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STUDIES ON LIGNIFICATION
IN WHEAT
(*TRITICUM AESTIVUM* VAR. THATCHER)

A thesis presented in partial
fulfilment of the requirements for the degree
of Master of Science in Botany at
Massey University

PATRICIA MARGARET LE GAL

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ABSTRACT

PART I

Transections of the stem of *Triticum* were examined after staining with dyes specific for functional groups within the lignin polymer. Anatomical observations suggest that the basis for the rapid increase in the lignin content of this plant 35 to 40 days after germination, is the differentiation of subepidermal sclerenchyma fibres in the stem at this time. The lignin formed in the fibre walls appears to have a higher methoxyl content than the lignin of the xylem vessels. A comparison of the development of lignification with stem elongation and flowering was made and the interrelationship of these processes discussed.

PART II

The role of p-hydroxyphenyllactic acid in lignification in wheat was investigated. ^{14}C-labelled tyrosine, p-hydroxyphenyllactic acid (HPLA), and ^{3}H-labelled HPLA were administered separately to the cut ends of shoots of *Triticum* and the incorporation of label into ethanol-soluble and ethanol-insoluble ferulic (and in some cases only, p-hydroxycinnamic) acid was measured. On the basis of the pattern of incorporation of label from the ^{14}C-tyrosine, experiments were carried out to determine the route by which HPLA is converted to lignin precursors. A failure to detect label from ^{3}H-HPLA in the cinnamic acids suggests that HPLA is not dehydrated directly to p-hydroxycinnamic acid and is not of regulatory significance in lignification in either 10 or 40 day-old wheat plants.

PART III

Information from several levels of organization within the plant is drawn together and discussed. Suggestions for further work investigating the controlling factors in lignification are included.

ACKNOWLEDGMENTS

The help, advice and support during the preparation of this thesis from many of my friends has been invaluable to me, and I am especially grateful to my supervisor Dr A.D.M. Glass and to Professor R.G. Thomas for their assistance and willingness to discuss my work with me at any time. Finally, thankyou Jessica, you have been perhaps the most enthusiastic helper of all.

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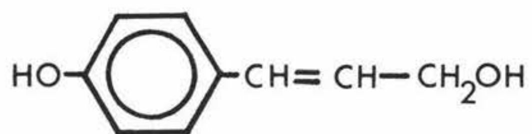
INTRODUCTION

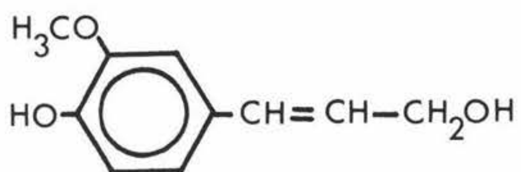
Lignin, the end product of the cellular process of lignification is, after cellulose, the second most abundant natural polymer (13). Over forty million tonnes being produced as a by-product of the pulp industry in 1968 alone (110). The problem of lignin waste and the resulting pollution has added impetus to research and the properties of lignin from economically important conifers are well known (131). The lignins of other plant groups are not as clearly defined and this is true of grass lignins in particular (14, 149).

The ability to synthesize lignin is restricted to the vascularized land plants (156) and it is likely that the properties of lignin and the ligno-cellulose complex enabled a more effective colonization of the land (5, 45). Tissues specialised for the transport of metabolites and for support contain most of the lignin although virtually any cell, except those of a meristem, may be lignified to a greater or lesser degree (159, 41, 160). As well as altering the permeability of the cell wall, adding to the compressive and, under certain conditions, the tensile strength of plant tissues (138, 46, 104, 132), lignification increases the resistance of the plant to microorganisms by welding the cells together and impeding the penetration of degradative enzymes (48, 132). The wounding of tissues for example induces the rapid synthesis of lignin precursors (39) and lignifying cells (87, 155, 43). Pathogenically infected tissue responds in a similar way (47, 48).

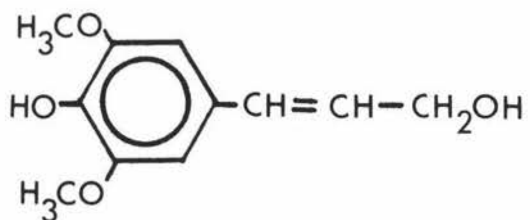
The complexity and variability of the lignin polymers makes the formulation of an accurate, inclusive definition difficult. A recent version of the structure of *Fagus silvatica* (beech) lignin (110) shows the polymer as being made up of three basic aromatic units (see Figure 1)



p-hydroxycinnamyl alcohol



coniferyl alcohol



sinapyl alcohol

FIGURE 1. The structure of lignin monomers.

trans *p*-hydroxycinnamyl alcohol

trans coniferyl alcohol

trans sinapyl alcohol

All three components may or may not be present in any one plant or even tissue simultaneously (145, 69, 131). Information published on the nature of lignin indicates that there are two main types (28, 131). *Guaiacyl* lignin contains almost exclusively coniferyl alcohol subunits and is found in the Gymnospermae, Pteridophyta and Cycadales, and *guaiacyl-syringyl* lignin contains both sinapyl and coniferyl alcohol subunits and is found in the Angiospermae and the Gnetales (31, 18). The Gramineae, of which wheat is a member, are unusual in that they contain substantial amounts of p-hydroxycinnamyl alcohol subunits (29, 145, 179).

After more than a hundred years of research into lignin formation[1] the details of biosynthesis are almost completely known (130). Recent enzymological studies made by M.H. Zenk and his co-workers (173) rival the early polymerization studies of Karl Freudenberg (45) and the later isotope incorporation studies of S.A. Brown, A.C. Neish and co-workers (13) as major contributions to our understanding of lignification. The regulation of the synthesis and deposition of lignin as it occurs in wheat is the primary concern of this study. Anatomical and biosynthetic information relevant to this problem is presented and the observations made are related to the growth and development of the whole plant.

During the development of the wheat plant, *Triticum aestivum* there is a period of rapid lignification that is initiated some 35 to 45 days (depending on the environmental conditions)

1 Lignin was first studied seriously by the French chemist and botanist Anselme Payen in 1838 (quoted by F.F. Nord and G. de Stevens, 1958 (111)).

after germination. This was first observed by M. Phillips _et al._ in 1931 (116) and later by J.E. Stone _et al._ in 1951 (154). Any major event regulating the formation and the deposition of lignin in the cell wall could reasonably be expected to occur at, or immediately prior to, this time.

Since the introduction of radioactive isotopes in the late 1940's, wheat has emerged as a popular plant for biosynthetic tracer studies, as it is easily grown and manipulated. However, apart from studies of embryo and early seedling anatomy with regard to vascular trace arrangement (4, 96, 11) and brief descriptions of the mature stem (40), little information is available on the developmental anatomy of the wheat plant through the time of rapid lignification. A detailed examination of the development of lignified tissue in the stem before, during, and after this time is made in Part I of this study. The information obtained by anatomical observation is extended and supported by lignin analyses of specific tissues. The relationship of lignification to other developmental processes in the plant is also explored. Information yielded from such investigations should prove invaluable in understanding the controlling factors in lignification, as these are likely to vary in different tissues and even in the same tissue at different stages of development.

The enzymes postulated by many to play the major role in controlling lignification, L-phenylalanine ammonia-lyase (PAL), and L-tyrosine ammonia-lyase (TAL) (grasses only), are highest in activity when the wheat plant is 7 to 10 days old (see Figure 2) (171). The lag between the time of maximum ammonia-lyase activity and the time of rapid lignification does not support the contention that these enzymes are alone responsible for controlling the biosynthesis of lignin. Before the discovery of PAL (92) and TAL

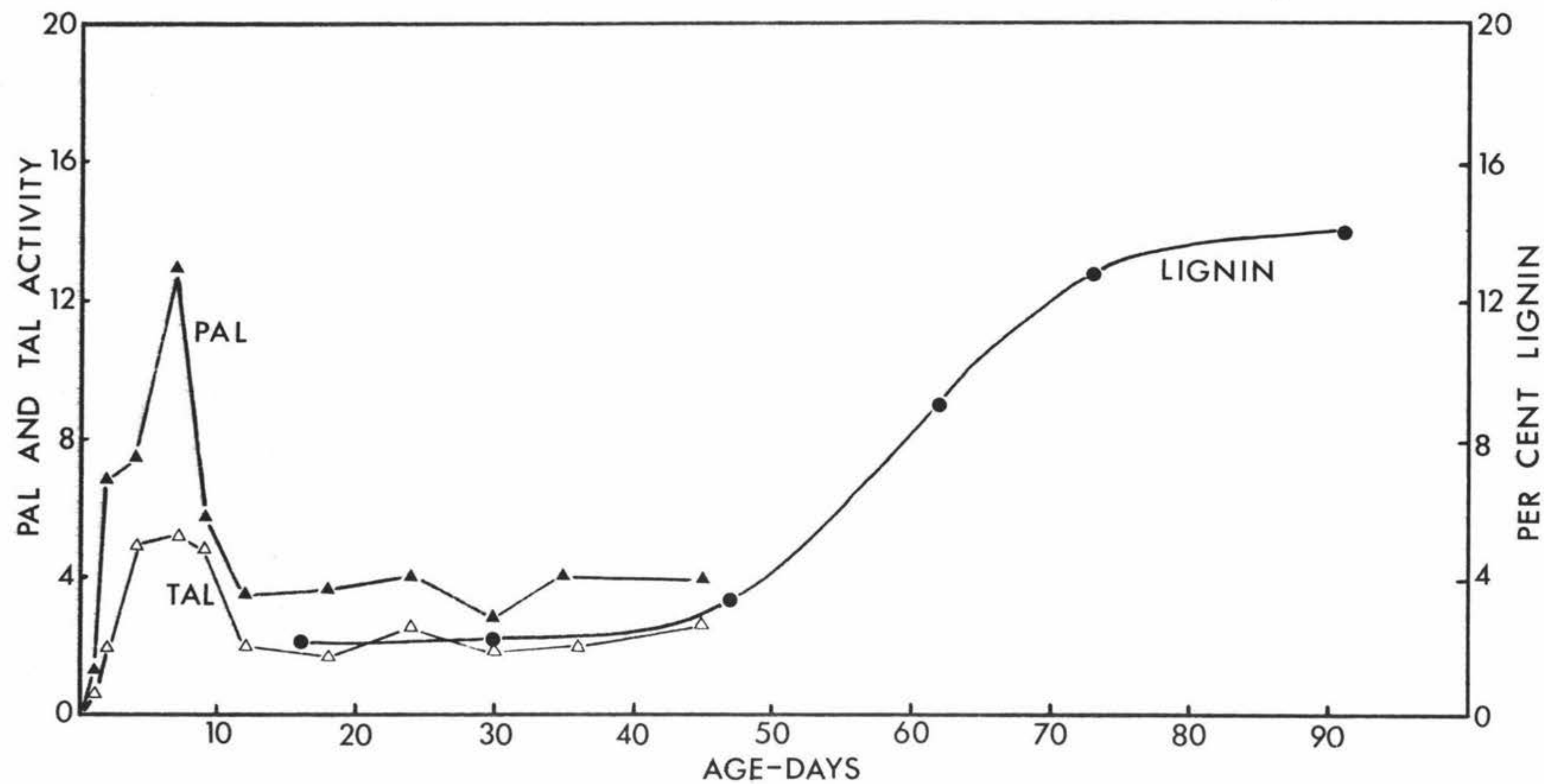


FIGURE 2. **Ammonia-lyase activity and the lignin content of wheat expressed as function of time. (From M. Young, 1966 and J.E. Stone** *et al,* **1951)**

PAL expressed as % turnover/0.2g acetone powder
TAL ''
LIGNIN expressed as % dry wt.

(109), the incorporation of phenylalanine and tyrosine into lignin was believed to take place via the phenylpyruvate and phenyllactate derivatives as shown in Figure 3 (167, 20). As all biosynthetic tracer work has so far been with ^{14}C-labelled precursors, the possibility that the phenyllactate to cinnamate and/or the hydroxyphenyllactate to hydroxycinnamate conversions exist has never been eliminated (82). Part II of this project is a time study investigating the role of hydroxyphenyllactate in lignification in wheat. The dehydration of this compound to p-hydroxycinnamic acid, if shown to take place, would be the third step in a series of reactions providing an alternative pathway to the deamination reaction; this may be significant in regulating biosynthesis, especially during the later stages of development when levels of the ammonia-lyases are known to be low.

Both anatomical and biochemical approaches to the problem of the control of lignification are necessary in order to realistically evaluate the information obtained from each. The theme of this study is reflected in the following quotation from the book "The Control of Growth and Differentiation in Plants". by P.F. Wareing and I.D.J. Phillips (163):

"Unless we attempt to relate the two approaches to each other, morphological and anatomical accounts of growth and differentiation must remain largely descriptive in nature, whereas our aim should clearly be to understand the processes underlying and controlling the structural changes. Conversely, physiological and biochemical studies which are not related back to developmental processes in the plant are liable to lose relevance and biological significance."

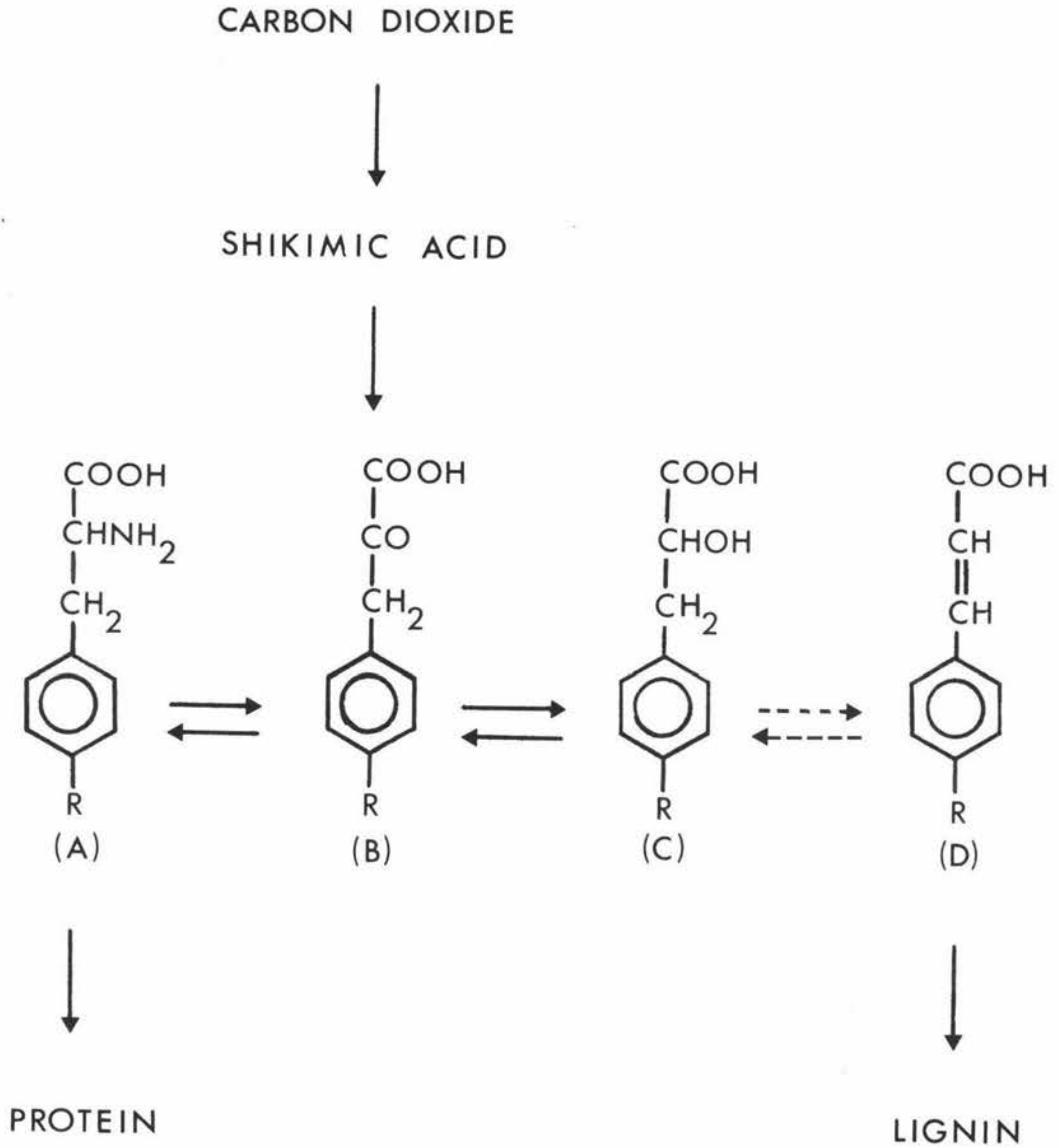


FIGURE 3. The pathway of lignification in wheat as proposed by D. Wright, S.A. Brown and A.C. Neish, 1958.

(A) R=H, phenylalanine; R=OH, tyrosine
(B) R=H, phenylpyruvate; R=OH, hydroxyphenylpyruvate
(C) R=H, phenyllactate; R=OH, hydroxyphenyllactate
(D) R=H, cinnamate; R=OH, hydroxycinnamate

PART I ANATOMICAL ASPECTS OF LIGNIFICATION IN WHEAT

I.1 INTRODUCTION

In the following study the pattern of lignification observed in the whole wheat plant by Stone et al in 1951 (154) is related to the anatomical changes within the wheat stem and the associated local changes in lignin content. Additional measurements of shoot dry weight and stem and internode length were made in an attempt to relate lignification to other developmental processes in the plant. The tissue arrangement within the stem of Triticum and similar monocotyledons is outlined in Section I.2 and provides information necessary to an understanding of the histochemical observations made. Also, an appreciation of the usefulness and an awareness of the shortcomings of the techniques used to locate, extract and measure lignin are critical to an evaluation of the results and a discussion of these precedes the experimental section.

I.2 THE ANATOMY OF THE WHEAT STEM

The basal portion of the immature wheat stem has been extensively studied (4, 96, 11) and there is some debate as to what the stem regions should be called. The first internode is recognized by L. Boyd and G.S. Avery (11) as being that region of the stem between the cotyledonary node and the point of divergence of the coleoptile (see Fig. 1 Ref. 11). The first internode does not elongate at all during the life of the plant. The second internode is immediately above the coleoptilar node and comparisons between plants of different ages in this study are based upon transections of this region variously stained for lignin. According to M.A. McCall (96), who has examined wheat seedling anatomy in detail, the internode immediately above the coleoptilar node is the third internode. The terminology used by Boyd and Avery in 1936 to describe the various parts of the stem is favoured in this study.

The vascular organization in the young stem is transitional between that of the root and that of the stem after elongation has occurred. The vascular bundles are not at this stage enclosed within bundle-sheath fibres.

The tissue arrangement within the mature Triticum stem (i.e. older than 40 days) is considered typical of a group of monocotyledons including, for example, Avena (oat), Hordeum (barley), Secale (rye) and Oryza (rice), where the vascular bundles are in two circles viewed in transection (41). Figure 4A is a diagram of the mature Triticum stem. The inner-circle vascular bundles are large and surrounded by bundle-sheath fibres and large thin-walled parenchyma cells, while the outer bundles are small, sometimes composed entirely of fibres, and embedded in a continuous ring of fibrous tissue (72, 41). An inner-circle vascular bundle is illustrated in Figure 4B. The transition from exarch to endarch xylem arrangement may be observed in some of the vascular strands of the inner bundles of the second internode (72). Immediately inside the epidermis of the lower internodes chlorenchyma may be present but in the upper internodes sclerenchyma fibres often extend to the epidermis. The stem is hollow along the length of the internode and solid at the node. Stem elongation takes place by means of an intercalary meristem at the base of the internode and the ammount of lignin in this region is less than that elsewhere (41).

The terminology describing those tissues that typically lignify has been developed for dicotyledons and gymnosperms and is confusing when applied to monocotyledons. Xylary fibres (those particularly associated with the xylem) and extraxylary fibres (cortical, perivascular and phloem fibres) are difficult to separately identify within the "cortically" positioned sclerenchyma of the wheat stem that contains both xylem and phloem. Bundle-

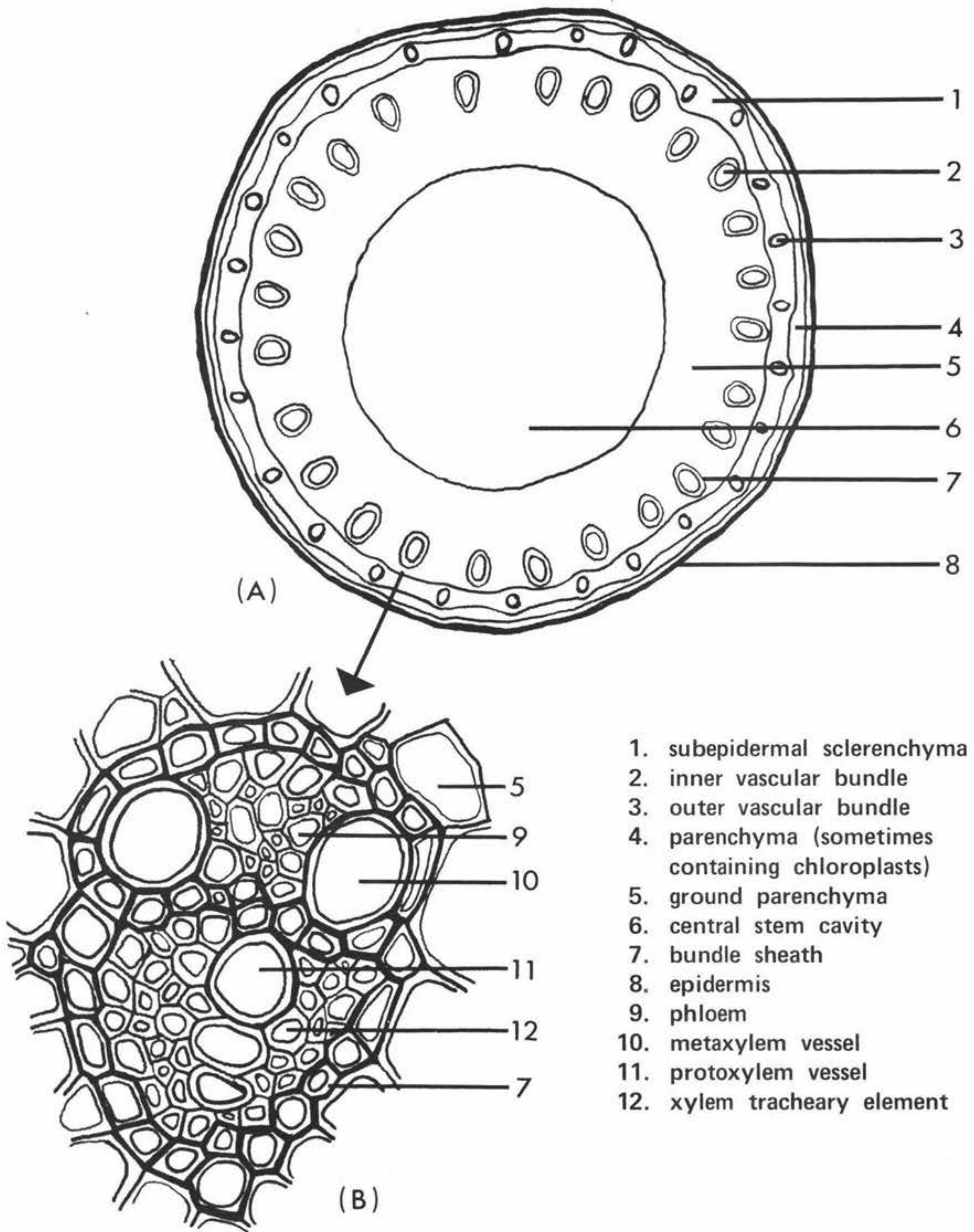


FIGURE 4. **Stem of *Triticum***

(A) Diagram of a transection of a mature internode

(B) Drawing of a transection of an inner vascular bundle.

sheath fibres are not easily classified as they may arise partly from the procambial vascular strands and partly from the ground parenchyma (141, 41), qualifying in the first instance as xylary and in the second as extraxylary fibres. Within the vascular bundle the protoxylem and metaxylem elements develop lignified secondary walls, while sieve and companion cells are not generally thought to lignify (41). There has, however, been a recent report from J. Kuo and T.P. O'Brien, 1974 (94) on the presence of lignified, functional (i.e. containing mitochondria and endoplasmic reticulum) sieve elements in Triticum. Tissues and cells are referred to in the text as they are labelled in Figures 4A and 4B.

The course of lignification in the monocotyledonous grasses has been characterized for Hordeum (117), Avena (118) and Phyllostachys (bamboo) (77) and in each case is similar to the pattern observed for Triticum (116, 154). Lignin and methoxyl content (which is an indication of the type of lignin present) were shown to be low initially, to increase rapidly at a time immediately before flowering and to reach a constant level when the plant was fully mature in Hordeum, Avena and Triticum. In Phyllostachys where sections along the length of the stem were used, the most basal section had the highest lignin and methoxyl content and the stem apical region the lowest. The stems of Triticum, Avena and Hordeum are known to be similar anatomically to Secale, Phleum (timothy grass) (145) and a number of others (120), all members of the Gramineae, that develop with increasing maturity an intensively lignified cylinder of sclerenchyma tissue. The distribution of lignin within the tissues of Phyllostachys (76, 77), Phleum (145), and Stipa, Festuca, Bromus and Agropyron (120) has been investigated using stains known to be specific for functional groups within lignin (see Section I.3.1). Xylem vessels become lignified early in

development, as shown by phloroglucinol-hydrochloric acid staining of transections in all the grasses studied, and in Phyllostachys early xylem vessels also stained red with Mäule's reagent indicating the presence of syringyl groups. Sclerenchyma, where present in older tissue was shown to contain varying amounts of lignin, depending upon the species of grass studied. In one case (120), phloroglucinol-HCl was used as the sole indicator for lignin and the results must be interpreted cautiously as this stain is specific for coniferaldehyde groups only.

As a general trend lignin formed early in development is of the guaiacyl type, in accordance with a positive reaction to the Wiesner (phl/HCl) test and a negative reaction to the Mäule test, and lignification appears to be limited to the xylem and some sclerenchyma fibres (131). Mature grasses may contain large quantities of syringyl as well as guaiacyl lignin and this is preferentially deposited in supportive fibrous tissue and, finally, in the walls of the ground parenchyma (76). Recent work by K.E. Wolter et al, 1974 (166) explores the possibility that the composition of lignin may alter depending upon the type of cell lignified. In Populus tremuloides (aspen) tissue cultures containing only mature vessels and undifferentiated parenchymatous cells, guaiacyl lignin only (as determined by infrared spectroscopy and degradation studies as well as the traditional histochemical tests for lignin), was found to be present. Cell and tissue-type differences in lignin are directly relevant to studies attempting to define the details of the regulation of lignin biosynthesis.

I.3 THE LOCATION, EXTRACTION AND MEASUREMENT OF LIGNIN

I.3.1 THE COLOUR REACTIONS OF LIGNIN

The presence of lignin may be detected by the treatment of plant tissue with various reagents that undergo colour reactions with specific groups within the lignin. The most common of these reagents is a phloroglucinol and hydrochloric acid mixture that associates with cinnamaldehyde units specifically and colours lignin a bright red-purple to red-orange (30, 113, 111). Figure 5 shows the reaction and chromogen thought to be responsible for the development of the colour. Pure coniferyl alcohol which does not colour upon treatment with phloroglucinol and hydrochloric acid (phl/HCl) is known to do so after mild oxidation (111). The colour fades with time (3 to 4 hours) and tissue sections stained with phl/HCl cannot be mounted permanently (90).

Lignins containing significant amounts of syringyl groups may not react positively to phl/HCl treatment and these may be detected by the Mäule, and Cross and Beaven reactions. The first of these involves treatment of the tissue successively with dilute aqueous permanganate, hydrochloric acid and ammonia and in the Cross and Beaven reaction, plant materials are chlorinated with saturated acidified calcium hypochlorite and then placed in a 1% sodium sulphite solution. Both tests are thought to have a similar chemical basis and appear to be specific for syringyl units (30, 111). The reaction is illustrated in Figure 6. Lignin containing a large number of syringyl units, such as some angiosperm lignins, stains a bright red with both treatments. Guaiacyl lignin turns yellow and in some cases, brown (30, 79). The colour developed in these reactions is also only temporary, fading after 35-45 minutes (90) A basic environment is necessary for the development of the colour and fading appears to be due to the sodium sulphite (or ammonia)

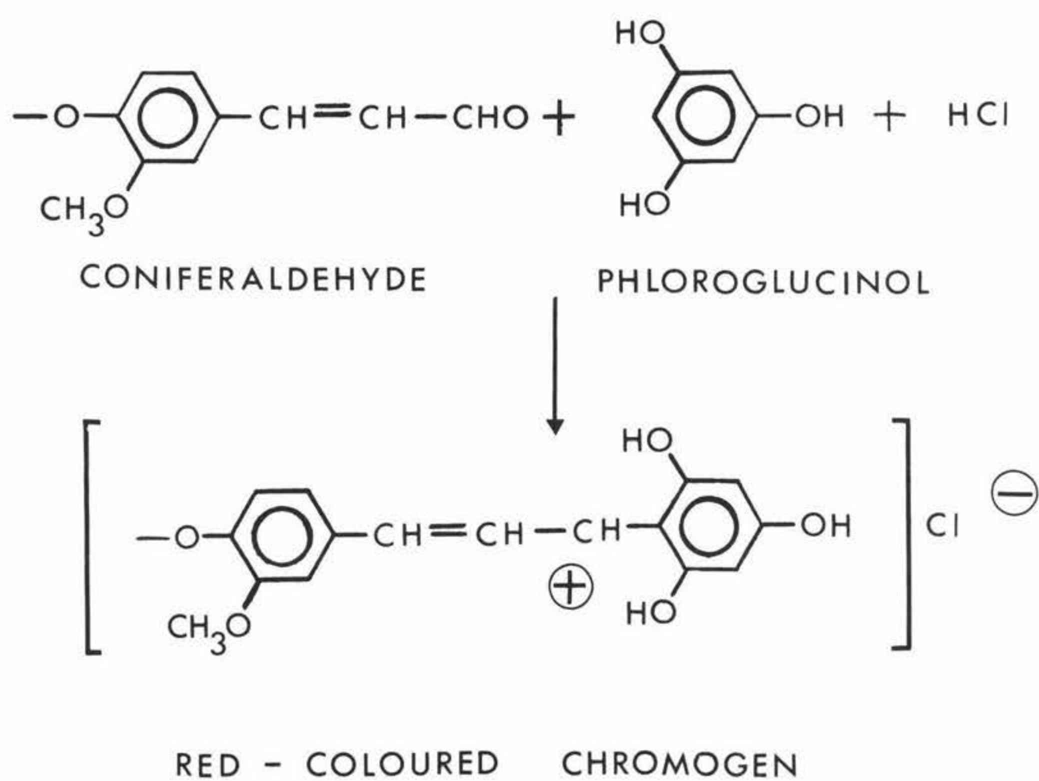
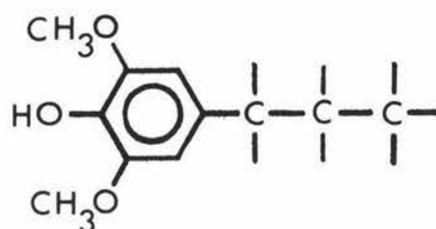
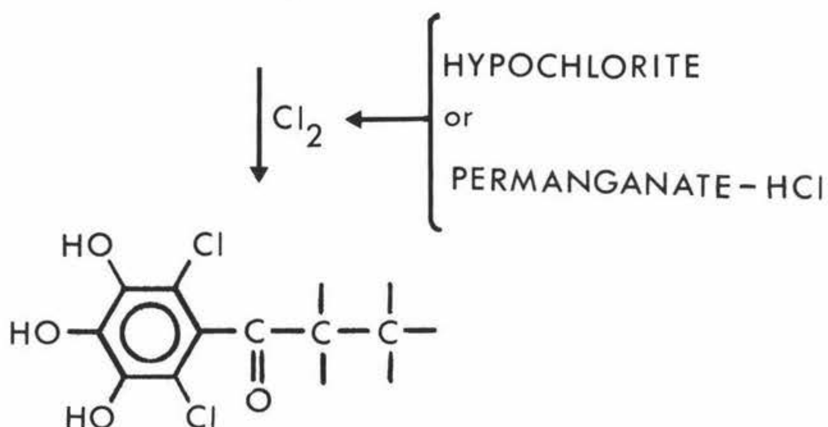
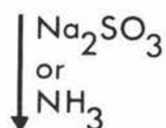


FIGURE 5. **Reaction of phloroglucinol with coniferaldehyde lignin units. (After T. Higuchi, 1971)**



SYRINGYL UNIT





RED COLOUR

FIGURE 6. Proposed mechanism of reaction for the ***Mäule*** **and** ***Cross and Beaven*** **tests for lignin. (After T. Higuchi, 1971; see also F.F. Nord and G. de Stevens, 1958)**

being slowly replaced by excess chlorine (24).

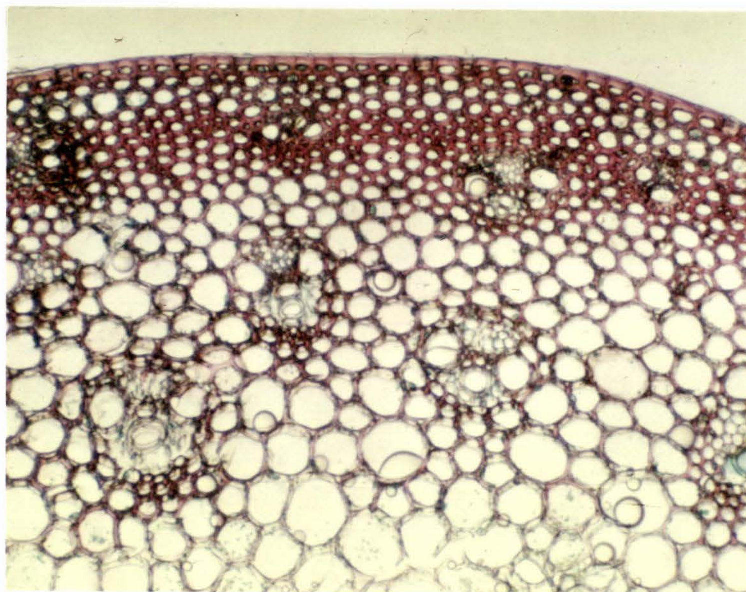
The specificity of guaiacyl and syringyl stains has been useful in taxonomic studies on plants (30, 28, 156). It has emerged that "primitive" (i.e. geologically ancient) plants tend to produce guaiacyl lignins and those more recent phylogenetically, such as the angiosperms produce both guaiacyl and syringyl-type lignins and may contain a high proportion of methoxyl groups.

Another stain often used in botanical histochemistry is the quinodoid dye, safranin. As a basic dye it stains phenolic hydroxyl groups generally (90), and basophilic cell contents other than lignin, for example the nucleoli and the chromosomes, take up this dye. Tissue sections are first overstained with safranin, washed in acid alcohol which removes dye not tightly bound, and then counterstained with fast green. Such a procedure shows up lignified cell walls clearly (see Plates 1A and 1B, p.17)[2] but as safranin is not specific for lignin other tests should be used simultaneously.

The colour reactions of concentrated acid and alkali are not well characterized. According to J.C. Pew (114, 115), the yellow colour that develops possibly results from the presence of substituted cinnamaldehydes, especially coniferaldehyde.

There is a difficulty in histological studies on lignified tissue in that although there has been a large amount of work carried out and there is an extensive literature on the subject the lignin polymer itself is not accurately defined (14, 15, 130). The structure of lignin from arborescent plants is still largely hypothetical (110) next to nothing being known of the three dimensional structure (69) and there are indications that grass lignins are substantially different from those of woody tissues

2 Note the faint pink of sieve-cell walls which are not generally thought to contain lignin.



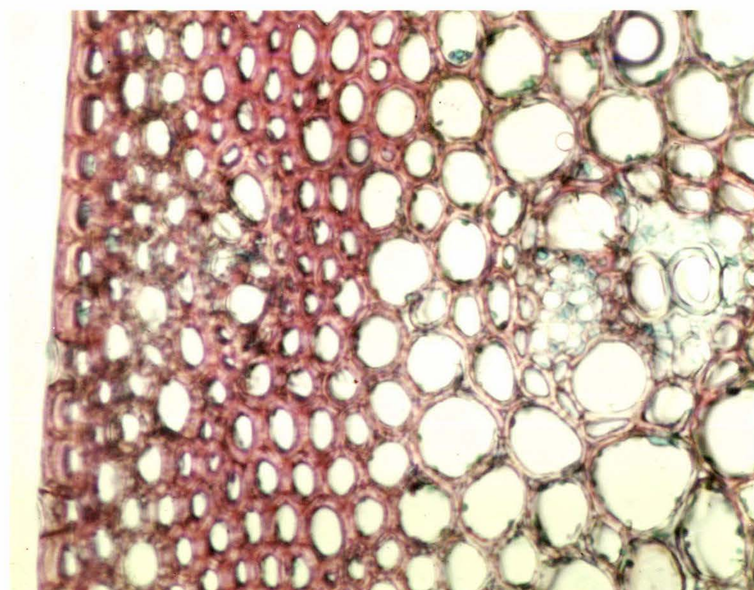


PLATE 1A (upper) Transection of 60 day-old <u>Triticum</u> stem stained in safranin and fast green (X40).

PLATE 1B (lower) Transection of 60 day-old <u>Triticum</u> stem stained in safranin and fast green (X100).

(143, 144).

Positive responses to Wiesner, Máule and safranin colour tests are not absolute proof of the presence of lignin, and are generally used along with a number of other physical and chemical criteria for positive identification (132). The reliability of histological stains however, is supported by the fact that an identical pattern of lignin distribution is obtained with methods based on ultra-violet light microscopy to that observed with phl/HCl staining in tracheids of Pinus radiata (161) and also by the observation that the functional groups responsible in the more specific Wiesner and Mäule tests for the development of colour, are part of the "lignin core" as defined by S.A. Brown in 1966 (14).

I.3.2 THE SOLVOLYSIS OF LIGNIN WITH BASE

Of the methods available for the estimation of tissue lignin content (132), base extraction followed by analysis of the lignin content of the solution is considered the most suitable for herbaceous plant material. The plant tissue is first dried and the ether and water soluble material removed. NaOH is added and the mixture heated to a temperature of 70°C for 16 hours. The unhydrolyzed residue is removed and the solution containing the lignin retained. This technique has been developed and used successfully by A. Bondi and H. Meyer, 1948 (9) and later by H.A. Stafford working with Phleum pratense (143, 144, 145, 146). J. Friend et al (48) have alsc used this method on potato tuber tissue. W.A. Jensen in his book Botanical Histochemistry (90), recommends the use of this method of extraction followed by spectrophotometry and analysis of the phenol content as a rapid and reliable means of estimating the absolute amount of lignin in small quantities of tissue, and as especially useful in developmental studies.

The reactions that take place within the lignin polymer upon treatment of the plant tissue with base (NaOH and other basic solutions) are discussed and illustrated fully by A.D.A. Wallis (158). The bonds thought to be broken are illustrated in Figure 7 on a small portion of Fagus silvatica (beech) lignin, the structure of which was proposed by H. Nimz in 1974 (110). These are briefly:

1 and 2, ether linkages between phenylpropane units with the formation of phenolic hydroxyl groups,

3, α to β carbon to carbon bonds in the propane side chain,

4, bonds attaching methoxyl groups to the C_9 unit via the aromatic ring, and

5, bonds attaching primary alcoholic groups to the β carbon of the side chain.

The bonds at positions 4 and 5 are more resistant to hydrolysis than bonds elsewhere, and soluble products resulting from demethylation are not common. Bond cleavage at positions 1, 2 and 3 may be seen to result in a reaction mixture containing the phenolic aldehydes, p-OH benzaldehyde, vanillin and syringaldehyde, and phenylpropane units. Dimeric and trimeric products have also been identified in the reaction mixture and unknown condensation reactions are thought to occur. While grass lignins may differ from those of other angiosperms and in particular, Fagus silvatica, chromatography of the ether soluble phenolic constituents of acidified alkaline extracts containing the lignin from Phleum (timothy grass) shows the presence of p-OH cinnamic, ferulic, syringic and vanillic acids and p-OH benzaldehyde, syringaldehyde and vanillin along with many other unidentified compounds (145). Spectrophotometry is carried out on this mixture.

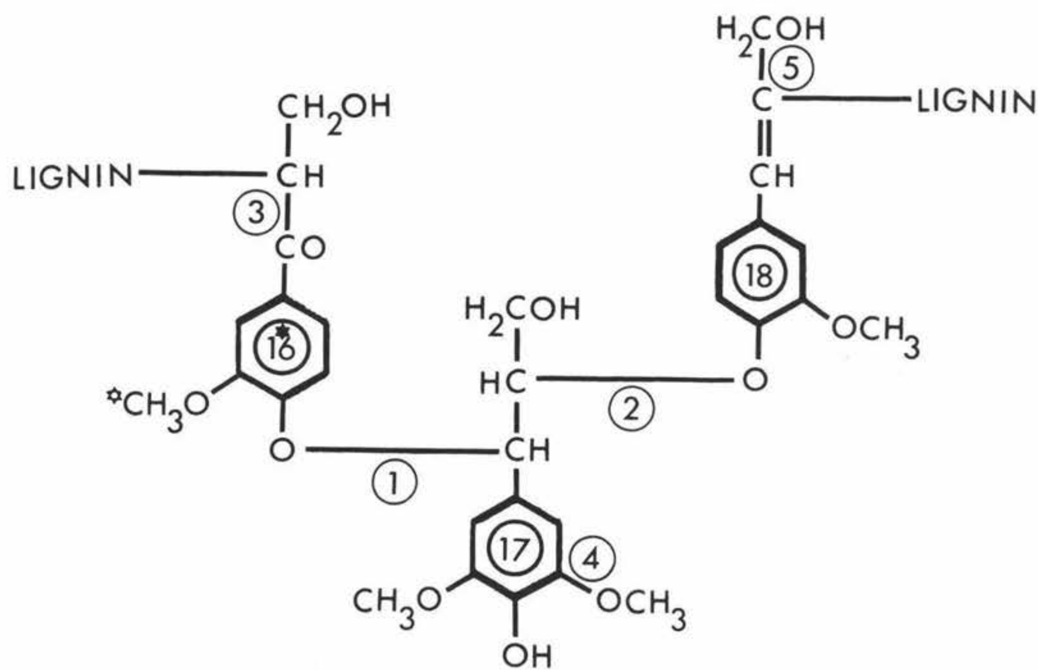


FIGURE 7. A small portion of beech lignin as proposed by H. Nimz, 1974 showing possible bond cleavage resulting from the treatment of lignin with alkali. (From A.F.A. Wallis, 1971)

1. α-ether cleavage
2. β-ether cleavage
3. α - β carbon to carbon bond cleavage
4. aromatic methoxyl group cleavage
5. primary alcohol group cleavage

* 16 and 18 are coniferyl alcohol derivatives and 17 is a sinapyl alcohol unit. The numbering is that used by H. Nimz.

☆ In grasses the methoxyl groups may be absent as p-hydroxycinnamic acid derivatives are thought to be present in significant amounts. (H.A. Stafford, 1962)

I.3.3 THE SPECTROPHOTOMETRIC ANALYSIS OF BASE-EXTRACTED LIGNIN

Lignin, because of the aromatic nature of its units (58), absorbs strongly in the ultraviolet region of the spectrum, and the spectra of lignin preparations, for example Eucalyptus regnans (161), characteristically show a maximum at 280mu. Free and etherified hydroxyl groups contribute significantly to this absorption maximum (58). If the solution is made alkaline, phenolic hydroxyl groups ionize and the maximum shifts to longer wavelengths (57). The shift is relatively slight and may be accentuated by recording the ionization difference spectrum using the neutral lignin solution as a blank.

The absorption maxima of alkali-lignin preparations are composite peaks due to the complex mixture of hydrolysis products present. The work done by L. Doub and J.M. Vandenbelt, 1947 with benzene derivatives (33), and by H.W. Lemon also in 1947 (95), on the absorption spectra of hydroxyaldehydes and hydroxyketones has helped to clarify this situation. p-OH benzaldehyde absorbs maximally at wavelengths 240 and 340mu, vanillin absorbs at 250 and 350mu and syringaldehyde at 250 and 360mu while the corresponding acids have absorption maxima between 280 and 300mu. More generally non-conjugated phenolic hydroxyl groups in alkaline solution absorb at a wavelength near 300mu while phenolic hydroxyl groups with large conjugated sidechains, including the cinnamic acid derivatives, absorb at wavelengths between 340 and 350mu (56).

Phleum lignin preparations, as previously mentioned (Section I.3.2), are a complex mixture of aromatic aldehydes and phenylpropane units. From the absorbing properties of these compounds the ionization difference spectra would be expected to contain maxima near wavelengths of 250, 300 and 350mu. This is found to be so. Low maxima are present for very young plants and

higher maxima (the increase is especially noticeable at 350mu) occur in preparations from the mature hay (143). Differences in spectra for internode, leaf sheath and leaf blade material were detected, indicating that the type of lignin present in each is different (145).

In the following experiments, "lignin" from the second internode of wheat plants aged between 10 and 71 days is extracted with NaOH and the absorption spectra recorded. The absorbance maximum at 345mu is taken as a measure of the conjugated, ionizable phenolic hydroxyl groups present. This value, for each sample, is assumed to be directly proportional to the lignin content of the tissue.

The contribution of protein and flavonoids not removed in the water and ether extracts to absorbance at this wavelength is unknown. M. Phillips et al (116) have shown that while the nitrogen content of wheat was high early in development it declined before the time of rapid lignification. Contamination of base-extracted lignin with protein may be expected to artificially increase the earlier values (measurements made on plants less than 40 days old) and have little effect on the later estimates. H.A. Stafford (143) felt that hydrolysed protein or flavonoids did not make any major contribution to the absorbance measured.

p-OH cinnamic acid and ferulic acid may be a further source of error as they are known to be present in unknown ester forms in grasses (145, 93) and there is uncertainly regarding their relationship to the lignin polymer. The majority of the free cinnamic acids would be removed from the plant material in the ether-water extractions and base treatment would release only those already incorporated into the wall.