Study of Lignin in Forages and Wood by ^{13}C CP/MAS NMR. 1. Some Evidence of Polymerization and Depolymerization

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A detailed investigation of the lignin resonance between 140 and 160 ppm by ^{13}C CP/MAS NMR on aspen wood and forage has been made. The spectra show that the breakdown of lignin as indicated by changes in the resonance in this region closely parallels changes in solubility and other properties of wood and forages. These changes also are related to digestive processes in ruminants. Changes in the spectra of timothy in this region as it matures indicate a concurrent change in the composition and molecular weight.

In the first complete experiments describing the NMR of solids by the CP/MAS technique (Schaefer and Stejskal, 1976), there was included a spectrum of wood as an example of a naturally occurring mixture of polymers, namely lignin and various carbohydrates such as cellulose. A large number of subsequent papers have described various aspects of the spectra of substances containing not only lignin and carbohydrates, as in the case of wood (Maciel et al., 1981; Schaefer et al., 1980; Kolodziejski et al., 1982; Haw et al., 1984b), but also forages containing variable amounts of protein as well (Himmelsbach et al., 1983; Elofson et al., 1984). In the latter study spectra were presented to show that the lignin content increased and the protein content decreased as a function of the maturity of the forage. To a first approximation, it has been found that the lignin content can be estimated by the size of the resonances between 140 and 160 ppm (Kolodziejski et al., 1982). On the basis of solution NMR studies (Ludemann and Nimz, 1973), the resonances in this region correspond to aromatic carbons substituted with OH or OR. These carbon atoms are unique to lignin among the main constituents of extractive free wood or forages. The only possible exception appears to be the carbon of the OH-substituted aromatic carbon of tyrosine, one of the amino acids making up the polypeptide chain of proteins, absent in wood but present in forages. Workers in the field agree that in forages with normal protein content, thus excepting seedling (>40% protein), tyrosine would not make a contribution above the noise level of the NMR spectrum (Himmelsbach et al., 1983).

The chemical structure of lignin has been under investigation for more than 100 years (Sarkanen and Ludwig, 1971). The first experiments showing clearly that lignin was a polymer of phenylpropane units were those of Harris and Adkins (1938), who showed that high-pressure catalytic hydrogenation produced reduction products derived from coumaryl, coniferyl, and sinapyl alcohols. Subsequent work has shown that these alcohols are apparently polymerized in a great number of ways as judged by the isolation of different fragments as the polymer is broken down (Ludemann and Nimz, 1973). In recent times, a computerized version of the structure of lignin has been proposed (Nimz, 1974), but no experimentally determined structure can be drawn at the present time. One difficulty is the probability that native lignin undergoes rearrangements and/or condensations under even the mildest conditions of isolation. One of the most important bonds holding the phenylpropane units together in lignin is the C_β–O–C4 linkage shown in Figure 1 (Sarkanen and Ludwig, 1971). These bonds are readily attacked by all the major delignification processes including sulfite, alkaline sulfide, and soda. A second important and recurring bond is the C_α–O–C4. This bond, since it is a benzyl ether, is cleaved as well by fairly mild acid treatment. In both cases, the result is the production of aromatic C4-OH groups substituted with methoxyl groups on the C3- and/or C5-carbons and to a small degree unsubstituted because of the occurrence of a few coumaryl units. From the work of Ludemann and Nimz it appears to be safe to conclude that the cleavage of these bonds should result in a shift of the NMR resonance from 150–155 to about 145–150 ppm. Based on this point, a decrease in the intensity of the resonance centered ca. 153 ppm together with an increase in intensity of the resonance centered ca. 147 ppm should indicate depolymerization of the lignin with an increase in solubility. Conversely, a decrease in the intensity of the high-field peak (ca. 147 ppm) in favor of an increase in the resonance in the low-field peak (ca. 153 ppm) should indicate an increase in molecular weight or cross-linking.

In 1984, Haw, Maciel, and Biermann (1984a) were able to show that the first part of this hypothesis was valid. They showed, by the application of CP/MAS technique to solid wood, that a sample of red oak wood submitted to increasng severity of steam temperature and pressure showed a clear and progressive shift in the intensity of the peak ca. 153 ppm to that ca. 147 ppm. They correctly attributed this change mainly to the cleavage of C_β–O–C4 and C_α–O–C4 bonds and the breakdown or depolymerization of the lignin. We propose to show in this paper the extension of both of these important hypotheses to the study of both wood and forages.

RESULTS AND DISCUSSION

In Figure 2 are shown some spectra of aspen wood and lignins derived from the wood by different procedures. Figure 2a is a typical spectrum of aspen wood. In the region 140–160 ppm, little, if any, high-field shoulder at 147 ppm can be seen. The resonance at 130 ppm is largely due to aromatic carbons substituted with hydrogen or other carbon atoms. The peak at 56 ppm is due to the carbon of methoxyl groups of the lignin. The resonances between 70 and 110 ppm are dominated by carbon of the carbohydrate complex. The resonance at 22 ppm and some of the resonance at 175 ppm are due to CH_3 and CO groups, respectively, of acetyl esters associated with hem-

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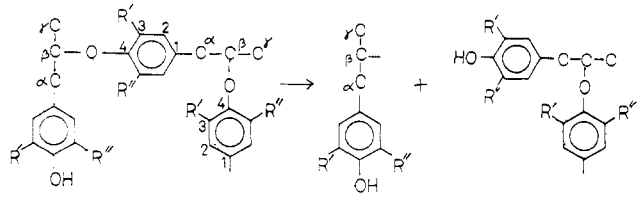

Figure 1. Basic structure of lignin.

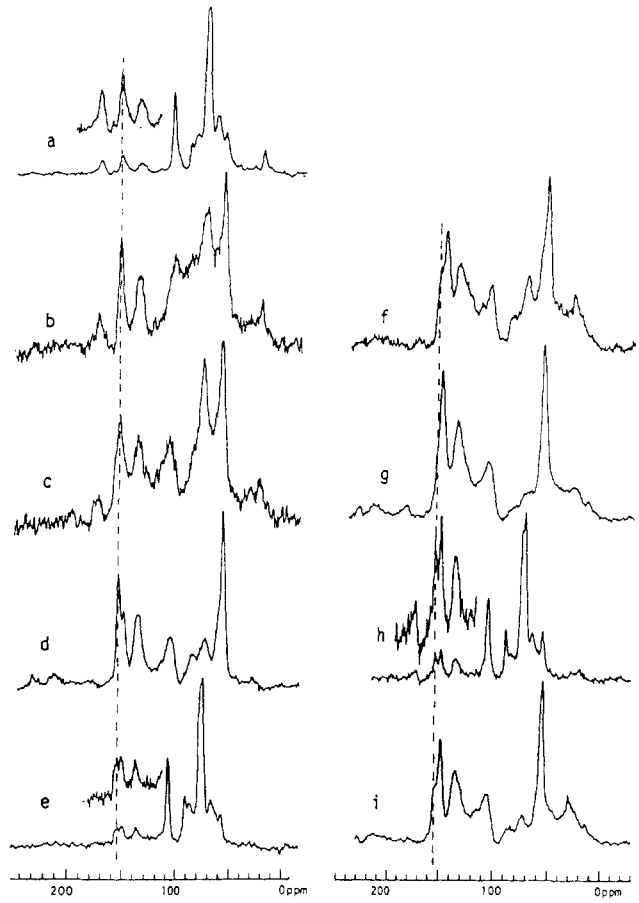

Figure 2. ^{13}C CP/MAS spectra of materials from aspen poplar: (a) untreated sawdust, (b) periodate lignin, (c) milled wood lignin, (d) dioxane lignin, (e) sawdust cooked in dilute H_2SO_4, (f) Klason lignin, (g) kraft lignin, (h) steam-exploded wood, (i) lignin extracted in alcohol from steam-exploded wood.

icellulose. Parts b and c of Figure 2 are spectra of a periodate lignin and milled wood lignin, both prepared from aspen, respectively. No shoulder at 147 ppm is apparent in either spectrum. In the case of the milled wood lignin the absence of the peak at 147 ppm is due to the fact that few if any C4-OH groups are released in the preparation of this lignin. In the case of the periodate lignin the absence of free phenolic groups correlates with the fact that the periodate procedure oxidizes any aryl hydroxyl groups with adjacent methoxyl groups to *o*-quinones (Adler and Hernestam, 1955) with subsequent destruction of the rings. A considerable amount of carbohydrate has remained with the lignin as judged by the peaks ca. 78 and ca. 105 ppm. Figure 2d is a spectrum of dioxane lignin. The presence of a peak at 147 ppm is clearly visible probably because the procedure to isolate this lignin exposes the wood to dilute HCl. Possibly the main cleavage has involved C_α–O–C4 bonds, which would be subject to hydrolysis by acid as previously mentioned. Figure 2e is a spectrum of aspen sawdust cooked in 1% sulfuric acid. Here, the intensity of the 147 ppm peak has approached that of the peak at 153 ppm. Possibly more C_α–O–C4 bonds than C_β–O–C4 bonds have been broken to produce phenolic OH groups under this still mildly acidic condition. Figure 2f is a spectrum of Klason lignin from aspen wood. Since this preparation involved treatment with 72% sulfuric acid followed by cooking in 3% sulfuric acid under 2 atm pressure, it is not surprising that the peak at 147 ppm has become dominant. Despite the apparent breaking of nearly all the C_β–O–C4 and C_α–O–C4 bonds, this lignin remains insoluble. This is widely believed to be due to acid-catalyzed condensations to produce acid-resistant C–C and other bonds. Therefore, little change in apparent molecular weight or solubility compared to native lignin has occurred despite the separation from the carbohydrate complex. Closer inspection of the resonance between 110 and 140 ppm reveals a broadening and intensification in this region compared with the previous isolated lignin spectra. This is indicative of the formation of additional unsaturated and aromatic C–C bonds. Previous workers (Kolodziejski et al., 1982) isolated lignin from lodgepole pine by different methods. They observed similar changes in Klason lignin, but no comment was made on the patterns of the peaks between 140 and 160 ppm. Close inspection of the spectra displayed in their paper does indicate that similar results were probably obtained despite the small scale of the reproduced spectra. Pine wood is known to contain little if any syringyl units in its lignin whereas hardwoods contain large amounts. Instead, the soft woods contain relatively large amounts of guaiacyl units. In the latter groups the hydrolysis of the C–O–C4 bonds involves a less significant change from 150 to 147 ppm in the chemical shift (Ludemann and Nimz, 1973). This probably accounted for the failure of the authors to comment on this matter. The spectrum of kraft lignin from aspen in Figure 2g shows only a single peak in the region of interest, and that is at 147 ppm. This accords with the known fact (Sarkanen and Ludwig, 1971, pp 654–657) that, in the kraft process, sulfide, present in the alkaline media, breaks virtually all the C_α–O–C4 and C_β–O–C4 bonds and the result is a highly phenolic and soluble (in such organic solvents as dimethyl sulfoxide) lignin. That steam alone may sever some of these bonds is shown by spectra h and i. In Figure 2h the spectra of a steam-exploded aspen wood show that the resonance in question is equally divided between the high-field and low-field sides of 150 ppm. Figure 2i is a spectrum of the lignin extracted from the above wood with ethanol and shows that almost all the resonance occurs at field higher than 150 ppm. This is NMR evidence of depolymerization. Dilute acetic acid is released by hydrolysis of acetyl groups previously mentioned. This acid may play a role in the depolymerization. These results agree with the findings of Marchessault and co-workers (1982) with aspen wood and of Haw et al. (1984a) with red oak wood although the roles of the acetic acid may have been greater for the steam explosion process. One of the reviewers pointed out apparent variations in the line width of the peak at 153 ppm (see Figure 2c,e). This may be due to creation of slightly different type of C–O bond or of different physical states. However, the signal to noise ratios of this region of the spectra were too low to make any reliable inferences.

Alkali alone can cause some destruction of the ether bonds in question. Figure 3a is a spectrum of a reasonably mature barley straw with a low protein content as indicated by resonances at 170–175 and 0–50 ppm (Elofson et al., 1984). This straw was then boiled for 1 h in 5% NaOH in water. The spectrum of the insoluble residue is shown in Figure 3b. It can be seen that the acetyl groups have disappeared completely due to saponification. Only a trace remains of the lignin peak at 140–160 ppm, and it is clearly

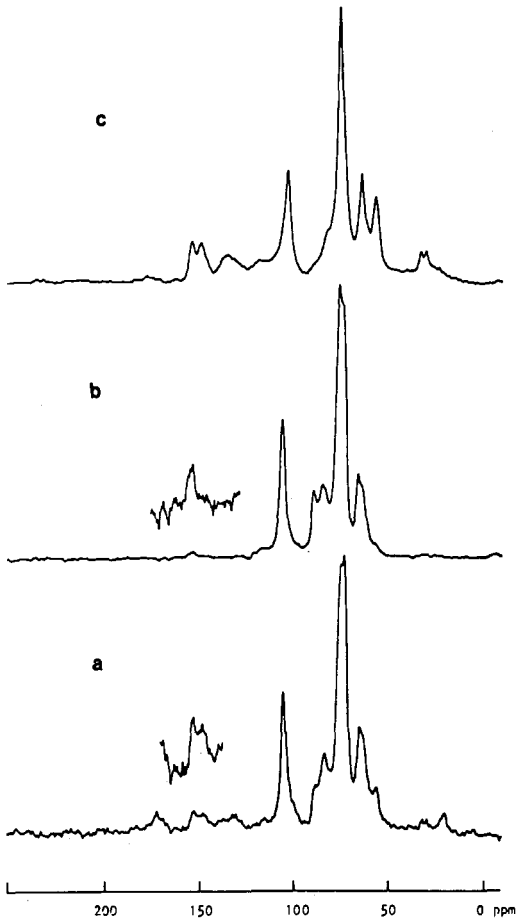

Figure 3. ^{13}C CP/MAS spectra of barley straw: (a) mature barley straw, (b) insoluble residue from barley straw boiled in 5% NaOH, (c) acid precipitate of 5% NaOH extracts from barley straw.

ca. 153 ppm. The remainder of the spectrum corresponds to a mixture of amorphous and crystalline cellulose (Atalla et al., 1980; Earl and VanderHart, 1980). The spectrum of the acid precipitate of the alkaline extract is shown in Figure 3c, corresponding to a mixture of hemicellulose and lignin (Kolodziejski et al., 1982). The presence of some insoluble peptides or protein may be indicated by the persistence of the resonance at field higher than 50 ppm. The well-defined peak at 56 ppm is mainly due to methoxyl groups in the lignin but may be in part due to small amounts of the α-carbons of still insoluble peptides (Himmelsbach et al., 1983). Two peaks at 147 and 153 ppm are visible, but the ratio of the peaks (147/153 ppm) is lower than that for the previously shown soluble lignins (Figure 2g,i). This indicates that several phenylpropane units remain chemically attached to each other through C–O–C4 bonds and the solubility depends on the reaction of alkali with the lignin. There was a weight loss of approximately 20% in this experiment. Some very low molecular weight fragments of the lignin soluble in the acidified solution as well as soluble peptides and amino acids may have failed to precipitate. This, together with some soluble sugars, presumably would account for the weight loss. Presumably the acid-soluble lignin would have its resonance at 147 ppm in the region of interest.

The other major process for delignifying wood is the sulfite process. In Figure 4 appear spectra of aspen wood exposed to SO_2 gas at 100 °C for 1 h (Figure 4a) and 20 h (Figure 4b), respectively, in sealed glass tubes. It is readily seen that the shoulder at 147 ppm has increased with longer exposure to SO_2 (compare with Figure 2a). These results are in qualitative agreement with results of

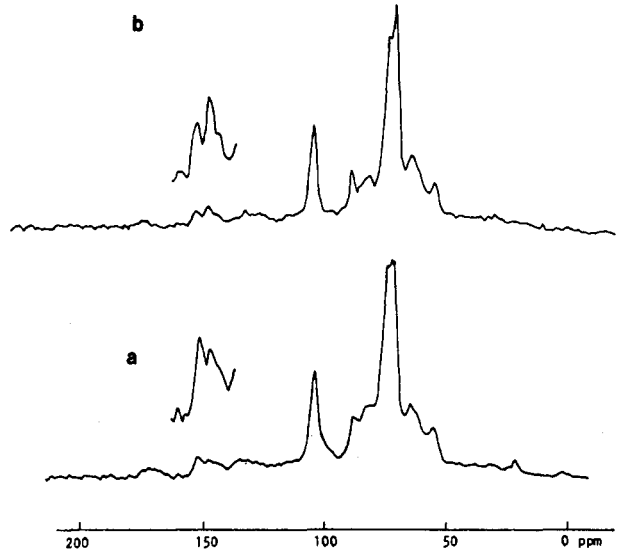

Figure 4. ^{13}C CP/MAS spectra of Aspen wood treated with SO_2 gas at 100 °C: (a) for 1 h, (b) for 20 h.

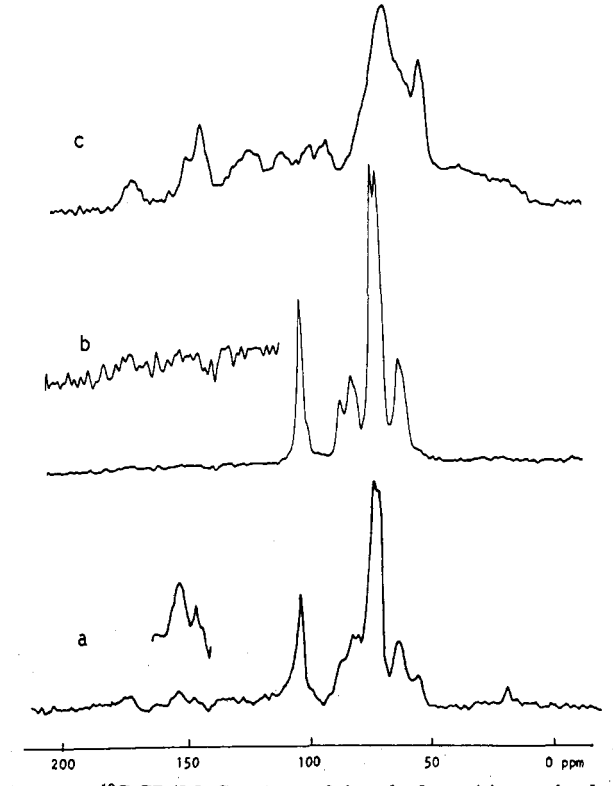

Figure 5. ^{13}C CP/MAS spectra of timothy hay: (a) overripe hay, (b) water-insoluble material after SO_2 gas treatment, (c) water-soluble material after SO_2 gas treatment.

Haw et al. (1984b) on sulfite pulps derived from loblolly pine. Similar results were observed when the above technique was applied to a mature forage (timothy). Figure 5a is a spectrum of an overripe sample of timothy hay. The dominant peak in the region of interest is at 153 ppm with possibly a shoulder at 147 ppm. Exposure to SO_2 gas for 1 h as before produced a fairly strong resonance at 147 ppm (spectrum not shown). A second sample, sealed under 1 atm SO_2 in glass inadvertently left for 5 years at room temperature, was examined as follows. Water was added to the opened tube, and the insoluble residue was filtered off and produced spectrum in Figure 5b. Only carbohydrate, apparently almost pure cellulose, can be identified. The water solution was evaporated, and the

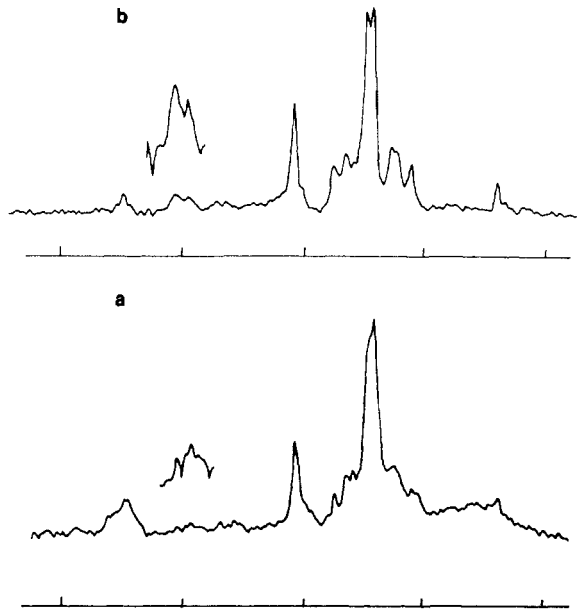

Figure 6. ^{13}C CP/MAS spectra of standard hay: (a) original, (b) fecal fiber after passage through steer.

residual solid produced the spectrum in Figure 5c. The resulting spectrum shows lignin, soluble sugar, sugar acids (72 and 174 ppm), and amino acids (0–50 and 174 ppm). Once again, the peak at 147 ppm dominates, but a shoulder still remains at 153 ppm, suggesting that not all the C_β–O–C4 and C_α–O–C4 bonds have been broken. It is well-known that treatment of low-quality forages with SO_2 enhances the feeding value (Ben-Ghedalia and Miron, 1983/1984). The later experiments done in this laboratory 5 years ago were originally prompted by the fact that a sample of oat silage treated with 1% SO_2 at the time of ensiling showed an enhanced peak ca. 147 ppm compared with an untreated sample of oat hay from the same field. Reflection on this latter result now suggests further investigation of the ensiling process because the lactic acid present in the warm silage might have hydrolyzed C–O–C4 bonds independent of the presence of SO_2.

The depolymerization of lignin by the many agents described in this paper does raise a problem of mechanism particularly for some of the mild chemical treatments. The chemical studies on lignin and model compounds have shown especially for alkaline sulfide (kraft) and sulfite processes that cleavage of the C_α–O–C4 and C_β–O–C4 bonds proceeds through rather complicated mechanisms involving quinone methides (Sarkanen and Ludwig, 1971, Chapters 15 and 16). For this mechanism to work it is postulated that the C4-carbon must have a free OH group rather than an OR or an OAr moiety attached to it. The question arises whether this indicates that depolymerization proceeds one phenylpropane unit at a time from the free end (C4-OH) of the polymer.

The peaks at 140–160 ppm seem to be of importance in the study of biological processes. In Figure 6a there is shown a spectrum of a standard hay (protein, 14.6%; NDF, 41.7%) that was fed to a steer. Figure 6b is the sample of fecal fiber after passage through the steer. It is apparent that the lignin content in the fical fiber is higher than in the original hay and the major peak is shifted to 153 from 147 ppm. While it is clear that the lignin has not been greatly degraded since it was still insoluble, it was apparent that all the carbohydrate except cellulose had been removed by the digestive processes (Elofson et al., 1984). It is possible that the change observed may be related in part to the breaking of C4–O–hemicellulose bonds. It is known

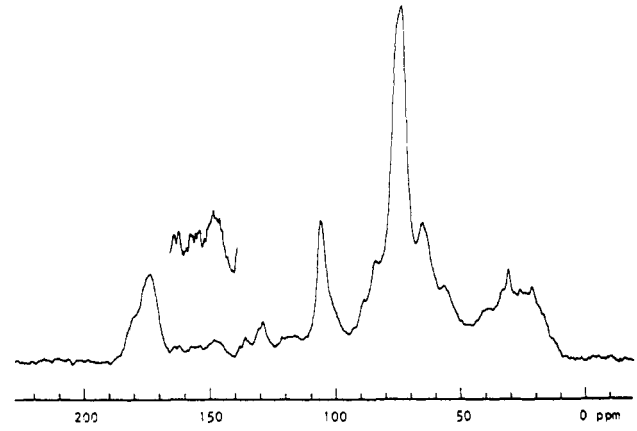

Figure 7. ^{13}C CP/MAS spectrum of young timothy hay.

that hemicelluloses are attached chemically to some extent to the lignin. This proposition seems to be supported by Kolodziejski and co-workers (1982) in their studies of enzymatic lignin and the lignin–carbohydrate complex (Sarkanen and Ludwig, 1971, pp 220–224).

Finally, in Figure 7 we show what is proving to be in our laboratory a very fruitful area for investigation in order to evaluate the nutritional value of forage. The spectrum is that of a green timothy hay with 13% protein. It shows that the peak ca. 147 ppm is actually stronger than that at ca. 153 ppm. This spectrum should be compared with that of mature timothy (only 2.8% protein) shown in Figure 5a. The nutritional value of this mature timothy is low not only because of the low protein content but also because of the low accessibility of the carbohydrate due to the increased lignin content. In this spectrum the peak at 153 ppm has become dominant. It is tempting to suggest not only that the lignin content has increased but also that its molecular weight has increased by cross-linking. This would support the well-known fact that digestibility of forages falls out of proportion to the increase in lignin content (Harkin, 1973). In the work described herein, lignin in the mature timothy depolymerized under SO_2 to such an extent as indicated by the change in ratio of the 147/153 ppm signals and the increase in solubility (Figure 5) as to indicate that this latter factor does not invalidate the first suggestion. In other words, the molecular weight or the amount of cross-linking in the lignin may be as important as its quantity in the measurement of the digestibility of a forage.

CONCLUSIONS

There can be little doubt from the work described here and by other workers in the field that ^{13}C CP/MAS NMR provides a unique opportunity to study lignin as it occurs in the natural state. As demonstrated in this paper, depolymerization and polymerization can be studied under the mildest and most severe chemical or biological condition. The study of Klason lignin is indicative of the possibility that the chemical structure of native lignin is less complicated than previously indicated. For wood the shape of the resonances in the 140–160 ppm range is quite clear. For forages more work is required to round out some of the suggestions outlined in this study. Detailed study of other resonances in these materials will undoubtedly yield very large dividends especially toward understanding the nutritional value of forages, a subject that is poorly understood (Ulyatt, 1973).

EXPERIMENTAL SECTION

^{13}C NMR spectra shown in Figures 5 and 6 were obtained on a Bruker CXP-180 at 45.3 MHz. All the other

spectra were obtained on a Bruker CXP-200 spectrometer at 50.3 MHz. A single, matched cross-polarization contact (Pines et al., 1973) of 2 ms was used with proton spin-temperature alternation (Stejskal and Schaefer, 1975). Several thousand free induction decays were collected over 1K memory and zero-filled to 8K before Fourier transformation. Andrew-type spinners made of boron nitride were spun ca. 4 kHz when Bruker probes were used, and cylindrical sapphire rotors with Kel-F caps were spun ca. 4–5 kHz when a Doty probe was used.

Periodate, milled wood, and dioxane lignins were prepared from aspen wood sawdust according to the procedures described by Browning (1967). Klason lignin was prepared by stirring 1 g of aspen wood sawdust in 10 mL of 72% H_2SO_4 for 2 h followed by dilution to 280 mL with H_2O and hydrolysis under 2 atm pressure for 1 h. Kraft lignin was precipitated from the pulping liquor (MacLeod and Cyr, 1983) with use of H_2SO_4. Steam explosion of aspen wood was done after the wood chips were held in steam at 612 psi for 50 s.

ACKNOWLEDGMENT

R.M.E. and G.W.M. are indebted to the Alberta Agricultural Research Council for support for some of this work. Thanks are extended to E. LeClair and J. Laidler for technical support.

Registry No. SO_2, 7446-09-5; lignin, 9005-53-2; Klason lignin, 8068-04-0; Kraft lignin, 8068-05-1.

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Received for review August 27, 1987. Accepted March 28, 1988.