, on the sample surface, the dimensions of which depend on the characteristics of the microscope objective. Very low power levels must be used to avoid laser-induced thermal effects. In our experiments, low power levels of the order of 5 mW were used. At higher irradiance levels, bands associated with lignin declined relative to those of cellulose; degradation or highly localized solubilization of lignin were suspected. The sensitivity of cell-wall lignin to radiation-induced effects was found to vary with the locations from which spectra were obtained, perhaps pointing to the variability in the chemical nature of lignin. Conditions leading to variations in the intensity of lignin bands were avoided in the present work.

Spectral intensities. Relative intensities (I′) of various bands in the spectra were measured relative to the peak height of the shoulder (2410 cm^{-1}) of the D_2O band. The O–D stretching modes of D_2O do not overlap with any cell-wall bands, and therefore serve as an ideal internal standard. It should, however, he noted that a minor contribution around 1200 cm^{-1} arises from the bending mode of D_2O. H_2O, on the other hand, has

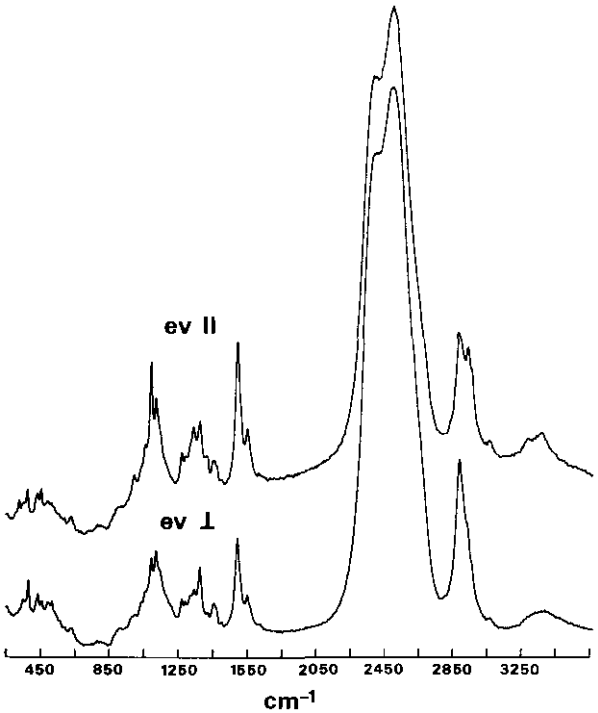

Fig. 3. Polarized Raman spectra of a spot lying in the secondary wall of a longitudinal section of *Picea mariana*

bands which interfere in regions overlapping with those of cell-wall constituents.

Repetitive scanning of a particular sample over long intervals of time indicated that the magnitude or the experimental error in the intensity values between sets of averaged spectra was no more than 7%.

Results

Molecular orientation. As noted above, Raman spectroscopy provides a valuable method for detecting molecular orientation. Interactions which can be described in terms of the derived polarizability tensors of a normal mode of vibration in a molecule, and the components of the electric vector of the incident radiation provide evidence concerning the orientation of molecules relative to the incident beam. This information can be obtained by analysis of the polarization of Raman-scattered light. In the present work well-known features in the spectra of both cellulose and lignin were studied. The Raman spectra were acquired from different points in the S_2 layers of cell walls of the woody tissue. Though the data presented are representative of latewood, no substantial differences between late- and earlywood spectra have been noted so far.

Although spectral data from several different representative points were gathered for each set compared, they were not sufficient for any meaningful statistical analyses. It is anticipated that multichannel detection will allow more rapid accumulation of data, and provide a data base sufficient to permit statistical analyses of the patterns of variation.

Representative spectra are presented in Figs. 3–7. Each includes two spectra recorded from the same point in a section of the S_2 layer in a particular cell. One spectrum is recorded with the electric vector of both exciting and scattered radiation parallel to the plane of the cell-wall surface (geometries I or III). The other spectrum is recorded with the electric vectors perpendicular to the plane (geometries II and IV). The key findings are reflected in the changes in the relative intensities of specific spectral features upon alteration of the recording geometry. Figures 3–5 show spectra recorded in geometrics I and II from longitudinal sections. Figures 6 and 7 were recorded in geometries III and IV from cross sections. Figures 5 and 7 are from delignified sections. Variations in specific spectral features associated with cellulose and lignin are discussed first with respect to their implications concerning molecular orientation and subsequently with respect to what they reveal about compositional variations.

Cellulose. Some of the well-characterized Raman spectral features of cellulose appear at 330, 380, 1098, and 2900 cm^{-1} (Atalla 1976; Atalla et al. 1980). Based on these earlier studies the band at 1098 cm^{-1} has been identified with one of the polarization- and orientation-sensitive skeletal modes of the chain. The methine C–H stretches of cellulose molecules are responsible for the band at 2900 cm^{-1}. These two bands have been found to be the most useful ones for exploring the orientation of cellulose molecules. Changes in the relative intensities of these bands occur when the direction of the incident polarization is changed relative lo the plane of the cell wall. Spectra obtained from longitudinal sections are shown in Figs. 3–5. When the electric vector (ev) direction of the incident beam is changed from parallel to perpendicular with respect to the cell wall, the features at 330 and 1098 cm^{-1} decrease in intensity while the 2900-cm^{-1} band shows an opposite trend. This implies that the native cellulose molecules arc preferentially oriented in the plane of the cell-wall and the methine C–H bonds are predominantly perpendicular to this direction. One exception to the trend noted has been observed for the 2900-cm^{-1} band. The anomaly may be the result of some contribution to the 2900-cm^{-1} region from aliphatic C–H's in lignin, especially from $-OCH_3$ and $-CH_2$

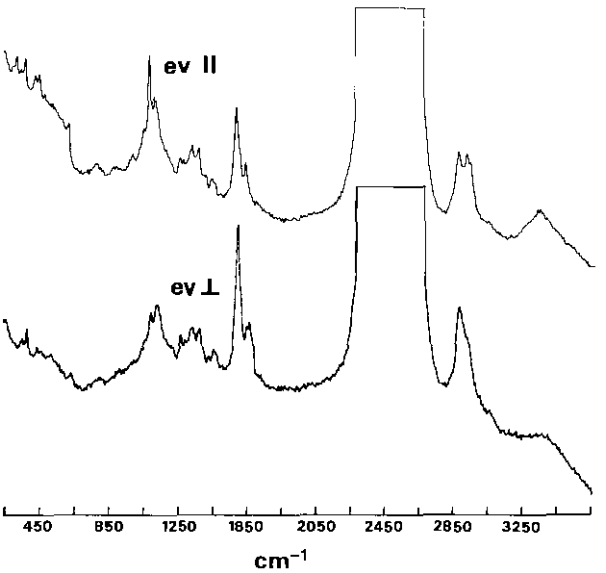

Fig. 4. Polarized Raman spectra of the secondary wall of a longitudinal section of *P. mariana*; 1600-cm^{-1} band is stronger in the perpendicular mode

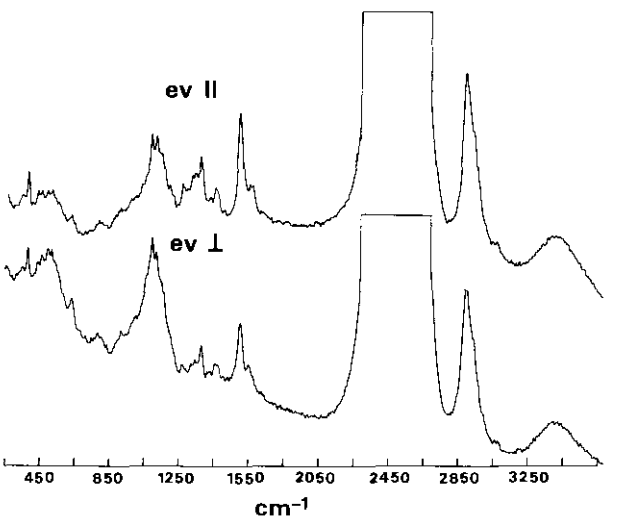

Fig. 6. Polarized Raman spectra of the secondary wall of a cross section of *P. mariana*

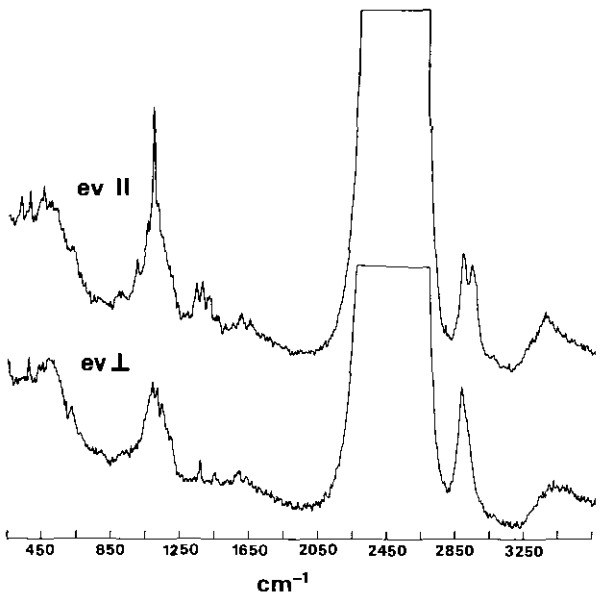

Fig. 5. Polarized Raman spectra of the secondary wall of a delignified longitudinal section of *P. mariana*

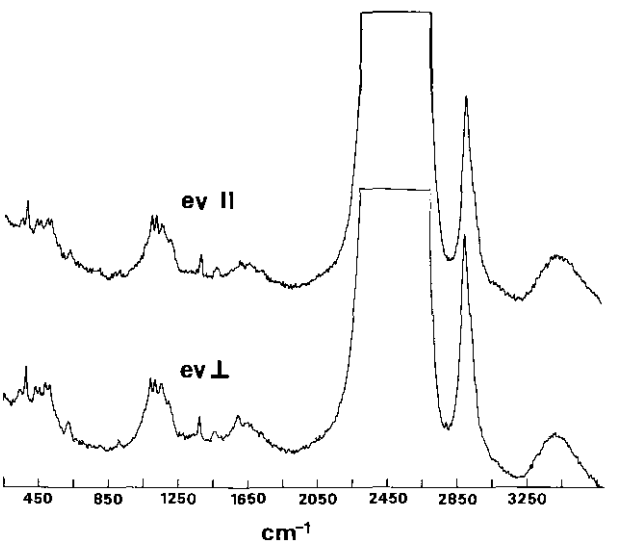

Fig. 7. Polarized Raman spectra of the secondary wall of a delignified cross Section of *P. mariana*

groups; rough estimates from the data on interunit linkages and functional groups indicate the presence of approx. seven aliphatic C–H's per phenylpropane unit in softwood lignins (Schaefer et al. 1981). This effect was assessed by examining spectra recorded from the delignified sections (Fig. 5); the lignin-removal process is known to have no major effect on cellulose orientation (Preston, 1974, p. 290). Two points are to be noted concerning the C–H stretch region of Fig. 5. First, that the 2900-cm^{-1} band is indeed stronger in the perpendicular mode than it is in the parallel mode, and second, that the doublet results from cellulose. The appearance of the rest of the region supports the conclusions drawn earlier, and confirms the observation that delignification does not bring about structural changes in cellulose. The intensity variation pattern for cell-wall cellulose is also

identical to that for Ramie fibers (Wiley and Atalla 1986); these fibers are known to have the cellulose chains parallel to the fiber axis, as noted in the discussion by French (1978).

A comment on the number of peaks in the C–H stretch region and their origin is in order. There are at least three peaks, at 2900, 2940 and 2960 cm^{-1}, when the sample is studied in geometry I. The presence of the higher-frequency peaks in the parallel mode in the C–H stretching region can be attributed to the methylene hydrogens at C–6 of the anhydroglucose ring. In ß-D-glucose, Raman bands at frequencies higher than those of methine C–H's have been assigned to CH_2 stretches (Wells 1977). Decline of the 2940-cm^{-1} feature upon sample rotation by 90° (Figs. 3–5) very likely reflects the $–CH_2OH$ conformation with respect to the chain.

Conclusions drawn from the studies on longitudinal sections are also supported by the spectra from the cross sections. These spectra are shown in Figs. 6 and 7. In these spectra the 330-cm^{-1} band is almost absent, and those at 380 and 1098 cm^{-1} do not change in intensity when the direction of polarization is changed. This observation is consistent with the chain axes being in planes which are perpendicular to the plane of the cross section. The apparent intensity variation in the 1098-cm^{-1} peak results from the broader part of the band and from small variations in the cellulose-chain organization. Some evidence of intensity variation is observed for the 2900-cm^{-1} band when lignin is present; the difference disappears upon delignification.

Finally, differences in Raman scattering between 3100 and 3600 cm^{-1} provide important information concerning H-bonding. The O–H stretching modes, particularly in Fig. 3, reflect alignment of crystalline cellulosic domains with the chain axis in the plane of the cell-wall surface. In Fig. 3, the spectrum recorded in geometry I includes two peaks superimposed upon the broad band in this region. These bands arise from intramolecularly hydrogen-bonded –OH groups which are parallel to the chain. Such peaks arc not observed in the other geometries wherein the electric vector of the exciting radiation is perpendicular to the chain direction. The –OH bands observed in these geometrics are usually quite broad and are associated with intermolecular H-bonding.

In summary then, the pattern of the spectral features associated with cellulose is consistent with two alternative schemes, in both of which the plane containing the chain axes of molecules is perpendicular to the plane of cross section. The orientation of the remaining two axes are either random, or they alternate from one fibril to the next, or from one lamella to another. While the X-ray evidence (Harada and Goto 1982) shows that the (101) and (101) planes in *Valonia* cellulases are oriented, the electron diffraction and X-ray results of Revol et al. (1982) on woody tissue favor the random orientation. More recently Wiley and Atalla (1986) found the random arrangement more consistent with data on ramie fibers. Thus the random arrangement appears the more plausible one for wood.

Lignin. The Raman band at 1600 cm^{-1} in the spectra of plant cell walls is similar to the aromatic-ring breathing modes of lignin model compounds (Erhardt 1986) and, therefore, has been identified with the aromatic part of the phenyl-propane units of the lignins. In addition, in the C–H stretch region, the aromatic C–H stretch usually occurs in the frequency interval 3000–3150 cm^{-1}. A very weak feature at 3075 cm^{-1} is indeed observed in Figs. 3 and 6. In the spectra from the longitudinal sections the lignin bands at 1600 and 1650 cm^{-1} are sensitive to polarization (Figs. 3, 4). This implies preferential orientation of lignin at these points. Though attention has been focused on the 1600-cm^{-1} band, the 1650-cm^{-1} band shows a similar pattern of variation. In Fig. 3 the 3600-cm^{-1} band is stronger when the electric vector of the exciting beam is parallel to the cell-wall; this pattern was typical of the majority of the points investigated in the S_2 region. Figure 4 represents an exceptional ease wherein the preferential orientation was perpendicular to the cell wall. Experiments carried out on cross sections are consistent with the patterns for the longitudinal sections. The decline in relative intensities of the 1600-cm^{-1} band in the spectra with polarization perpendicular to the cell wall indicates that the aromatic rings are preferentially oriented in the plane of the cell wall surface.

The finding of preferential orientation should not be surprising, since there is considerable evidence that both cellulose (Frey-Wyssling 1976; Preston 1974) and hemicelluloses (Marchessault and Liang 1962; Page et al. 1976) in cell walls are rather highly organized. Although orientation of the aromatic rings in the plane of the cell wall is detected most often, spectra from some locations showed a lower degree of preferential orientation. In one or two exceptional cases the spectra showed a more intense 1600-cm^{-1} band in the perpendicular mode as in Fig. 4. This variability indicates the occurrence of nodes in the patterns of orientation of lignin.

Compositional variability. Figures 3–7 contain typical sets of spectra obtained from different locations in the secondary wall of *P. mariana* wood. In order to evaluate the total amount of cellulose or lignin present within a domain, the relative peak intensities in both modes (parallel and perpendicular) were added. This was necessary to ensure that any minor variations in the organization of the two constituents did not confuse the trends associated with the compositional variability.

Here again the skeletal mode at 1098 cm^{-1}, and the methine C–H stretches at about 2900 cm^{-1} were selected as indicators of the cellulose. It was observed that the sum of relative peak intensities for both the 1098- and the 2900-cm^{-1} features in different locations changes in a similar manner, as would have indeed been expected. Additional evidence was derived from the studies on transverse sections of spruce. Because of the lower intensity in the 1098-cm^{-1} peak in these spectra, the data associated with the 2900-cm^{-1} peak were preferred.

Only the strong lignin band at 1600 cm^{-1} was used in the comparisons with the intensity of the cellulose bands. These comparisions demonstrate substantial variation in the lignin content in the secondary wall.

In order to test whether or not the variability in composition was of a similar magnitude around an individual cell as between cells, spectra were recorded at different points in the cross section of a single cell. Although the ratio of the 1600-cm^{-1} band intensity to that of the 2900-cm^{-1} band varied by a factor of two, the range of variation was less than that between adjacent cells.

Results in summary. The spectral studies carried out on the cell walls with the help of the Raman microprobe provide information concerning structural organization at the molecular level. Moreover, the microprobe can be used to explore the distribution of lignin and cellulose within the walls. Preliminary data indicate that both cellulose and lignin are oriented. The chains of cellulose are oriented in planes perpendicular to the cross section, and the orientation of lignin is such that in most instances the aromatic rings are parallel to the plane of the cell-wall surface. Further, the spectral evidence indicates variation in the relative amounts of cellulose and lignin. It also indicates the presence of varying amounts of these components in different locations of the secondary wall. However, more information is needed from a wide variety of morphological regions of plant tissue in order to establish the patterns of organization of both cellulose and lignin, as well as their interrelationships in the cell wall.

Discussion

Given the complexity of cell-wall morphology, it would have been surprising indeed if we had not observed evidence of molecular organization. The evidence for the orientation of cellulose was not unexpected, for although we believe ours to be the first direct evidence of molecular orientation of cellulose in the walls of woody plants, it has been recognized for many decades that cellulose is highly organized in the architecture of seed hairs and bast fibers.

Nor should it be surprising that evidence of orientation of the phenyl rings of lignin was detected, nor that the pattern of orientation is not uniform through out the cell walls. For, given that the polysaccharides of the cell wall are highly organized, it would indeed seem unlikely that the organization of the lignin component would be random.

The problem which lies ahead is an understanding of the type of order and organization which prevail in native plant tissue. When the organization of cell walls is characterized within the conceptual framework available based on traditional chemical methods, the categories which usually come to mind are those of crystallinity and of amorphous character. Yet much of the organization in cell-walls does not lend itself well to description in terms of the simple translational symmetries of crystallography. Nor, however, is it homogeneously disordered, which is a characteristic usually implicitly attributed to amorphous structures. It is our hope that the methodology we have developed on the basis of Raman microprobe spectroscopy will enable us not only to detect organization at the molecular level, but also to develop the data base necessary to define the categories of order which prevail in the cell wall.

The question also arises whether the methodology we have developed is applicable to investigation of components of cell walls other than those upon which we have focused our attention in the present work. Additional components of interest would be the hemicelluloses, the pectins, and perhaps proteins. Our experience with other polysaccharides indicates that the skeletal motions of most of the hemicelluloses will result in fairly broad bands, unless they are so organized that they have a high degree of repetitive order. Functional groups that result in highly localized modes may, however, result in the distinctive spectral features

that can he used to derive organizational information. Thus the carboxyl groups of pectic substances and some of the hemicelluloses could he investigated. Most proteins also have some localized modes that can serve as the distinguishing feature.

Application of the methodology to other tissue types is another logical extension of our work. Even at the present limit of resolution of about 1 μm it is possible to develop new information on tissue of more complex architecture. Development of a microprobe system with multichannel detection will greatly enhance the facility with which the methodology can be applied.

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