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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

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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.

CONTENTS

PAGE

ABSTRACT ii

ACKNOWLEDGMENTS iv

LIST OF FIGURES viii

LIST OF PLATES xi

LIST OF TABLES xiii

INTRODUCTION 1

PART I ANATOMICAL ASPECTS OF LIGNIFICATION IN WHEAT 8

I.1 INTRODUCTION 8

I.2 THE ANATOMY OF THE WHEAT STEM 8

I.3 THE LOCALIZATION, EXTRACTION AND MEASUREMENT OF LIGNIN 13

1. THE COLOUR REACTIONS OF LIGNIN 13

2. THE SOLVOLYSIS OF LIGNIN WITH BASE 18

3. THE SPECTROPHOTOMETRIC ANALYSIS OF BASE-EXTRACTED LIGNIN 21

I.4 MATERIALS AND METHODS 23

1. CULTIVATION OF THE PLANTS 23

2. SECTIONING OF THE PLANT MATERIAL 23

3. STAINING OF THE PLANT MATERIAL 24

1. PHLOROGLUCINOL-HYDROCHLORIC ACID 24

2. MÄULE REACTION 24

3. SAFRANIN-FAST GREEN 24

4. ACID AND BASE TREATMENT OF TISSUE SECTIONS 25

4. PHOTOMICROGRAPHY 25

5. LIGNIN EXTRACTION AND SPECTROPHOTOMETRY 25

6. GROWTH MEASUREMENTS 26

PAGE

I.5 RESULTS 27

1. GROWTH MEASUREMENTS 27

2. HISTOCHEMICAL OBSERVATIONS 29

1. 20-DAY STEM SECTIONS 29

2. 30-DAY STEM SECTIONS 30

3. 35-DAY STEM SECTIONS 30

4. 40-DAY STEM SECTIONS 30

5. 50-DAY STEM SECTIONS 31

6. 60 and 70-DAY STEM SECTIONS 31

3. SPECTROPHOTOMETRIC ANALYSIS OF LIGNIN CONTENT 40

I.6 DISCUSSION 46

I.7 SUMMARY 52

I.8 CONCLUSION 53

PART II BIOCHEMICAL ASPECTS OF LIGNIFICATION IN WHEAT 54

II.1 INTRODUCTION 54

II.2 THE REGULATION OF LIGNIN BIOSYNTHESIS 55

1. AN OUTLINE OF THE PATHWAY 55

2. THE ROLE OF PHENYLLACTIC AND HYDROXY-PHENYLLACTIC ACIDS 64

II.3 MATERIALS AND METHODS 69

1. CULTIVATION OF THE PLANTS 69

2. SOURCE OF THE ISOTOPES USED 69

3. ADMINISTRATION OF THE COMPOUNDS 69

4. EXTRACTION AND PURIFICATION OF THE CINNAMIC ACIDS 70

5. MEASUREMENT OF RADIOACTIVITY 73

II.4 RESULTS 74

II.5 DISCUSSION 79

II.6 CONCLUSION 83

PAGE

PART III AN OUTLOOK ON THE CONTROL OF LIGNIFICATION 84

APPENDIX 89

BIBLIOGRAPHY 92

LIST OF FIGURES

FIGURE		PAGE
1.	The structure of lignin monomers.	2
2.	Ammonia-lyase activity and the lignin content of wheat expressed as a function of time. (From M. Young, 1966 and J.E. Stone et al, 1951).	5
3.	The pathway of lignification in wheat as proposed by D. Wright, S.A. Brown and A.C. Neish, 1958.	7
4.	Stem of Triticum (A) Diagram of a transection of mature internode (B) Drawing of a transection of an inner vascular bundle.	10
5.	Reaction of phloroglucinol with coniferaldehyde lignin units. (After T. Higuchi, 1971).	14
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).	15
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).	20
8.	The relationship of lignification in the second internode of wheat to shoot dry weight, stem elongation and flowering.	28

FIGURE		PAGE
9.	Absorption spectra of a crude lignin preparation from 10 day-old wheat at pH 7.0 (A) and pH 12.3 (B) and the difference spectrum (C).	41
10.	Absorption spectra of a crude lignin preparation from 71 day-old wheat at pH 7.0 (A) and pH 12.3 (B) and the difference spectrum (C).	42
11.	The change in lignin content with time of second internode tissue (I-2) and of the whole shoot (W).	44
12.	The pathway of lignification as thought to occur in the Gramineae and an indication of the involvement of the cinnamic acids in secondary metabolism.	56
13.	The possible routes for the conversion of tritiated p-hydroxyphenyllactic acid to p-hydroxycinnamic acid. (T = tritucum)	67
14.	The incorporation of ^{14}C-tyrosine with time into ethanol-soluble ferulate or p-hydroxycinnamate for wheat plants aged 10, 20, 40 and 60 days.	75
15.	Change in activity in ethanol-soluble and ethanol-insoluble ferulate with age after 2 hours incorporation of ^{14}C-tyrosine.	75
16.	The role of HPLA and PLA as "pool" metabolites contributing to the origin of tyrosine and phenylalanine (O.L. Gamborg and A.C. Neish, 1959).	80

FIGURE PAGE

17. (A) Radioactivity in segments cut from a narrow strip after chromatography of the reaction mixture in 2% acetic acid. The HPLA standard is shown above.
(B) Chromatography of an aliquot of purified ^{3}H-HPLA in 2% acetic acid and the scan of this strip for radioactivity.
(C) Chromatography of an aliquot of purified ^{3}H-HPLA in benzene:acetic acid:water (10:7:3, v/v) and the scan of this strip for radioactivity. 90

LIST OF PLATES

PLATE		PAGE
1A	Transection of 60 day-old *Triticum* stem stained in safranin and fast green (X40)	17
1B	Transection of 60 day-old *Triticum* stem stained in safranin and fast green (X100)	17
2A	Transection of 20 day-old *Triticum* stem stained in phloroglucinol and HCl (X40)	32
2B	Transection of 20 day-old *Triticum* stem stained in Mäule's reagent (X40)	32
3A	Transection of 30 day-old *Triticum* stem stained in phloroglucinol and HCl (X40)	33
3B	Transection of 30 day-old *Triticum* stem stained in Mäule's reagent (X40)	33
4A	Transection of 35 day-old *Triticum* stem stained in phloroglucinol and HCl (X40)	34
4B	Transection of 35 day-old *Triticum* stem stained in chlorine-sodium sulphite (X40)	34
4C	Transection of 35 day-old *Triticum* stem treated with 0.5N NaOH (X40)	35
5A	Transection of 40 day-old *Triticum* stem stained in phloroglucinol and HCl (X40)	36
5B	Transection of 40 day-old *Triticum* stem stained in Mäule's reagent (X40)	36

PLATE		PAGE
6A	Transection of 50 day-old *Triticum* stem stained in phloroglucinol and HCl (X40)	37
6B	Transection of 50 day-old *Triticum* stem stained in Mäule's reagent (X40)	37
7A	Transection of 60 day-old *Triticum* stem stained in phloroglucinol and HCl (X40)	38
7B	Transection of 60 day-old *Triticum* stem stained in Mäule's reagent (X40)	38
8A	Transection of 70 day-old *Triticum* stem stained in phloroglucinol and HCl (X40)	39
8B	Transection of 70 day-old *Triticum* stem stained in Mäule's reagent (X40)	39

LIST OF TABLES

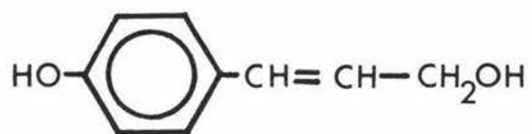
TABLE		PAGE
I	R_f values	72
II	The activity in ethanol-soluble and ethanol-insoluble ferulate after the uptake of ^{3}H-HPLA by wheat shoots.	78

INTRODUCTION

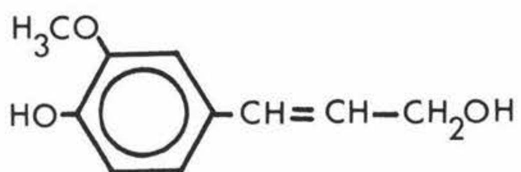
Lignin, the end product of the cellular process of lignification is, after cellulose, the second most abundant natural polymer (13). Over forty million tonnes being produced as a by-product of the pulp industry in 1968 alone (110). The problem of lignin waste and the resulting pollution has added impetus 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).

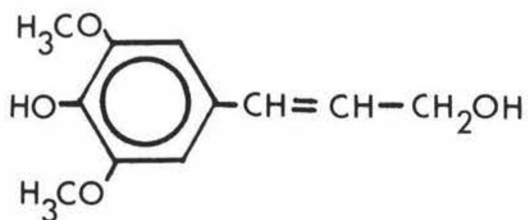
The complexity and variability of the lignin polymers makes the formulation of an accurate, inclusive definition difficult. A recent version of the structure of *Fagus silvatica* (beech) lignin (110) shows the polymer as being made up of three basic aromatic units (see Figure 1)



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

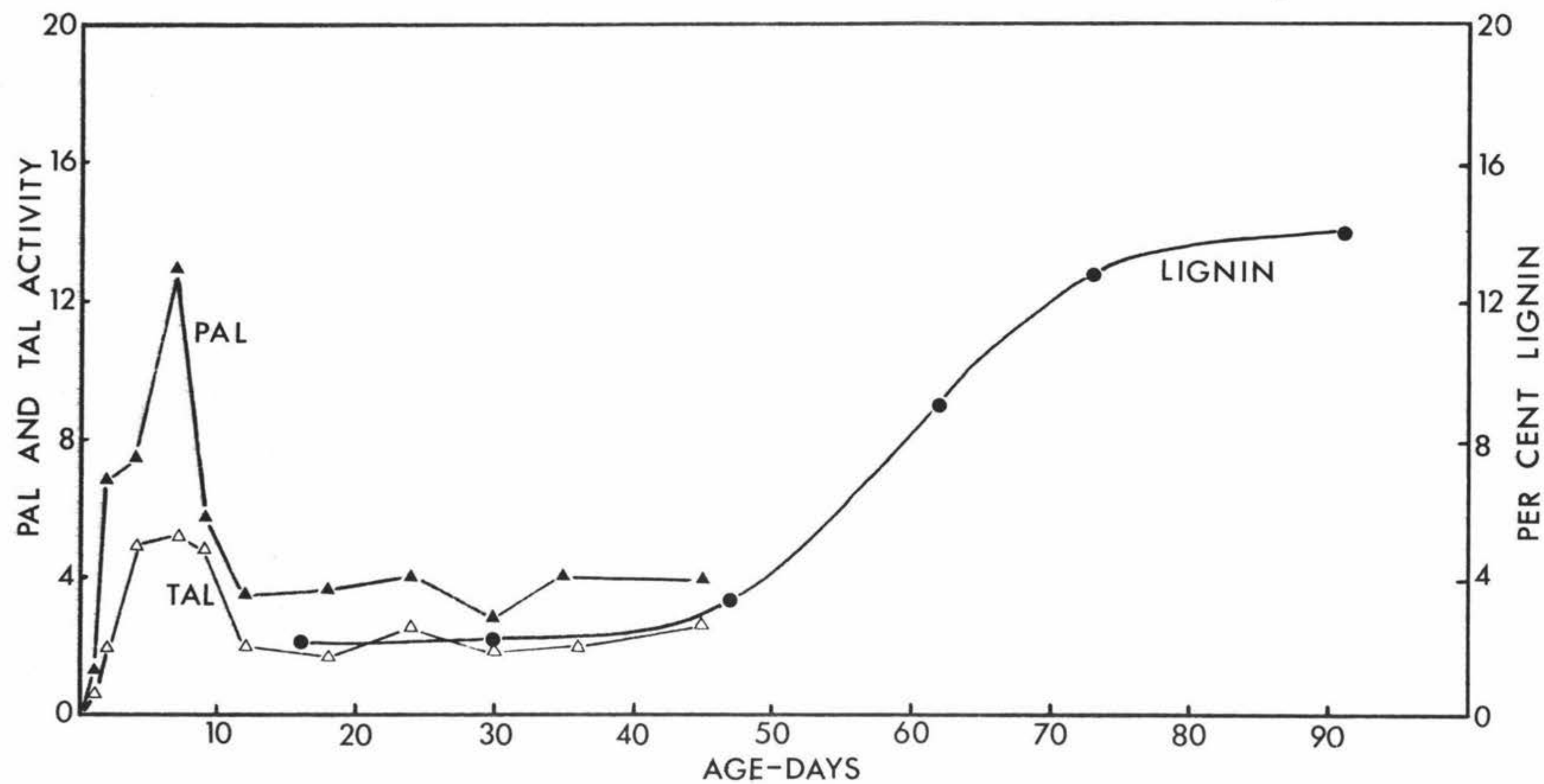


FIGURE 2. **Ammonia-lyase activity and the lignin content of wheat expressed as function of time. (From M. Young, 1966 and J.E. Stone *et al,* 1951)**

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."

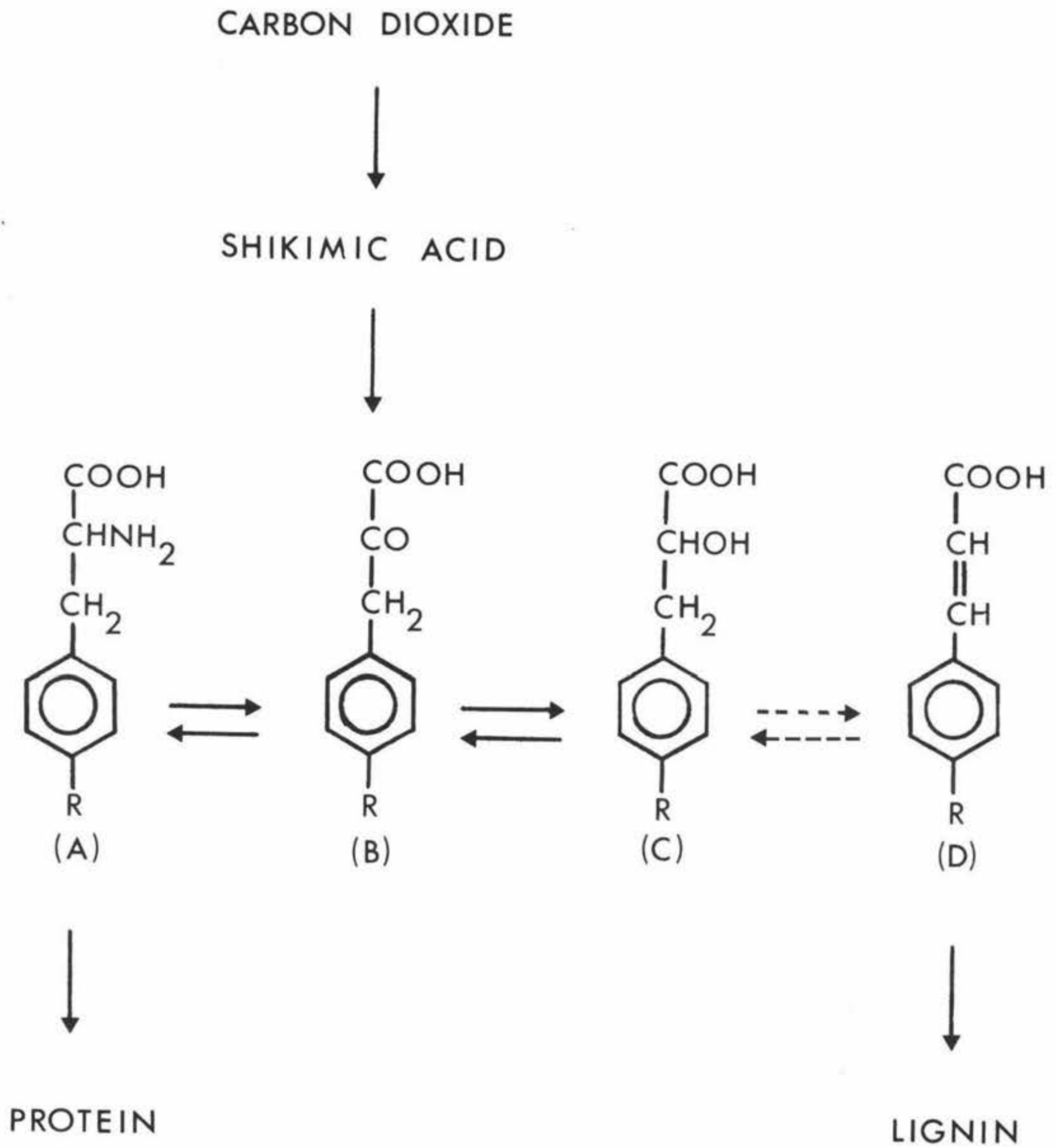


FIGURE 3. The pathway of lignification in wheat as proposed by D. Wright, S.A. Brown and A.C. Neish, 1958.

(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-

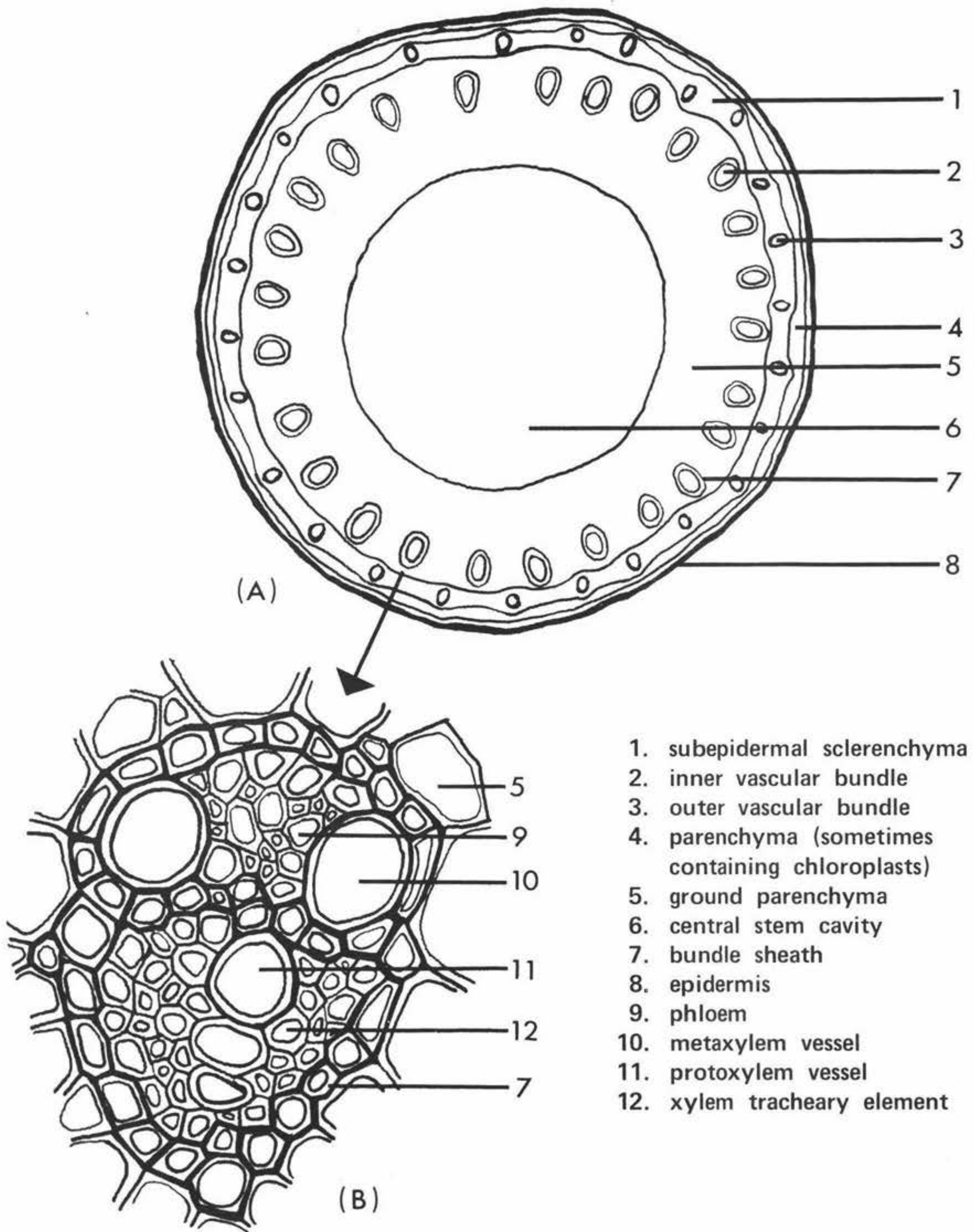


FIGURE 4. **Stem of *Triticum***

(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)

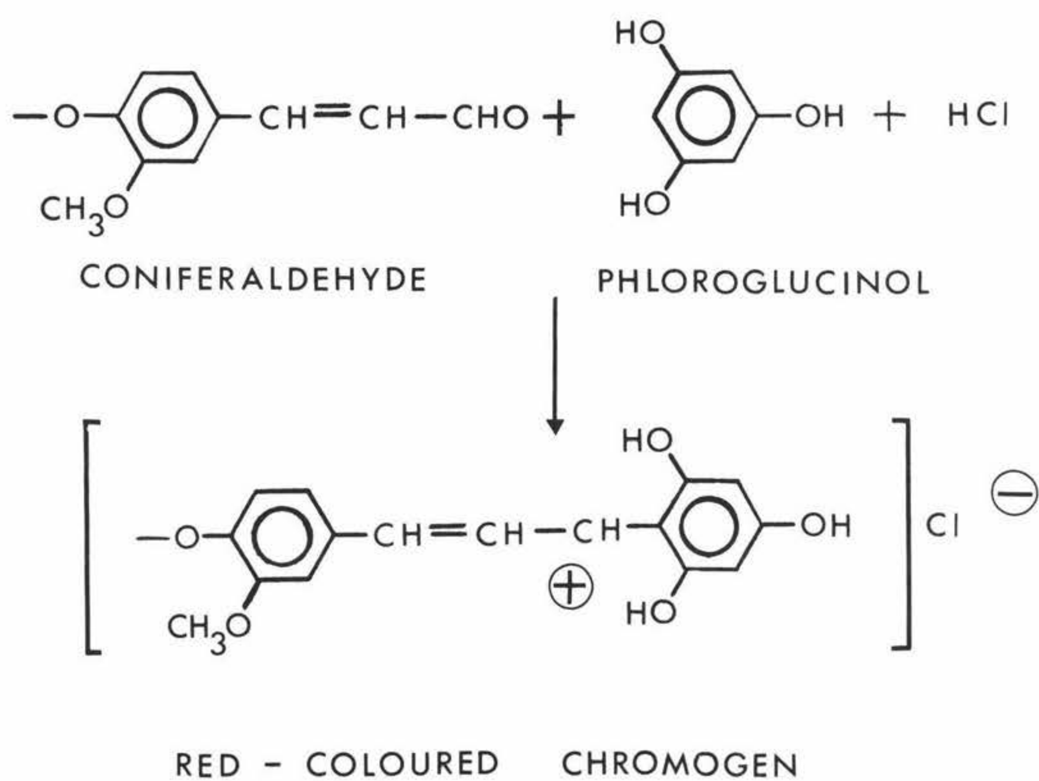


FIGURE 5. **Reaction of phloroglucinol with coniferaldehyde lignin units. (After T. Higuchi, 1971)**

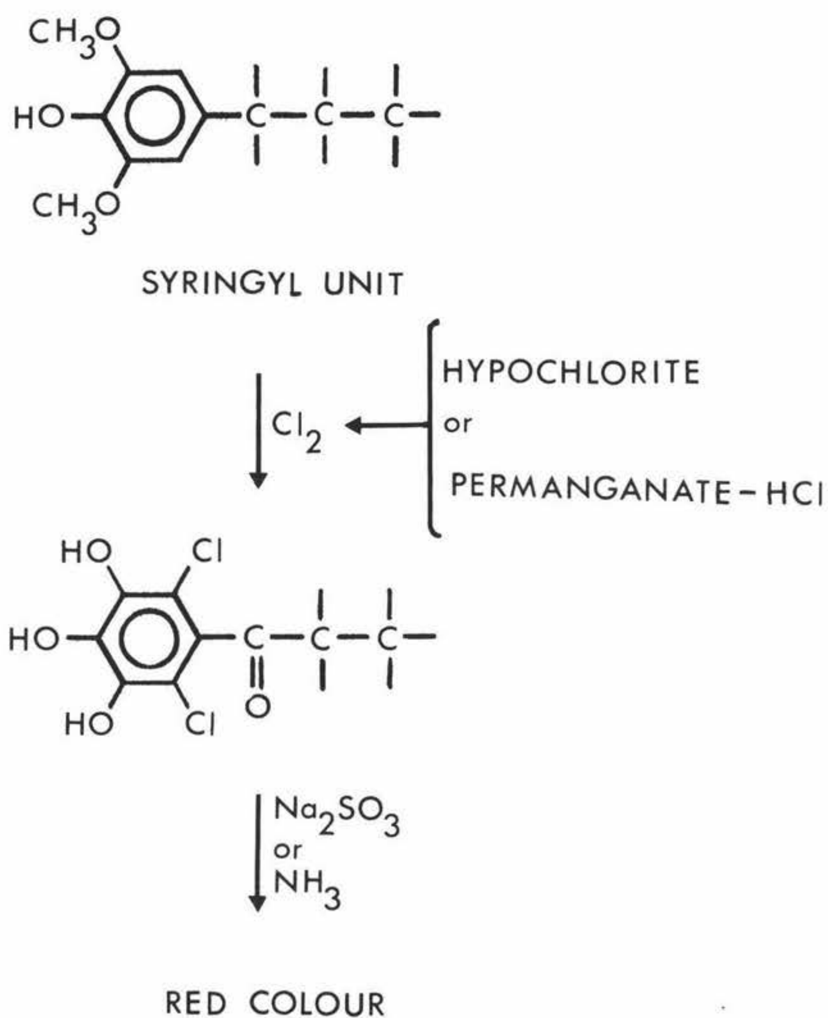


FIGURE 6. **Proposed mechanism of reaction for the** ***Mäule*** **and** ***Cross and Beaven*** **tests for lignin. (After T. Higuchi, 1971; see also F.F. Nord and G. de Stevens, 1958)**

being slowly replaced by excess chlorine (24).

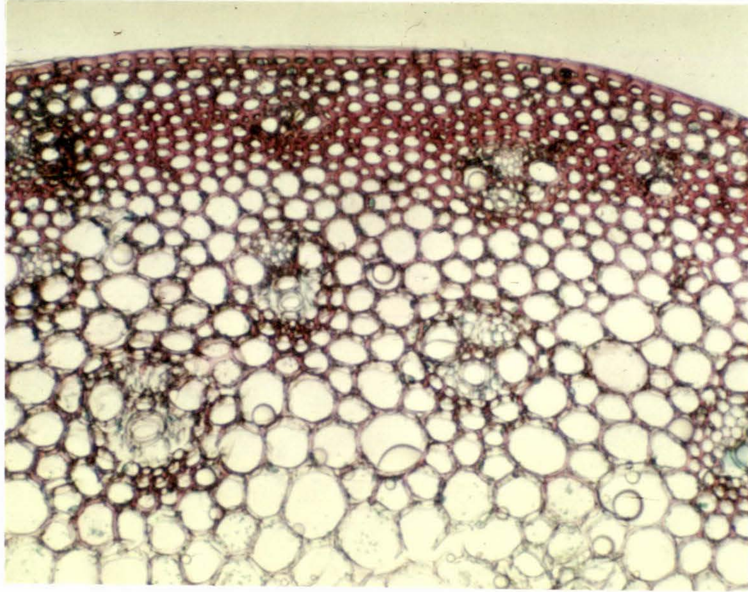
The specificity of guaiacyl and syringyl stains has been useful in taxonomic studies on plants (30, 28, 156). It has emerged that "primitive" (i.e. geologically ancient) plants tend to produce guaiacyl lignins and those more recent phylogenetically, such as the angiosperms produce both guaiacyl and syringyl-type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



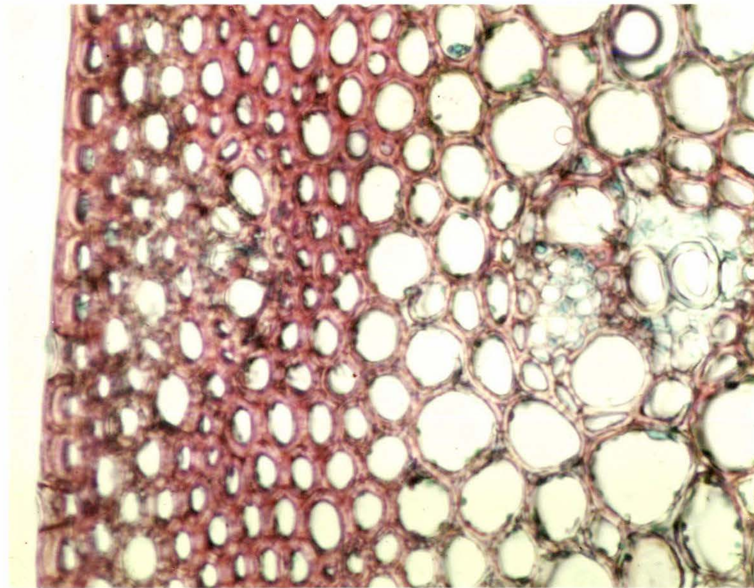


PLATE 1A (upper) Transection of 60 day-old Triticum stem stained in safranin and fast green (X40).

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

(143, 144).

Positive responses to Wiesner, Mäule 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 also 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.

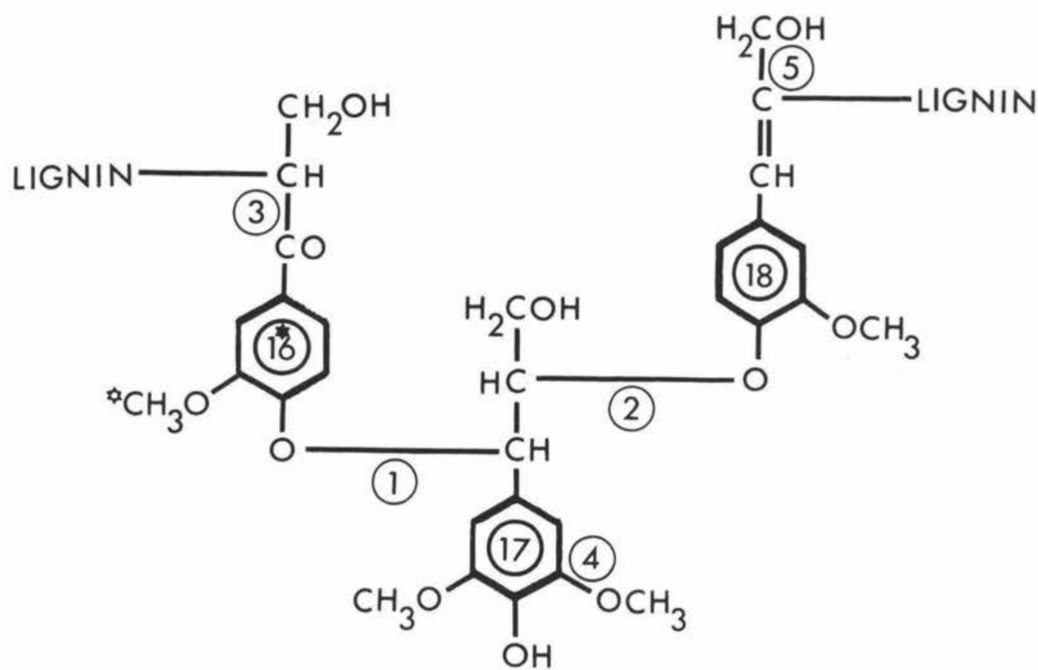


FIGURE 7. A small portion of beech lignin as proposed by H. Nimz, 1974 showing possible bond cleavage resulting from the treatment of lignin with alkali. (From A.F.A. Wallis, 1971)

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.

I.4 MATERIALS AND METHODS

I.4.1 CULTIVATION OF THE PLANTS

Wheat (Triticum aestivum var. Thatcher) plants were grown in a peat and Horticultural Perlite (50:50) potting mixture. To every half bushel of this mix, 50 ml osmocote (NPK saturated resin), 35 ml superphosphate, 7 ml uramite and 250 ml ground white lime were added before planting. Each tray of plants was supplied with nutrient in a liquid form at three-weekly intervals. The plants were irradiated with light for 14 hours a day from 10 X 40 watt cool white fluorescent reflector tubes and 8 X 15 watt incandescent bulbs with a combined intensity of approximately 13500 lux. The temperature of the growth cabinets fluctuated between 21°C and 25°C.

I.4.2 SECTIONING OF THE PLANT MATERIAL

Fresh tissue sections were used in every case. Sections 30 microns thick were cut from I-2 of the main wheat stem on a freezing microtome and placed immediately in water. They were then transferred with a pasteur pipette to microscope slides, stained, rinsed with distilled water, and mounted in glycerol before photomicrography. This method of sectioning has three main advantages:

1. the plant tissue is as close as possible to its natural state,

2. sections are of uniform thickness - this is necessary for the comparison of stain intensity,

3. it is rapidly carried out.

Transections of 35 day old stems only were hand sliced with a razor blade.

I.4.3 STAINING OF THE PLANT MATERIAL

I.4.3.1 PHLOROGLUCINOL - HYDROCHLORIC ACID

A saturated solution of phloroglucinol was prepared by dissolving one gram of phloroglucinol in 100 ml of water with slight warming. An equal quantity of conc. HCl was then added slowly to the measured liquid. A large drop of this solution was then placed on a slide over tissue sections and left for about 10 minutes for the colour to develop. Fading of the colour occurs after 3 to 4 hours so photomicrography was always carried out within 30 minutes of staining.

I.4.3.2 MÄULE REACTION

Tissue sections were first treated with a drop of aqueous permanganate (approx. 0.1 gm $KMnO_4$ dissolved in 50 ml water) and rinsed with distilled water. A drop of 2N HCl was then placed on the slide and left for a few moments before rinsing again with water. Finally the tissue was treated with ammonia solution, rinsed with water and mounted. The red colour faded very quickly and photomicrographs were taken immediately.

I.4.3.3 SAFRANIN - FAST GREEN

The tissue was treated with a drop of 1% safranin solution for a few seconds and rinsed thoroughly with distilled water. A very dilute solution of fast green was used to counter-stain the sections. The fast green contained a small amount of clove-oil and the slide had to be rinsed several times to remove this before mounting as usual in glycerol.

I.4.3.4 ACID AND BASE TREATMENT OF TISSUE SECTIONS

The lignin was tested for solubility in 72% H_2SO_4 by placing a drop of this over tissue sections for 10 minutes after which the slide was observed under the microscope. The colour reaction to 0.5N NaOH of tissue sections was also noted.

I.4.4 PHOTOMICROGRAPHY

Kodak tungsten (3200K) high speed ektachrome reversal film with an ASA rating of 125 was used in all cases. A blue filter was inserted for all photographs except Plate 4A.

I.4.5 LIGNIN EXTRACTION AND SPECTROPHOTOMETRY

The lignin was extracted according to the method of H.A. Stafford (145). A. Bondi and H. Meyer, who first used this technique considered base extraction to be quantitative for young annuals (9).

For whole shoot estimates one complete shoot was used and chopped finely before drying. For second internode estimates segments 5 mm long were removed from the base of the main stem in plants less than 50 days old. The second internode of the main stem of plants 50 days and older had extended to a length greater than 0.7 mm so that samples of 50, 60 and 71 day old plants were made up of segments from this region.

All plant material was dried for 24 hours at 90°C. 50 mg of dry tissue was weighed out for each sample and moistened with distilled water. The tissue was ground in a pestle and mortar with ether until all the chlorophyll was removed. Approximately 5 ml of distilled water was added and the suspension centrifuged. After resuspension in water and a second wash, the residue was extracted with 4 to 5 ml of 0.5N NaOH in a water bath at 70°C for 16 hours. Centrifugation to separate the alkali-soluble lignin

from unhydrolyzed wall material was then carried out and 2 X 4 ml washes of the residue with 0.5N NaOH were added to the supernatant. The solution was neutralized to pH 7.0 with HCl and the volume made up to 25 ml with 0.05M phosphate buffer at pH 7.0 in a volumetric flask.

Ultraviolet absorption spectra (220 to 360mu) were determined using an Hitachi recording spectrophotometer on aliquots, one diluted with 0.5M phosphate buffer at pH 7.0 and the other with 0.5N NaOH at pH 12.4. The difference spectra were obtained by subtraction using the neutral solution in each case as a blank. Duplicate samples were made for each age of plant analyzed. Re-extraction of the final residue with base yielded no further u.v. absorbing material and the residue after drying was tested for materials reacting with phl/HCl and Mäule reagents.

I.4.6 GROWTH MEASUREMENTS

Weight and length values recorded in the results are the average of measurements made on 10 plants for young plants and on 5 plants for wheat older than 40 days.

Plant material was dried for 24 hours at 90°C for dry wt. estimates. Stem and internode measurements were made on the main axis only of each plant and the appropriate parts of these plants were included in the samples for lignin extraction.

I.5 RESULTS

I.5.1 GROWTH MEASUREMENTS

Information on the change in stem length and the dry weight of the shoot with time is presented in Figure 8. An increase in sample size would probably have resulted in smoother, more accurate curves and these results represent no more than preliminary observations. An increase in dry weight occurs at 35 to 40 days which parallels the increase in lignin of the portion of the stem measured (see Section I.5.3 for an explanation of the lignin measurements). The rapid increase in stem and internode length does not however occur until some 5 to 10 days after the increased lignin content is detected. Measurements of the stem length were made from the base of the main stem to the node at the bottom of the flower stalk and could include up to seven internodes. Dry weights included the weight of the whole shoot and rose sharply after 70 days (values not plotted on Fig. 8), presumably with the onset of grain filling.

The lignin content of the second internode remained low until the age of approximately 30 days and rapidly increased after this. The main flower head was well extended before the lignin deposited at the base of the plant reached a constant maximum level. The term "apex elongation" refers to the change in shape that the apex undergoes prior to spikelet initiation and is the first morphological sign that the plant is about to flower. "Anthesis" describes the plant some 25 days later when stamens from the main flower head extend from individual spikelets. The floral morphogenesis of Triticum aestivum has been described in detail by C. Barnard, 1954 (7), and apex elongation was found to take place about 10 weeks after sowing in autumn in the field, and any time from 4 to 6 weeks, depending upon the variety, in glass house conditions. Apex elongation occurred in the growth conditions of this study between 4 and 5 weeks after germination.

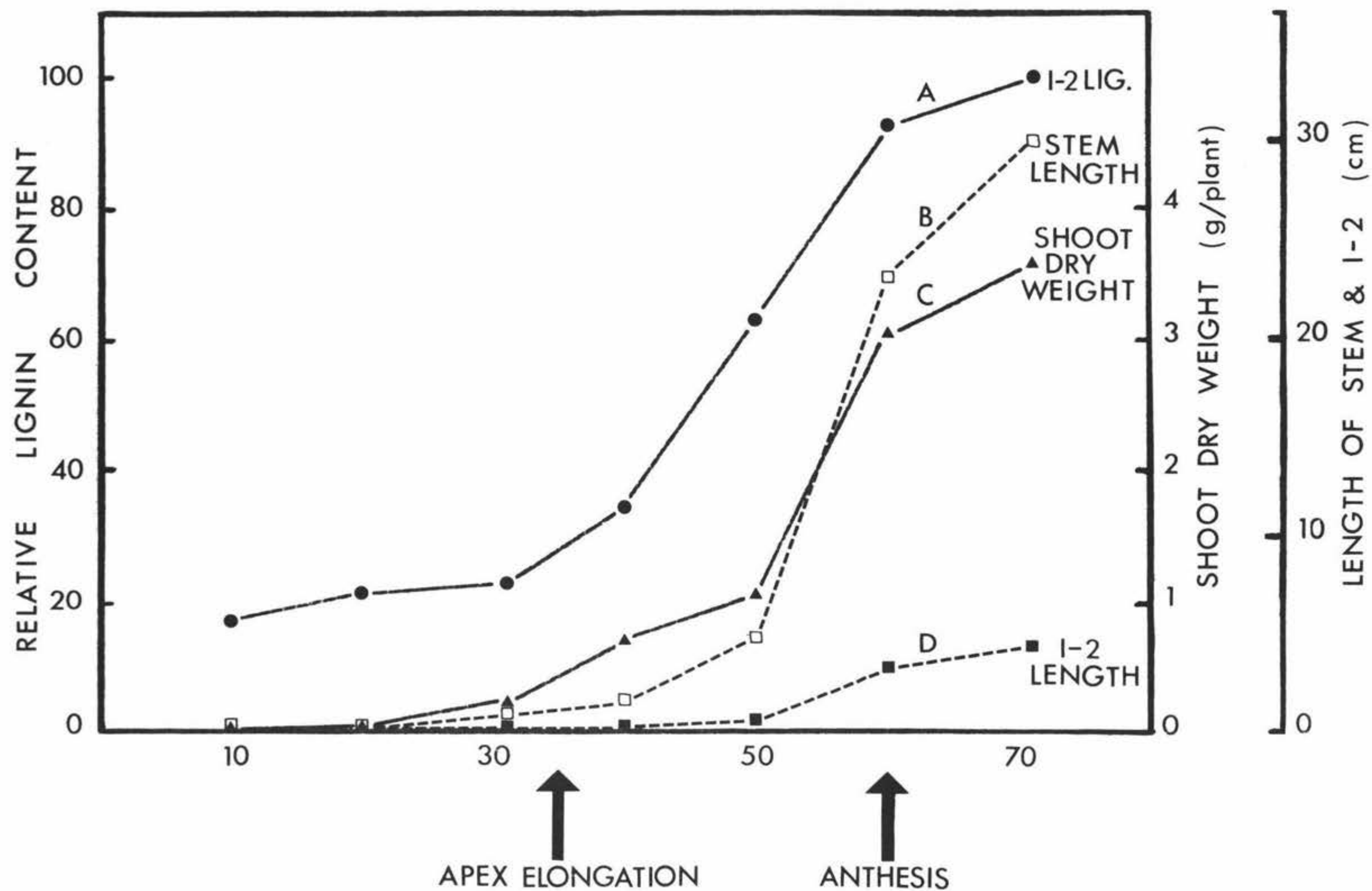


FIGURE 8. The relationship of lignification in the second internode of wheat to shoot dry weight, stem elongation and flowering. (I – 2 = second internode)

I.5.2 HISTOCHEMICAL OBSERVATIONS

A brief preliminary investigation of leaf and internode tissue showed that the base of the stem becomes the most heavily lignified region of the plant and detailed studies were limited to the second internode (I-2) of the stem. Histochemical tests applied to transections of tissue revealed that there are differences with increasing age in the lignins deposited in the walls of the proto-xylem, metaxylem, subepidermal sclerenchyma, bundle sheath fibres and epidermal cells. The responses to Wiesner (phl/HCl) and Mäule ($KMnO_4/HCl/NH_3$) reagents are illustrated in Plates 2 to 8 (pp 32 to 39). Plate 4B is a photomicrograph of 35-day-old wheat stained with Cl_2/Na_2SO_3 and Plate 4C is a section of the same age treated with base.

The presence of lignin containing guaiacyl units (coniferaldehyde and coniferyl alcohol) is shown by the development of a red colour with phl/HCl and a brown colour with $KMnO_4/HCl/NH_3$. Syringyl lignin stains dark-red with Mäule's reagent, and not at all with phl/HCl. Cl_2/Na_2SO_4 stains guaiacyl lignin yellow and syringyl lignin rose-red while thickened, even slightly lignified walls become yellow in 0.5N NaOH. Before a positive colour response is visible, the cell wall may, in some cases, appear more distinct with one stain than another (e.g. compare Plates 2A and 2B).

I.5.2.1 20-DAY STEM SECTIONS

At 20 days some of the vessels were lignified, showing as a pale brown-pink in phl/HCl and brown in $KMnO_4/HCl/NH_3$ (Arrow 'V', Plates 2A and 2B). There was an area distinct only after the syringyl group test (Arrow 'S', Plate 2B), containing thick-walled parenchyma and differentiating vascular elements, corresponding to the sclerenchyma ring of older tissue. The epidermis (Arrow 'E',

Plate 2A) was thin-walled at this age. A few bundles of lignified fibres were situated at intervals around the edge of the stem and extended into the epidermis.

I.5.2.2 30-DAY STEM SECTIONS

The situation at 30 days was similar to that at 20 days except that the xylem appeared to take up slightly more stain. The appearance of the vessel walls as dark brown rather than red in some places (Plate 3A) is due to the diaphragm on the microscope being incorrectly adjusted. Lignified bundle sheath fibres were visible in the leaf sheath (Arrow 'LBS', Plate 3B).

I.5.2.3 35-DAY STEM SECTIONS

The subepidermal sclerenchyma tissue was easily visible at 35 days (Arrow 'S', Plate 4B) but the walls did not yet colour to indicate that they contained lignin. These cells are distinct from thick-walled collenchyma associated with the vascular bundles (Arrow 'C', Plate 4B). Phl/HCl showed the presence of increased amounts of guaiacyl lignin in the xylem but, at this stage, there was no trace of the cells that would become supportive fibrous tissue after treatment with this stain (Plate 4A). The reaction of 35-day-old stem tissue to 0.5N NaOH (Plate 4C) was similar to the reaction to the test for syringyl groups (Plate 4B).

I.5.2.4 40-DAY STEM SECTIONS

At 40 days lignification of the sclerenchyma was proceeding rapidly and sufficient coniferylaldehyde groups were present for the middle lamellae of sclerenchyma fibres to stain very faintly pink in phl/HCl (Arrow 'S', Plate 5A). The greater sensitivity of these cells to the Mäule test when compared with the Wiesner test was more noticeable at this age than at any other (compare Plates 5A and 5B). The bundle sheath (Arrow 'BS', Plates

5A and 5B) and epidermal cells (Arrow 'E') were also more sensitive to the syringyl stain at this age.

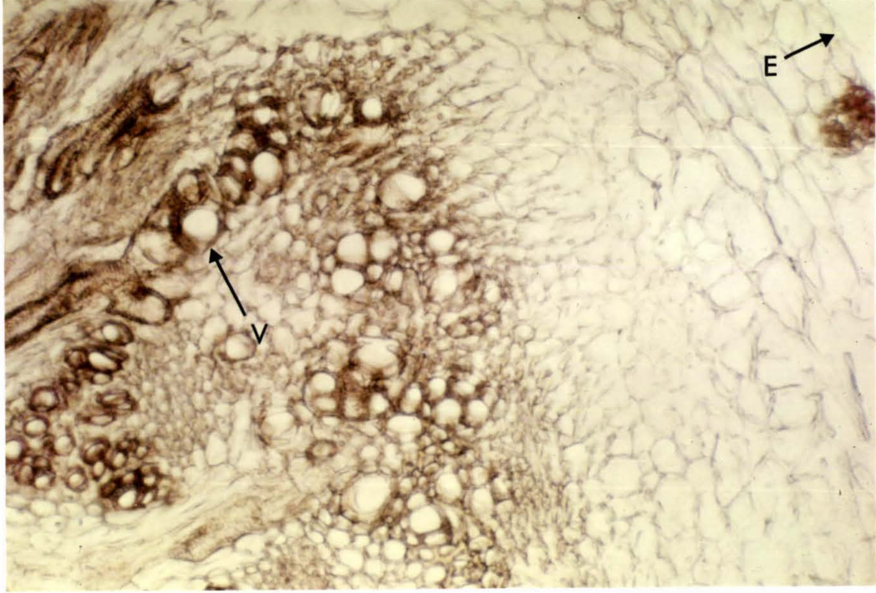
I.5.2.5 50-DAY STEM SECTIONS

At 50 days the internode of the main stem was at least 0.7 mm long and the outlines of both inner and outer vascular bundles were more clearly visible than in younger stems. The walls of the sclerenchyma, epidermis and bundle sheath stained a dark red-brown in $KMnO_4/HCl/NH_3$ indicating that syringyl lignin was present in significant amounts (Plate 6B). The bundle sheath, epidermis, metaxylem and sclerenchyma all stained to a greater or lesser degree with phl/HCl (Plate 6A). Protoxylem at all ages stained only weakly with each of the treatments used.

I.5.2.6 60 AND 70-DAY STEM SECTIONS

60 and 70 day-old sections were similar, the metaxylem, bundle sheath, epidermis and sclerenchyma responding to both stains. The ground parenchyma had started to lignify and the sclerenchyma fibres extended to the epidermis, although regions of thinner-walled cells were still visible beneath the epidermis. A major difference in the staining between guaiacyl and syringyl tests was the restriction of a strong positive reaction to the middle lamellae in the former case and the more even staining of the middle lamellae, primary and secondary walls in the latter. As the cell walls had become very heavily lignified at the later ages, sectioning using the freezing microtome after embedding the tissue in gelatine was difficult, the tissue tearing in places.

Both guaiacyl and syringyl lignin increased with age but their distribution varied both between tissues and between regions of the cell wall. At all stages the bundle sheath, epidermis and sclerenchyma stained more intensely with Mäule's



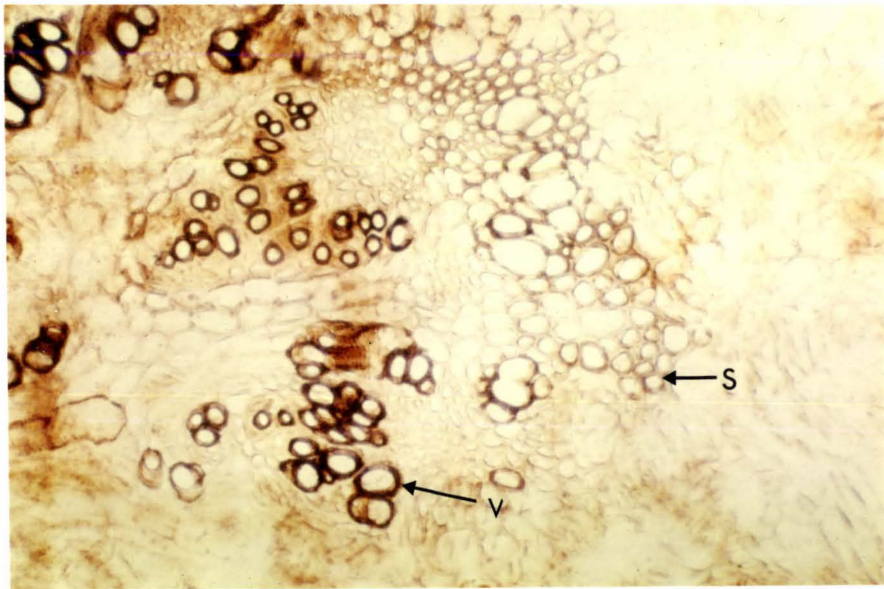
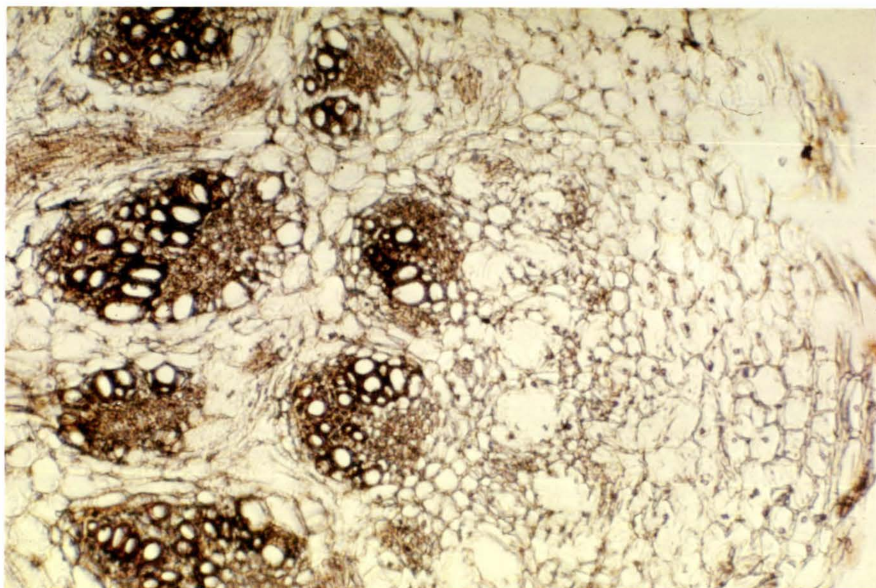


PLATE 2A (upper) Transection of 20 day-old _Triticum_ stem stained in phloroglucinol and HCl (X40).

PLATE 2B (lower) Transection of 20 day-old _Triticum_ stem stained in Maüle's reagent (X40).
(V = vessel, S = sclerenchyma, E = epidermis



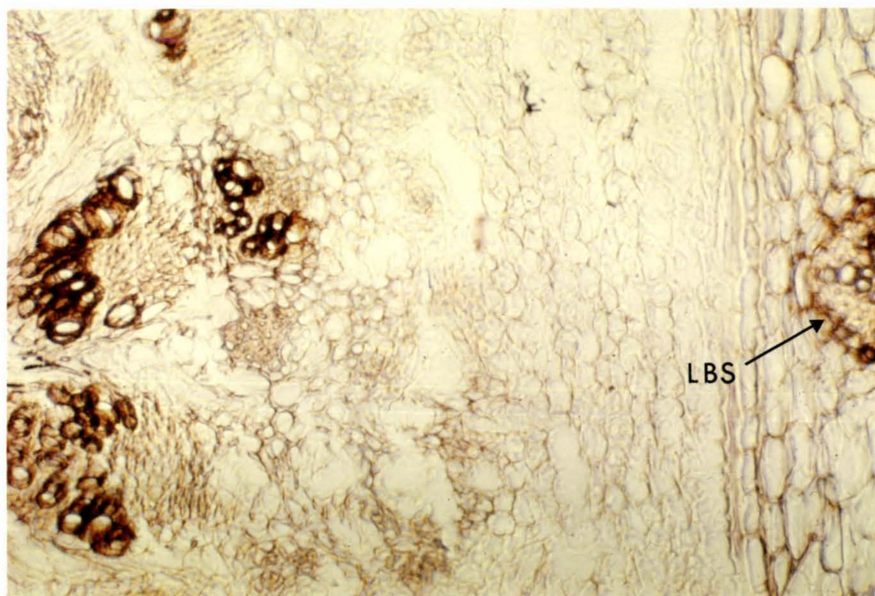
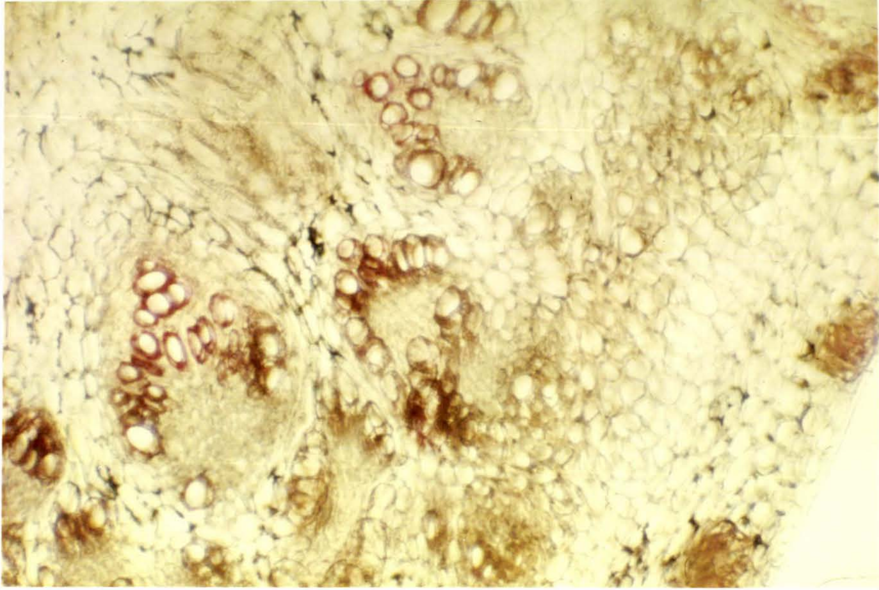


PLATE 3A (upper) Transection of 30 day-old Triticum stem stained in phloroglucinol and HCl (X40).

PLATE 3B (lower) Transection of 30 day-old Triticum stem stained in Mäule's reagent (X40). (LBS = leaf bundle sheath)



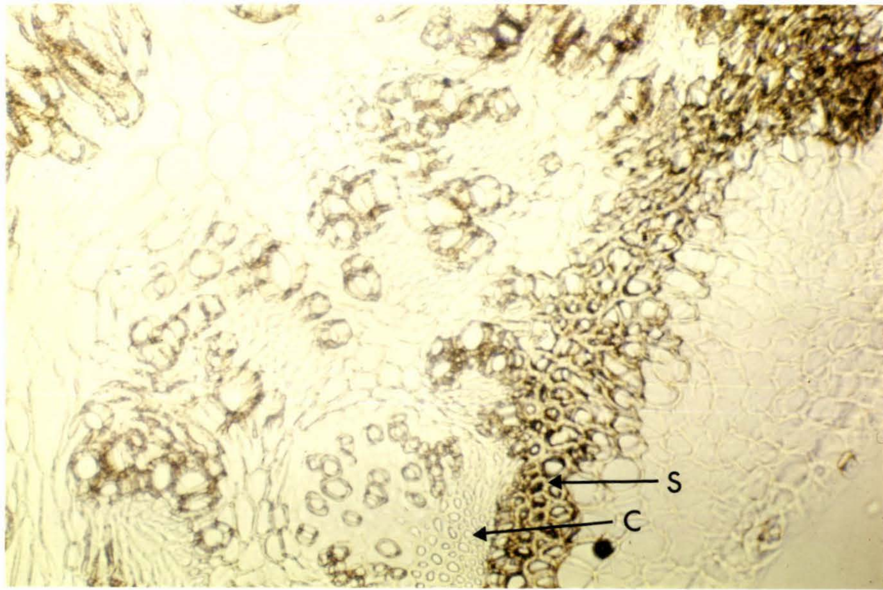


PLATE 4A (upper) Transection of 35 day-old *Triticum* stem stained in phloroglucinol and HCl (X40).

PLATE 4B (lower) Transection of 35 day-old *Triticum* stem stained in chlorine-sodium sulphite (X40). (S = sclerenchyma, C = collenchyma)

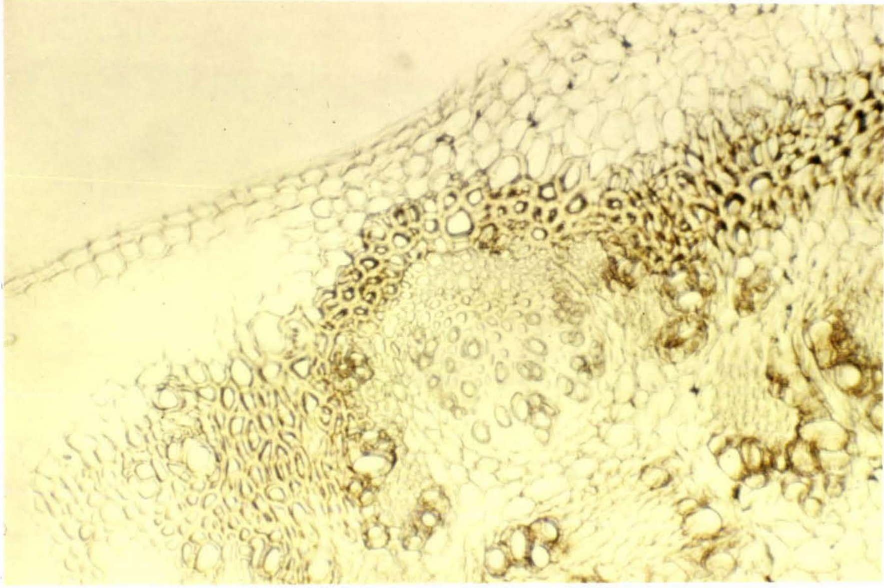
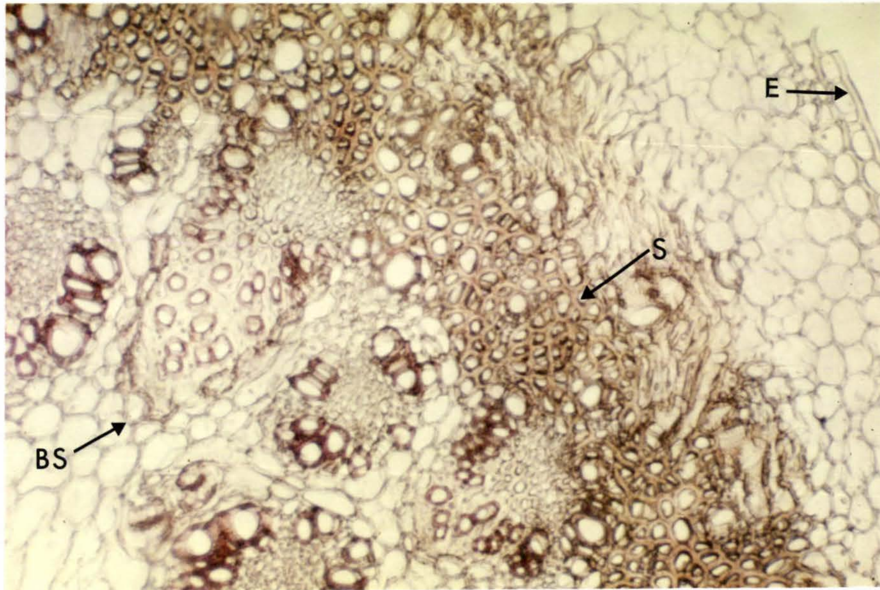


PLATE 4C Transection of 35 day-old <u>Triticum</u> stem treated with 0.5N NaOH (X40).



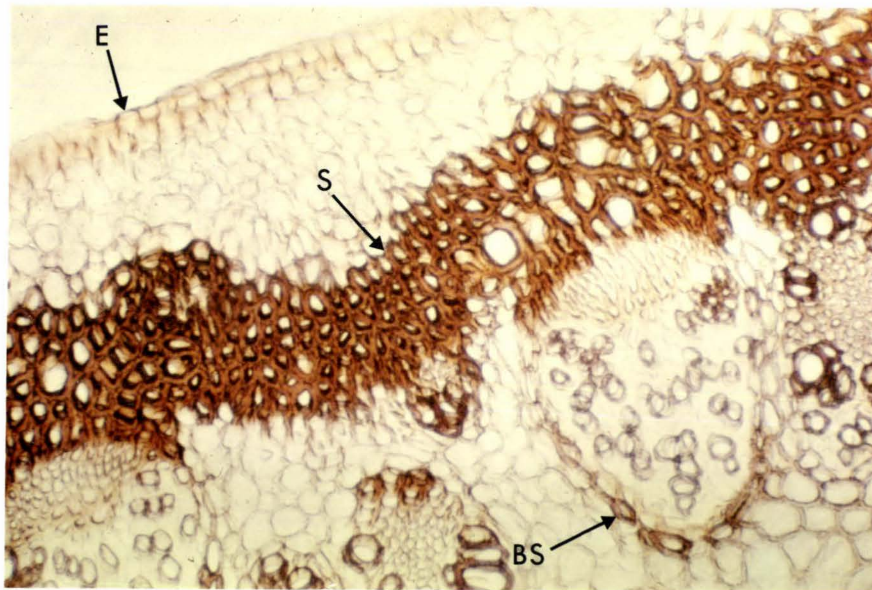
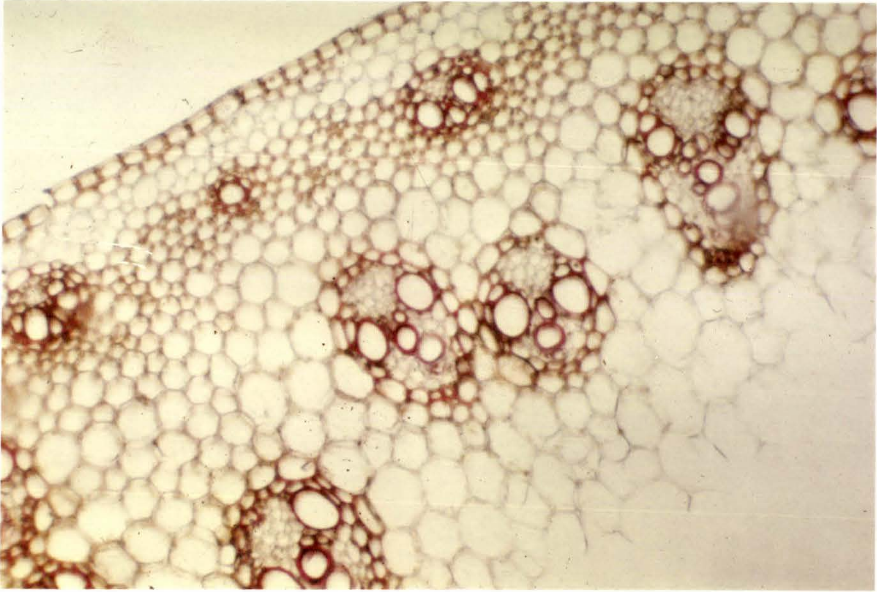


PLATE 5A (upper) Transection of 40 day-old <u>Triticum</u> stem stained in phloroglucinol and HCl (X40).

PLATE 5B (lower) Transection of 40 day-old <u>Triticum</u> stem stained in Mäule's reagent (X40). (S = sclerenchyma, BS = bundle sheath, E = epidermis)



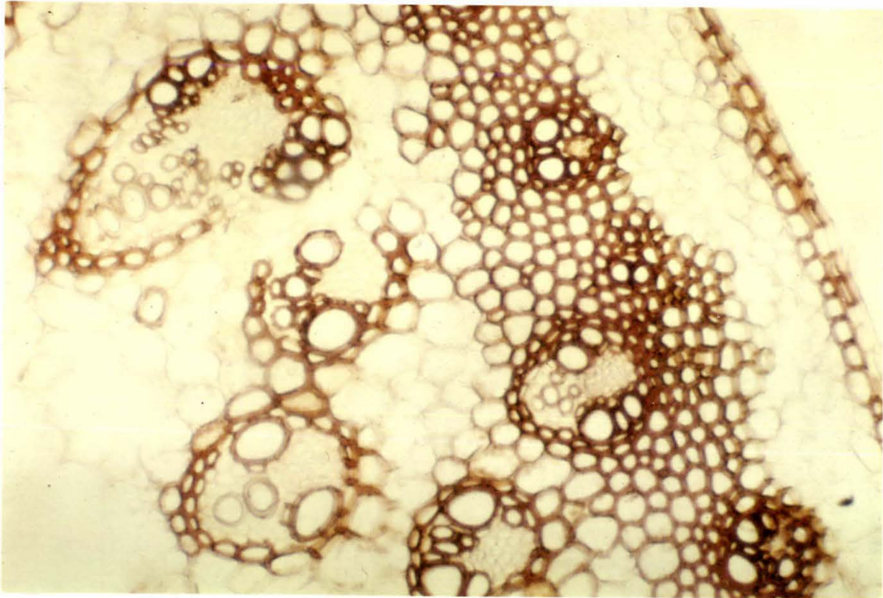
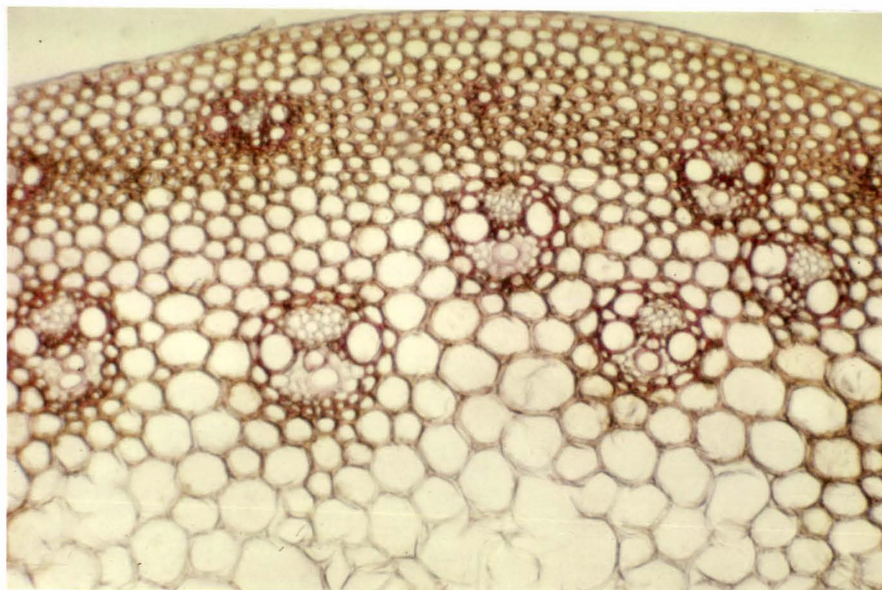


PLATE 6A (upper) Transection of 50 day-old <u>Triticum</u> stem stained in phloroglucinol and HCl (X40).

PLATE 6B (lower) Transection of 50 day-old <u>Triticum</u> stem stained in Mäule's reagent (X40).



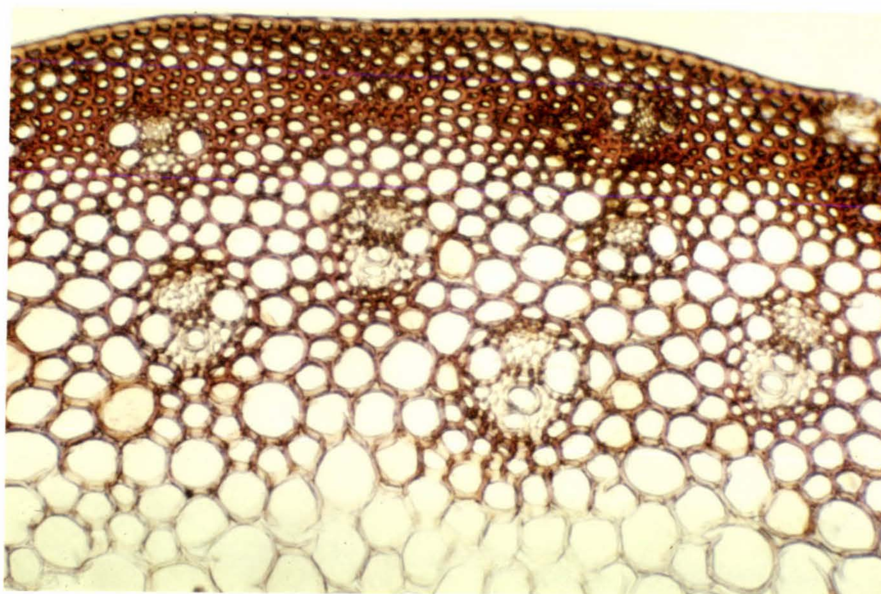
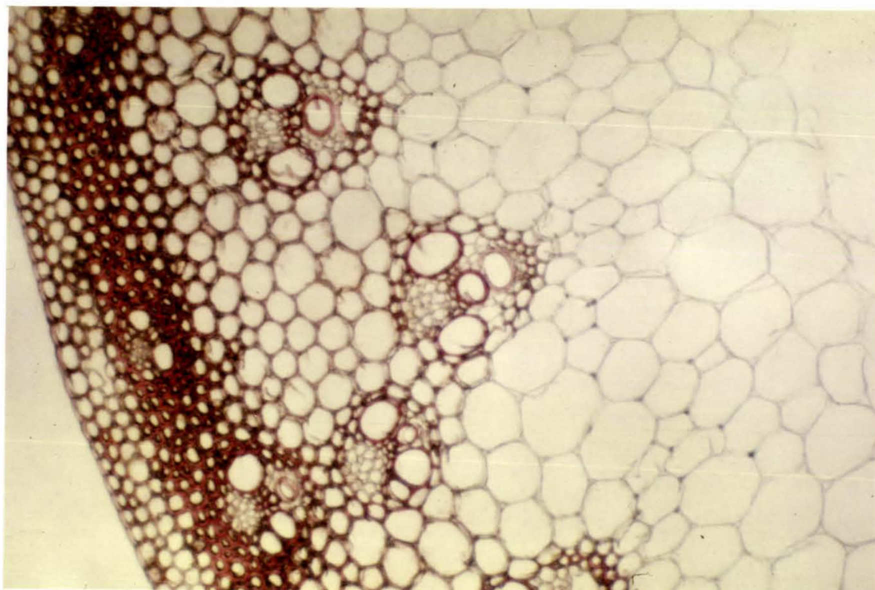


PLATE 7A (upper) Transection of 60 day-old *Triticum* stem stained in phloroglucinol and HCl (X40).

PLATE 7B (lower) Transection of 60 day-old *Triticum* stem stained in Mäule's reagent (X40).



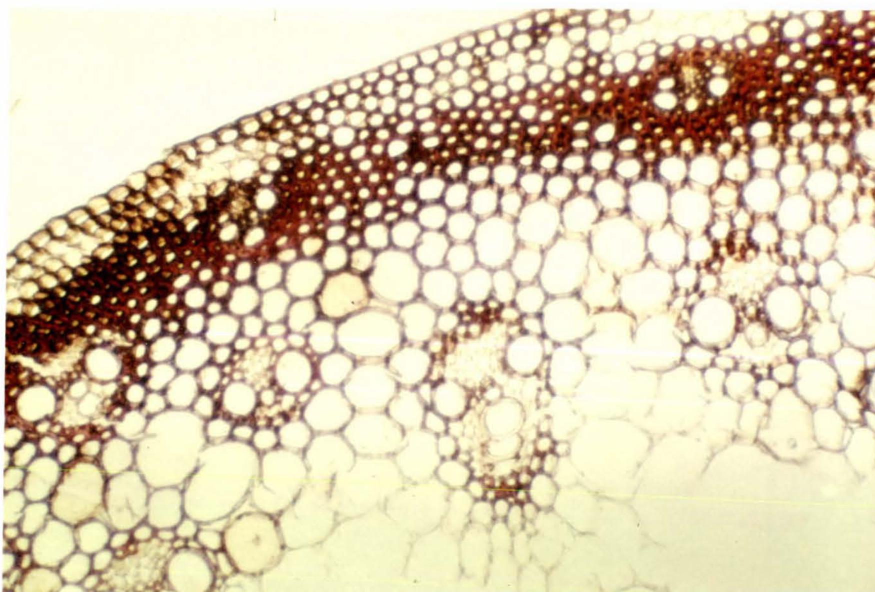


PLATE 8A (upper) Transection of 70 day-old Triticum stem stained in phloroglucinol and HCl (X40).

PLATE 8B (lower) Transection of 70 day-old Triticum stem stained in Mäule's reagent (X40).

reagent than they did with phl/HCl and an increase in sensitivity to this stain was especially noticeable between 35 and 40 days (compare Plates 4B and 5B). The majority of the lignin deposited at this time appeared to be in the subepidermal sclerenchyma fibres.

The placement of tissue sections in 72% H_2SO_4 to test the lignin for solubility, was not successful as a further means of identification. At ages earlier than 50 days the acid appeared to "dissolve" all the lignin present apart from sections of the subepidermal sclerenchyma and the occasional metaxylem vessel. This was not because the lignin shown to be present by staining dissolved, but rather the pit areas tended to break down and distribute small pieces of lignified wall over the slide. Fine pieces of pale yellow lignified wall could be found after careful examination of the preparation. It was not until the bundle sheath lignified to form an enclosure around the vascular bundle that the thin-walled cells were actually seen to dissolve away leaving a skeleton of lignified walls behind.

Safranin/fast-green staining was carried out on 20 and 60 day-old tissue only. In the younger tissue xylem only stained red and the results of the 60 day staining are shown in Section I.3.1, Plates 1A and 1B. This stain is of limited usefulness in the localization of lignified cell walls.

I.5.3 SPECTROPHOTOMETRIC ANALYSIS OF LIGNIN CONTENT

Anatomical observations showing that the base of the stem where most of the lignification takes place were supported by measurements of the relative lignin content of I-2 and whole-shoot samples. Figures 9 and 10 contain the absorption spectra for the youngest and oldest I-2 tissue analyzed. Curve (A) in each is the solution at pH 7.0 and shows the characteristic maximum for neutral lignin preparations at 280mu. Curve (B) illustrates how

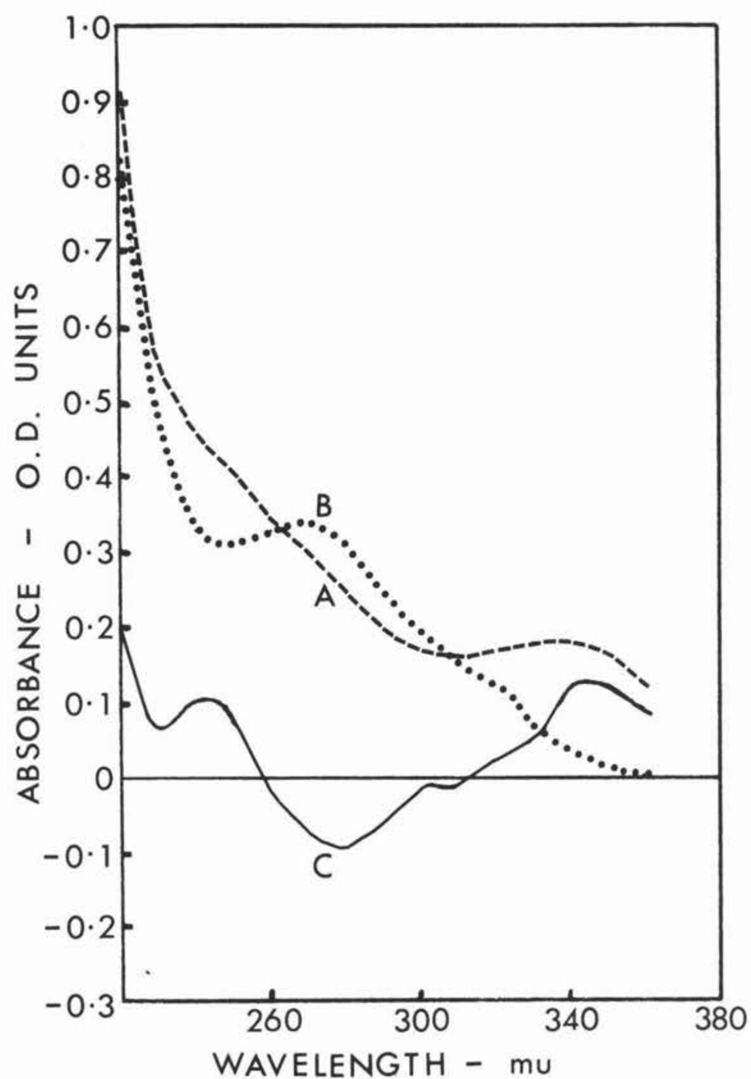


FIGURE 9. Absorption spectra of a crude lignin preparation from 10 day-old wheat at pH 7.0 (A) and pH 12.3 (B), and the difference spectrum (C). The solution in the cuvette contained lignin extracted from 200 μg of plant material per ml.

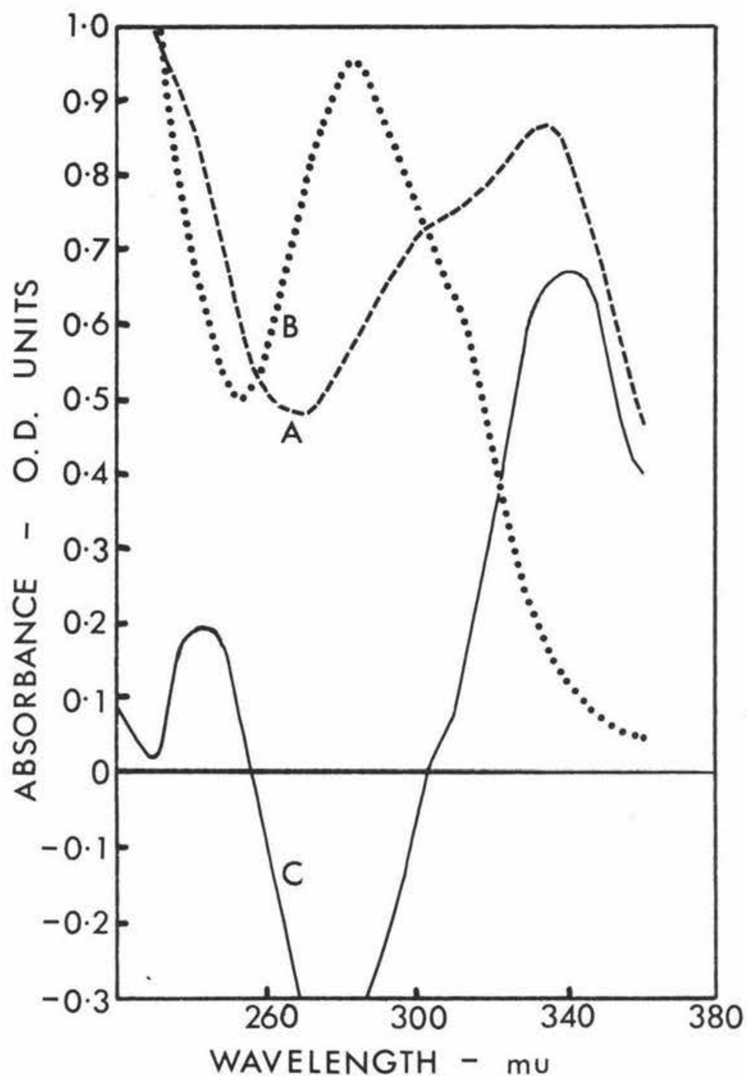


FIGURE 10. Absorption spectra of a crude lignin preparation from 71 day-old wheat at pH 7.0 (A) and pH 12.3 (B), and the difference spectrum (C). The solution in the cuvette contained lignin extracted from 200 μg of plant material per ml.

the maximum shifts under alkaline conditions to a longer wavelength. The ionization difference (ΔE_i) spectra, Curves (C), describe more clearly than the original absorption curves the composition of the lignin preparation. The ΔE_i spectra show maxima at 245 to 250mu and at 345mu, and a minimum at 270mu. A shoulder appears at 300mu that is more pronounced in the younger I-2 preparation. As explained in Section I.3.3 the shape of the ΔE_i curve is due primarily to the absorbance of ionized phenolic hydroxyl groups. Non-conjugated phenolic hydroxyl groups cause the shoulder at 300mu (in some lignin preparations there is a large peak at this wavelength (57)), and conjugated phenolic groups cause the much larger maximum at 345mu and also the maximum at 250mu. The proportion of conjugated aromatic hydroxyl groups is seen to increase with age more than other species in the hydrolysate, although the absorbance at 300mu is interferred with by the larger peak next to it, and is difficult to measure.

The ΔE_i spectra for 20, 31, 40, 50, and 60 day-old wheat samples were recorded in a similar way to 10 and 71 day-old samples and the absorbance at 345mu of each translated into the values that appear in Figure 11. Relative lignin content is expressed as a percentage of the absorbance of the ΔE_i curve at 345mu of 71 day-old I-2 tissue, i.e.

$$\frac{O.D._{345mu}\ \text{sample}}{0.67} \cdot 100$$

The procedure was duplicated for each age of plant and on no occasion did the individual values differ more than 7% from the mean.

On a percentage dry weight basis the lignin content of the whole shoot was much less than that of the second internode. At maturity, samples from the whole shoot contained only 28.1%

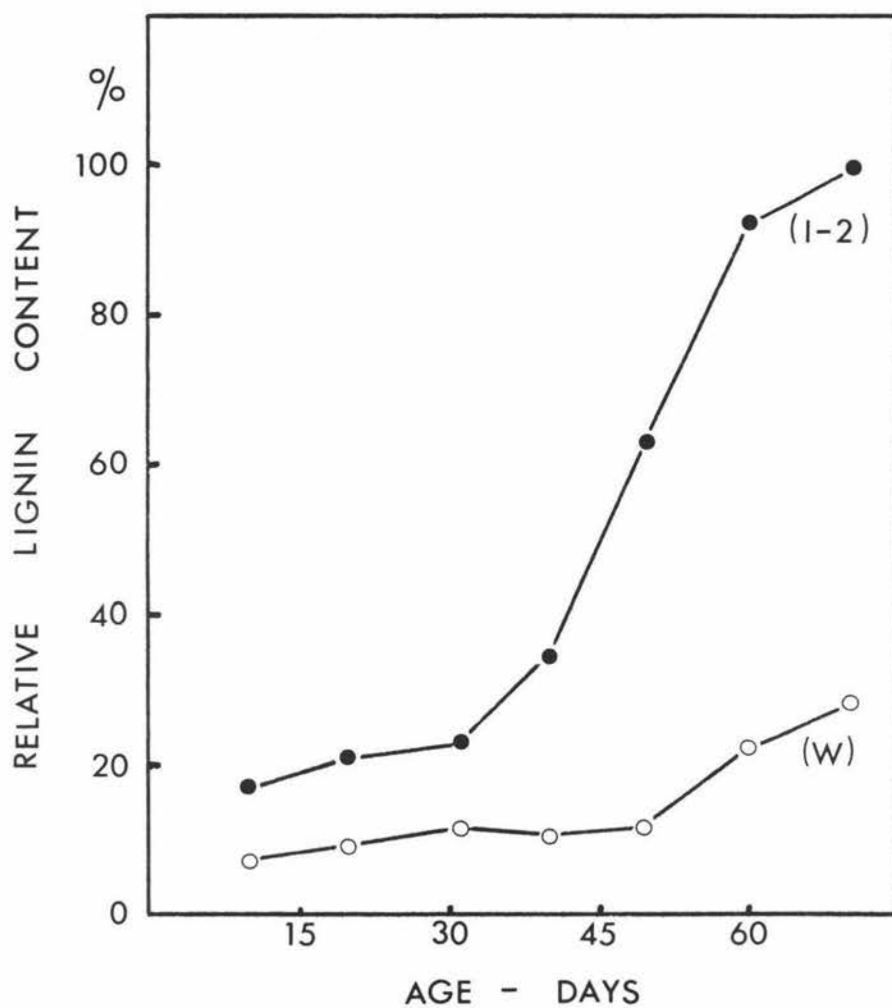


FIGURE 11. The change in lignin content with time of second internode tissue (I–2) and of the whole shoot (W).

Each point on the graph represents an absorbance value expressed as a percentage of the absorbance of 71 day-old second internode tissue, which was 0.67 O.D. units measured at $\lambda = 345$ mu (see Fig. 10).

of the lignin of the I-2 sample. At younger ages (less than 40 days) whole-shoot samples were almost entirely leaf material as the total stem length was less than 0.5 mm. A slight rise in lignin content is observed beyond 50 days as stem tissue makes a contribution to the sample. The stem is observed histochemically to contain more lignin than the leaf for although bundle sheath fibres are lignified earlier in the leaf, the mesophyll parenchyma remain thin-walled throughout development to function photosynthetically.

I.6 DISCUSSION

It is evident from the results that major developmental changes occurred in the wheat plant at a time 30 to 35 days after germination. Before this time the plant was still very small, leaf material contributing mainly to the dry weight of the shoot as the stem had not yet elongated; lignin content was low and restricted almost entirely to the vascular tissue. After this time lignin content of the stem and shoot dry weight increased rapidly and the plant "bolted" with the onset of flowering. A heavily lignified cylinder of sclerenchyma fibres developed within the stem. The sensitivity of these fibres to the test for syringyl groups is a qualitative indication of the increase in methoxyl content of the lignin formed. From the work of M. Phillips et al it is also known that in wheat (116), barley (117) and oat (118), each of which have similar lignification kinetics, total plant nitrogen is decreasing during this growth period (in wheat total plant nitrogen is highest at 7 days and at 35 days contains less than 50% of the maximum value) and the increase in cellulose parallels that of lignin. Causal factors involved in initiating the changes outlined above are obscure and much of the discussion of the problem is necessarily speculatory until the relationship of lignification to whole plant development is more closely defined.

The histochemical observations made in this study support the suggestion by K.E. Wolter et al, 1974 (166) that the guaiacyl and syringyl lignins in angiosperms are compartmentalized with guaiacyl-syringyl lignin in the middle lamellae and cell corners. R.A. Jeffs and D.H. Northcote, 1966 (88), working with bean callus and O.L. Gamborg, 1967 (49), working with potato, found that the lignin produced in tissue cultures differed from the lignin produced within the plant in that it contained no syringyl groups.

This may have been because fibres were included in the control samples for nitrobenzene oxidation whereas the cultured material would have only contained vessels and tracheids. However, while guaiacyl lignin is in the main restricted to xylem vessel walls and middle lamellae and syringyl lignin is deposited in the developing sclerenchyma and bundle sheath fibres (illustrated most clearly in Plates 5A and 5B), this is not exclusively so in wheat. T. Higuchi (76) found syringyl as well as guaiacyl lignin to be present in vessels formed early in development in bamboo. These differences could indicate that the factors controlling variation in lignin composition may not be as rigid, or even the same, in grasses as in dicotyledons. Differences in the hemicellulose component of the cell wall, for example, are known to exist between some monocotyledons and dicotyledons (21) and this may be related to differences in the lignin as hemicelluloses are thought to be the means by which lignin attaches to the wall (159).

The development and lignification of fibres within the stem is almost certainly responsible for the large increase in lignin content observed at 40 days in wheat. Fibre development is most advanced at the base of the stem and Figure 11 shows that this part of the plant has the highest extractable lignin content.

Phillips et al (116) and Stone et al (154) both observed a rise in methoxyl content comparable to lignin content with increasing age. Stone et al also observed very similar increases in vanillin and syringaldehyde (i.e. after nitrobenzene oxidation) with time, indicating that a mixed guaiacyl-syringyl type of lignin continues to be formed until the wheat plant is mature. A study of the oldest stem sections reveals very little difference in the response of the sclerenchyma to each stain apart from a greater reaction of the middle lamellae to phl/HCl (Plates 8A and 8B).

Histochemical studies are limited by the sensitivity of the stains used and the intensity of a reaction is variable with thickness of the sections. Sectioning was initially carried out at 10 day intervals on the freezing microtome and when it was found that the critical time of change was between 30 and 40 days, hand sections were made of 35 day-old stems and checked against different reagents. Judging by the reaction of the xylem to Cl_2/Na_2SO_3 (Plate 4B), this stain appears less sensitive to syringyl groups than $KMnO_4/HCl/NH_3$, keeping in mind that this was a hand-cut section of unknown thickness.

The similarity of Triticum anatomy and pattern of lignification to Avena (41, 118) and Hordeum (41, 117) suggests that most of the lignin in these plants is also in fibrous tissue and the factors initiating rapid lignification may be similar.

While the significance of lignin compartmentation is elusive, there are many developmental, morphological and functional differences between fibres and vessels (41) any one of which could result in different lignin content. Work by T.L. Shininger, for example, in 1970(136) has shown that the differentiation of xylem fibres in Xanthium internodes is halted by removing the bud and young leaves, while cambial derivatives still form and xylem vessel walls thicken and lignify. Xylem fibres are thin-walled and parenchymatous until they are formed simultaneously with the development of a new leaf. When the leaf passes the stage of rapid expansion it no longer has an effect on fibre wall-thickening and lignification. Although Xanthium is unusual in that cambial division occurs in the absence of leaves and buds or exogenously applied hormones (cf. the findings of P.F. Wareing (162) and A. Hejnowicz and M. Tomaszewski (73)), this study does indicate that the development and lignification of the secondary walls of vessels and fibres may be controlled by separate factors.

The interaction of the 'physiological' plant with individually lignifying cells and tissues is relatively unexplored (see section in the review by S.A. Brown, 1966 (14)). Plant growth substances are known in some instances to stimulate lignification (8, 44, 126, 165, 67) and in others to inhibit it (139, 44, 165); the role of hormones in controlling lignification is unclear. At the cellular level the indications are that the changing physiological balance of the cell with time bears direct relevance to the production of lignin precursors. Cytokinins, for example, may be involved in increasing lignin synthesis indirectly by causing cells in tissue culture to stick together (67). O.L. Gamborg and his coworkers working specifically with tissue culture material are currently investigating the importance of the cell cycle and the rate of cell division in the biosynthesis of secondary metabolites (51). Lignification is thought to promote cellular senescence (140) and though there are exceptions (94) it is true that cells with lignifying walls die and become empty transport channels or rigid supportive shells. A change from anabolic to a catabolic process in the cell could result in the products of protein break-down (e.g. phenylalanine, tyrosine, S-adenosyl methionine) becoming available for lignin biosynthesis. An interesting parallel may be made here with the onset of whole plant senescence in the change from the vegetative to the reproductive stage of growth in wheat and the formation of a rapidly lignifying block of tissue. Early xylem vessels would lignify in a meristematic environment, similar to that in tissue cultures, and the fibres amidst regions of cells approaching senescence. In Brassica napo-brassica (swede) root the parenchyma of the secondary xylem remain unlignified until the time of rapid expansion of the inflorescence when they undergo rapid lignification. These same cells lignify in response to the

ethylene treatment of discs of tissue cut from the root (123).

The relationship between flower initiation, the development of the fibres and the elongation of the wheat stem is of particular interest. Figure 8 shows that the elongation of the shoot is delayed approximately 10 days from the start of lignification. (The increase in total stem length with time (Figure 8) coincides with the increase in whole-shoot lignin (Figure 11) but this comparison is invalid as the samples for the lignin estimates did not contain only stem material.) The elongation of the internode <u>after</u> an increase in lignin content is the reverse of the procedure as it is thought to occur in individual cells. S.M. Siegel and F. Porto (140) suggested that lignification limited cell expansion by acting antagonistically towards growth promoters such as auxin for example, and A.B. Wardop (159) has suggested that lignification may limit the enlargement of plant cells by immobilizing the hemicellulose matrix, thereby preventing surface growth. F.W. Whitmore (165) however, was unable to obtain conclusive evidence to support the notion that the lignin of wheat coleoptile cell walls limits growth.

The walls of cells that continue to elongate for some time after becoming lignified, do so in a pattern (e.g. spiral thickening) that allows for extension and this supports the idea that lignin immobilizes wall components. An electron microscope study by P.K. Hepler <u>et al</u> (74) shows that both lignification and secondary wall development may proceed together in wound vessel members in <u>Coleus</u> and that lignin deposition may be initiated before the cell has finished growing. Lignin deposition may therefore be in a position to limit expansion. The situation may differ in fibres as growth may be intrusive and the middle portion of the cells may become lignified before the tips do so (41). Fibres

that grow intrusively are thought to do so only after the surrounding cells have completed growth or are only slightly expanding and again, an increase in lignin content would be expected to follow an increase in internode length.

The growth of the internode is by the elongation of cells produced by the intercalary meristem(41), a region of active cell division immediately above the node. Cells already present prior to activation of the meristem may become lignified before the elongation of the stem is measurable and in this way artificially create the delay seen in Figure 8. It seems unlikely that increased lignification and fibre development induce rapid stem elongation and more reasonable to suggest that they are all a part of similar physiological conditions at the time of change from vegetative to reproductive growth. As wheat is daylength sensitive it is likely that the signal to the plant to flower is transmitted via the leaves to the nodal area. Cell division at the node would be followed by elongation and subsequently, differentiation and lignification.

I.7 SUMMARY

1. The rapid increase in lignin content observed in wheat 35 to 40 days after germination is primarily the result of the development and lignification of a cylinder of sclerenchyma fibres at this time and fibre differentiation is most advanced at the base of the stem. Xylem vessel wall thickening and lignification is seen as contributing to the early low lignin content.

2. Lignin formed early in development is mainly of the guaiacyl type and later formed lignin incorporates syringyl units.

3. The changes in lignin quality and quantity are accompanied by the major developmental events of rapid stem elongation ("bolting") and flowering.

I.8 CONCLUSION

While it is clear that the anatomical basis for the rapid increase in lignin content in wheat is extensive fibre differentiation, the events initiating the change in tissue development and the type and quantity of lignin present are not. A relationship is indicated between flowering, stem elongation and lignification that is worthwhile exploring and defining more closely. Some experiments were attempted, for example, investigating the influence of IAA, GA_3 and 6-amino furfuryl purine on excised portions from the base of 35 day-old wheat stems but these did not yield any useful information because of technical difficulties. Even though our chemical and biosynthetic knowledge of lignification is extensive and enzymological studies are at present being carried out by several groups of research workers (these aspects of lignification are discussed more fully in Part II of this project) lignification remains a physiological enigma. Information on the way in which the environment is able to influence when, where and what type of lignin is formed would provide a useful basis for future work and, more importantly, a fuller understanding of the balance of the controlling factors in lignification.

PART II BIOCHEMICAL ASPECTS OF LIGNIFICATION IN WHEAT

II.1 INTRODUCTION

This study is primarily concerned with the regulation of lignin biosynthesis in higher plants, in particular, wheat. An evaluation is made of the evidence supporting suggestions in the literature that regulation is at any one point in the pathway, special attention being given to the supposed role of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase in controlling lignification. The evidence for a possible minor pathway involving phenyllactic acid and hydroxyphenyllactic acid is discussed and an outline of experiments designed to test for the presence or absence of the hydroxyphenyllactate pathway both before and after the initiation of rapid lignification in wheat, is presented.

II.2 THE REGULATION OF LIGNIN BIOSYNTHESIS

II.2.1 AN OUTLINE OF THE PATHWAY

The pathway of lignin biosynthesis has been elucidated by in vitro polymerization studies, experiments using isotopic tracer techniques, enzyme studies on tissue extracts and cytological observations attempting to relate biosynthetic events to structural change (14, 15). An abbreviated version of the currently accepted pathway of lignin biosynthesis in plants with both L-tyrosine ammonia-lyase (TAL) and L-phenylalanine ammonia-lyase (PAL), is presented in Figure 12. While individual experiments concerned with a portion of the pathway may be highly significant, attention is drawn to the fact that each is a part of a long complex pathway, much of which is still uncertain and along which there may be many opportunities for control.

The incorporation of $^{14}CO2$ into lignin was first studied by J.E. Stone in 1952 (153) using wheat and this work was continued by S.A. Brown, K.G. Tanner and J.E. Stone, 1953 (19). By measuring the activity in lignin degradation products it was found that once ^{14}C had been bound into lignin (as represented by syringaldehyde) it did not re-enter the respiratory pathways showing that lignin is a stable end-product and that the syringyl component at least, is not re-metabolized in wheat.

The work of B.D. Davis, 1951 (32) established shikimic acid as an obligatory intermediate in the biosynthesis of aromatic amino acids from carbohydrate in microorganisms and the idea that aromatization may also occur via shikimimate in higher plants was subsequently tested. S.A. Brown and A.C. Neish (16) were able to show that ^{14}C - shikimic acid and ^{14}C-L-phenylalanine were incorporated into the lignin polymers of Triticum vulgare and Acer negundo (maple) with comparable efficiency. Shikimic acid

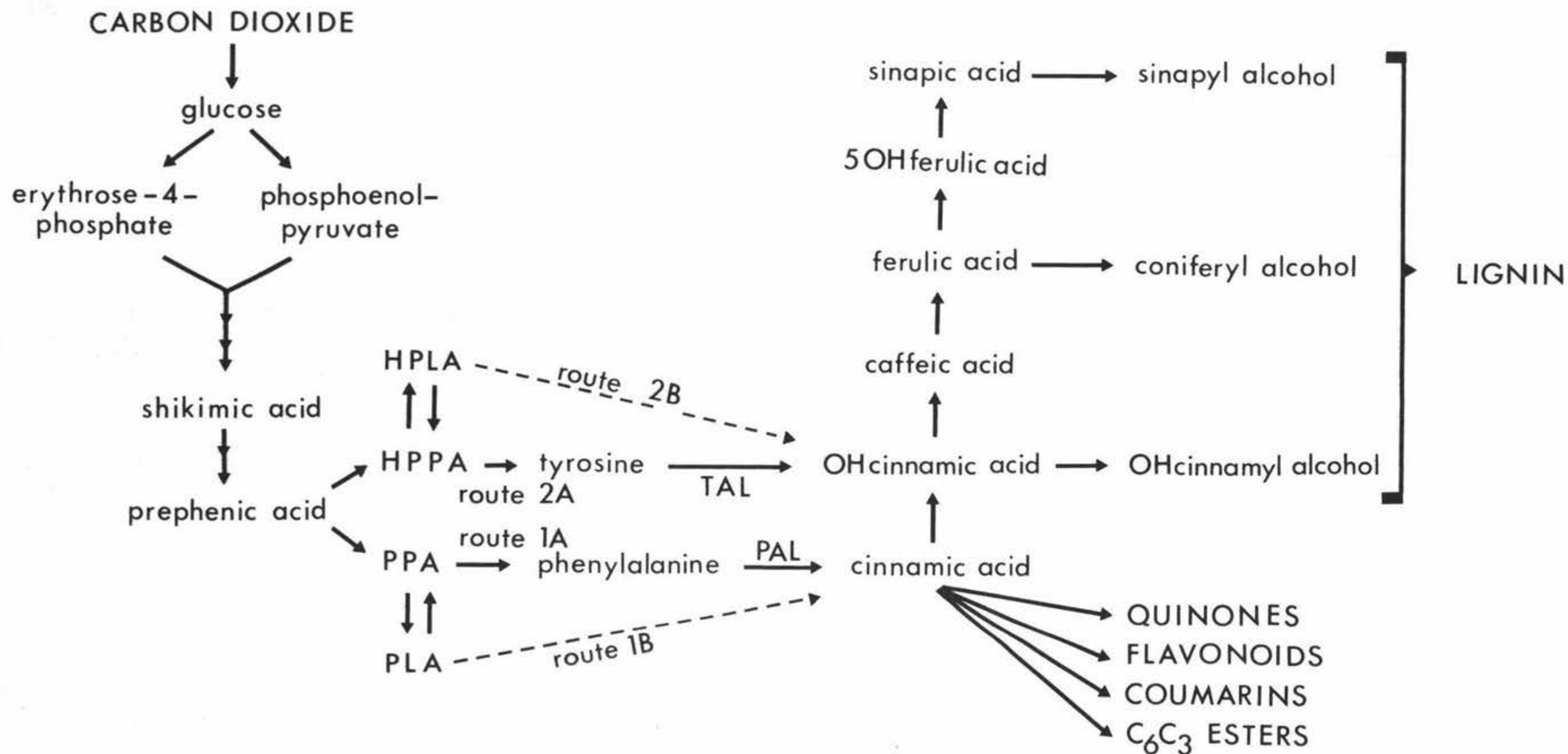


FIGURE 12. The pathway of lignification as thought to occur in the Gramineae and an indication of the involvement of the cinnamic acids in secondary metabolism.

labelled at positions 2 and 6 of the cyclohexene ring was shown by G. Eberhardt and W.J. Schubert (35) to be incorporated into the lignin of Saccharum officinarum (sugar cane) without randomization of the label and in Salvia splendens (97), Triticum vulgare and Fagopyrum tataricum (50) ^{14}C-shikimic acid was found to be a precursor of both phenylalanine and tyrosine. The involvement of shikimic acid has also been demonstrated in the biosynthesis of caffeic acid (98) and quercetin (157). Following work with microorganisms once more (137) D-glucose was identified as the carbohydrate source for lignin biosynthesis in higher plants (134, 2). S. Yoshida and G.H.N. Towers found in 1963 (170) that shikimic acid is formed from phosphoenolpyruvate and erythrose-4-phosphate in Pinus resinosa. Thus the main links of the shikimic acid pathway have been established for higher plants. While it is likely that this pathway does function, and precursor incorporation studies have been supported by the detection of the corresponding enzymes in plant tissue extracts, shikimate kinase has yet to be detected in higher plants (168), and there is some evidence that quinic acid is also involved in the biosynthesis of aromatic amino acids (164, 49).

L-phenylalanine has been shown by a number of workers (16, 17, 18, 20, 98) to be a good precursor of lignin in a wide range of plants while L-tyrosine incorporation has been demonstrated for members of the Gramineae only (17, 20).

Although the activity of the enzymes of the shikimate pathway has been shown to increase in conditions promoting the synthesis of polyphenols (e.g. after wounding (105)), and to be high in lignifying regions of the plant (83), it is unrealistic to look at changes in the activity of these enzymes as being of specific regulatory significance in lignification as they are equally involved in protein metabolism (106) and in the biosynthesis

of a wide range of secondary metabolites present in plant tissues (168, 149).

Likely candidates for the control over secondary metabolite production are PAL and TAL which provide the precursors cinnamate (92) and p-OH cinnamate (109) for the formation of flavonoids, quinones, lignins and cell wall esters (108, 149). They are situated at the metabolic branch point diverting the flow of aromatic amino acids from protein through to the phenolic compounds. PAL has been shown to be present in the higher cryptogams (172), Basidiomycetes (121, 6) and Streptomyces (38), while TAL is, in the main restricted to members of the Gramineae.

The role of PAL in phenolic biosynthesis has been studied in detail (reviewed by E.L. Camm and G.H.N. Towers, 1973 (23)) and the synthesis (or prevention of inactivation) of PAL has been shown to regulate the synthesis of chlorogenic acid in potato tuber tissue (174) and to be correlated with the accumulation of flavonoids in strawberry leaf discs (27), grapefruit (101) and strawberry fruits (85). The activity of PAL may be photoinduced via phytochrome but enzyme levels may also be insensitive to the light (23, 149), indicating the involvement of more than one form of PAL or of regulatory factors other than phytochrome. Evidence for the light-induced control of ammonia-lyase activity is not common in monocotyledons but has been reported to exist in Hordeum vulgare where it is also correlated with the production of flavonoids (133, 99, 100). The wide distribution of the ammonia-lyases along with studies correlating increased enzyme activity with increased lignification in Hordeum (109), Fagopyrum (buckwheat) (169), Phyllostachys (78), Pisum sativum (dwarf pea) (25) Coleus and Glycine (soybean) (127), supports a role for these enzymes as regulators of lignin biosynthesis. Work by P.H. Rubery and

D.H. Northcote (128) localizing PAL in the xylem of Acer (sycamore), Apium (celery) and Pisum also supports the idea that PAL activity is closely linked to lignification. PAL activity was absent in Phaseolus (bean) callus tissue and Parthenocissus (Virginia creeper) crown gall. However the evidence so far for the involvement of PAL and/or TAL in the control of lignification is correlative rather than definitive and is to be contrasted with studies where high ammonia-lyase activity is recorded early in development. M. Young (171) recorded maximum PAL and TAL activity 7 days after germination in Triticum (see Figure 1) and PAL levels are highest at the time of maximum growth in cell suspension cultures of Glycine (65) and Petroselinum (parsley) (66). Also, it is true that while S. Yoshida and M. Shimokoriyama (169) observed increased activity of PAL in the more mature and lignified parts of the buckwheat stem, the highest enzyme levels were recorded at the shoot apex. The work of C.K.C. Cheng and H.V. Marsh (25) investigating the effect of GA_3 on dwarf pea is sometimes cited as evidence for the regulation of lignification by PAL (127, 59) but in fact maximum PAL activity occurred 16 to 18 days before an increase in lignin content was observed. These latter observations support only an indirect involvement of the ammonia-lyases with lignification and it may be that another pathway (e.g. the direct dehydration of phenyllactate) provides the phenylpropanoid precursors later in development or that the products of deamination are stored until they are required.

It is possible that the ammonia-lyase activity in young tissue may be related to the differentiation of early xylem tissue and that the coincidence of enzyme activity with maximum protein synthesis (116, 172) is a result of extracting the whole plant rather than specific tissues. It is unlikely that a probable high concentration of phenylalanine at this time is responsible for

inducing the enzyme (174) (cf. suggestion in (169))and more likely that the high PAL (and TAL in wheat) levels do regulate the formation of some, as yet unknown, secondary metabolite(s) or alternatively, are involved in early xylem differentiation. Unfortunately the work of M. Young on PAL and TAL in wheat is not continued into the time of rapid lignification and it is not known whether or not enzyme activity increases at this time, approximately 40 days after germination. P.H. Rubery and D.E. Fosket (127) working with cultured Coleus internodes and soybean callus observed a temporal separation between maximum protein synthesis and the peak of ammonia-lyase activity. The latter reflected the pattern of xylogenesis. This study focuses on the problem of claiming a single factor as responsible for the regulation of differentiation and lignification as identical manipulation of the balance of hormones supplied exogenously to Coleus and Glycine provoked a different response from each. The following explanation is offered by the authors " ... PAL is synthesized as a part of the genetically programmed sequence of xylem differentiation which is initiated in these systems by the addition of a limiting factor which allows the appropriate set of internal conditions for differentiation to be reached." The function then of PAL and TAL as rate-limiting enzymes in lignin biosynthesis is uncertain.

The association of ammonia-lyase activity with a variety of cellular organelles and cell fractions and the range of values for the molecular weight of purified forms of PAL and TAL (150) suggest that the pathways to various phenylalanine and tyrosine- derived end-products may be distinct spatially and biochemically. A. Boudet et al (10, 3) for example, have isolated two forms of PAL from Quercus (oak) roots and this appears to be the way in which the C_6C_3 and C_6C_1 pathways of biosynthesis are regulated separately.

One form, associated with the microsomal fraction, is sensitive to feedback inhibition by cinnamic acid and the other, associated with the fraction including mitochondria amd microbodies, is benzoate-sensitive. D.B. Harper, D.J. Austin and H. Smith (71) have also obtained evidence that suggests flavonoid synthesis in Pisum, may be regulated by a spatial separation of precursor "pools" destined for different end-products. At the cellular level, J.D. Pickett-Heaps (119), combining the techniques of electron microscopy and auto-radiography, observed the accumulation of administered tritiated cinnamic acid in the golgi vesicles of differentiating xylem vessels and the aggregation of these vesicles near the bands of wall thickenings. A scheme relating the subcellular and biochemical events in the formation of C_6C_3 phenolic compounds and the accumulation of esters, flavonoids and lignins is presented by H.A. Stafford in a recent review of the subject (150). It is proposed here that several multienzyme complexes are involved in regulating secondary metabolism and while such a view is largely speculatory it is a possible interpretation of the evidence so far.

The formation of p-hydroxycinnamic acid either directly from tyrosine (109) or from cinnamic acid (107) is followed by the hydroxylation of this substance to caffeic acid, methylation to ferulic acid, hydroxylation at the 5 position on the benzene ring to 5-OHferulate and finally, methylation to sinapate (15). Although it was originally thought that this sequence involved modification of the "ethanol-soluble" cinnamate derivatives, from tracer studies during which either the free acids or salts of these were incorporated (98), the evidence is now in favour of the "ethanol-insoluble" cinnamic acid esters as the natural intermediates in lignification in wheat (37, 36). The nature of these metabolically active esters is not known but it has been suggested they

may be cinnamoyl-CoA-protein derivatives (37, 173). The situation in grasses is complicated by the presence of large quantities of p-OHcinnamic acid and ferulic acid esters (145, 93) that do not appear to be part of the lignin "core" (14). After extensive study of natural and induced lignins in Phleum and species from other families H.A. Stafford (145, 146) has concluded that there are two types of lignin present in grasses. One is essentially a polymer of ferulic acid and varying amounts of p-OHcinnamic acid, and the other is based on coniferyl alcohol, coniferyl aldehyde and sinapyle-type units.

It is likely that regulation of the later stages in the lignification pathway does occur to ensure that the methoxyl content of lignin is modified according to cell-type (166) but the activity of caffeic acid O-methyltransferase has been shown to be low at the time of rapid lignification (53). The same enzyme is thought to be responsible for the methylation of 5-hydroxyferulate in some species (135). A partial reversal of the 5-hydroxyferulate to sinapate reaction (81) as well as the conversion of sinapate to p-hydroxycinnamate (37) may take place in young wheat plants, but the significance of demethoxylation in the regulation of lignin composition has not been assessed. The availability of methionine, known to be the methyl group donor for ferulate and sinapate formation (22, 135), may be a factor influencing the type of lignin formed (i.e. guaiacyl cf. guaiacyl-syringyl type lignin).

p-Hydroxycinnamic acid, ferulic acid and sinapic acid (see Figure 12) may accumulate as end-products independantly of each other (150), and each is reduced to the corresponding alcohol before incorporation into the lignin polymer (15). This reduction was demonstrated for ferulic acid by T. Higuchi and S.A. Brown in 1963 (81). The higher specific activity of

isolated coniferylaldehyde than coniferyl alcohol suggested that this substance was intermediate in the conversion. The activated forms of cinnamic acids, the cinnamoyl-CoA esters, were prepared and characterized in 1966 by G.G. Gross and M.H. Zenk (62) and the in vivo reduction of cinnamic acid, firstly to cinnamaldehyde and then to cinnamyl alcohol was first shown to occur in Neurospora crassa, a non-lignin-producing fungus (60, 63). In 1970 K. Hahlbrock and H. Grisebach (64) were able to demonstrate the formation of cinnamoyl-CoA esters with enzyme preparations from parsley cell cultures and in 1972 R.L. Mansell et al (103) published evidence that the CoA ester participated as an activated intermediate in the reduction of ferulic acid to coniferyl alcohol in higher plants. p-OH cinnamic acid appears to be similarly reduced (34, 152). The three enzymes involved in the reduction of ferulic acid to coniferyl alcohol were extracted from a higher plant by G.G. Gross et al, 1973 (61) and cinnamyl alcohol dehydrogenase has since been purified and characterized from a wide variety of taxonimically different plant groups and plant tissues revealing a possible correlation between the activity of this enzyme and lignification (102). In a series of papers M.J.C. Rhodes and L.S.C. Wooltorton (122, 123, 124) associate an increased synthesis of a lignin-like material during the ageing of swede root discs with increased activity in the enzymes involved in phenolic biosynthesis and suggest a role for the CoA synthetase enzyme in lignin biosynthesis (125).

Before they are incorporated into lignin the p-OHcinnamyl, coniferyl and sinapyl alcohols undergo an enzyme-initiated radical formation (45, 15). The enzyme thought to be responsible for this is peroxidase which acts in the presence of H_2O_2 (75, 70). Radical formation allows a variety of linkages between each of the phenylpropanoid units (130). Subsequent polymerization of these

units is thought by some to be spontaneous (15). Others suggest however that additional regulatory mechanisms may operate even later than the enzyme-mediated oxidation of lignin monomers as often cells with an adequate supply of phenolic precursors and peroxidase do not form lignin (146, 149). The short half-life (of the order of a minute) of the monomer radicals makes it clear that their polymerization most probably occurs within the matrix of the wall at the site of lignin deposition and it is possible that both the sequence and availability of points of attachment for the lignin polymer may be a regulatory factor. The polymerization of lignin appears to be the final process in the formation of the cell wall as a living, growing structural material.

II.2.2 THE ROLE OF PHENYLLACTIC AND HYDROXYPHENYLLACTIC ACID

Prior to the discovery of L-phenylalanine ammonia-lyase (PAL) by J. Koukol and E.E. Conn in 1961 (92) and of L-tyrosine ammonia-lyase (TAL) by A.C. Neish, also in 1961 (109), lignification was thought to proceed via the phenylpyruvate(PPA) and phenyllactate (PLA) derivatives as shown in Figure 3. This scheme resulted from work by D. Wright et al (167) in which the radioactive stereoisomers of phenyllactate were compared with L-phenylalanine as lignin precursors in five plant species. In all species (-)-phenyllactate was a relatively good precursor, in no case being used more than three times less efficiently than phenylalanine and in one case, that of Salvia, was incorporated more efficiently into the lignin aldehydes (vanillin, syringaldehyde and p-hydroxybenzaldehyde).. Further work with ^{14}C-labelled phenylalanine, tyrosine, p-hydroxyphenylpyruvic acid (HPPA) and p-hydroxyphenyllactic acid (HPLA) established that wheat was able to use all these compounds efficiently in the biosynthesis of lignin while Fagopyrum tataricum (buckwheat) and Salvia splendens (salvia) used phenyl-

alanine only (20). A comparison of a number of species led to the suggestion that grasses possess a specific tyrosine-metabolizing enzyme or that non-grasses lack tyrosine transaminase or oxidase or the enzyme required to convert HPPA to the lignin monomers (17).

In 1958 S.N. Acerbo et al (1) found that carboxyl-labelled HPPA was incorporated into Sacchrum officinarum (sugar cane) lignin without randomization of the label. After fifteen days of metabolism 71% of the introduced activity was recovered in the isolated lignin which is an unusually high value if the pathway involves the obligatory formation of tyrosine. The channelling of this amino acid into protein and phenolic derivatives other than lignin might be expected to account for more than 29% of the total. However the sugar cane plants used were "mature" and possibly lignifying rapidly, if this species, which is also a member of the Gramineae, follows the pattern of lignification observed by J.E. Stone et al for wheat (154). An investigation by D.R. McCalla and A.C. Neish in 1959 (98) into the capacity of a number of ^{14}C-labelled compounds to act as precursors for the phenolic acids thought to be intermediates in lignification (p-OH cinnamic acid, caffeic acid, ferulic acid, sinapic acid), re-affirmed the then current thinking by showing that (-)PLA may be an efficient precursor of lignin monomers in Salvia.

With the isolation and characterization of the two enzymes PAL and TAL, which are able to by-pass the sequence of conversions involving transamination, reduction and dehydration, the incorporation of PLA and HPLA into lignin is explained first by their conversion to the keto- acid and so to phenylalanine and tyrosine respectively by transamination. Subsequently it was shown that extracts from salvia, wheat and a number of other plants, readily oxidize PLA and HPLA to the corresponding keto- acids (52). Also O.L. Gamborg and A.C. Neish had already found in 1959 (50)

that PLA and PPA were easily converted to both free and bound phenylalanine and similarly HPLA and HPPA to tyrosine so that such a back-conversion is clearly possible. Thus PLA and HPLA were relegated to a role as "pool", rather than "intermediary" metabolites in lignification.

However T. Higuchi and S.A. Brown in 1963 (82) explored the phenylpropanoid biosynthetic pathway in lignifying wheat plants using isotope competition techniques and concluded that as phenylalanine and tyrosine are converted to PLA and HPLA respectively, this route remains a possible alternative to the established routes involving the ammonia-lyase conversions. Attempts to show the reversal of the biosynthetic pathway from the cinnamic acids to phenylalanine and tyrosine have not been generally successful (23) but in one case (97) detectable amounts of cinnamic acid, and in another (50), p-OH cinnamic and cinnamic acids, have been incorporated into the free amino acids. This could indicate the involvement of a minor, reversible alternative pathway equally as well as the partial reversibility of the ammonia-lyase conversions.

As the C_6C_3 - skeleton is incorporated intact into the lignin polymer (35, 1) the use of ^{14}C-labelled precursors has not shown definitively either the presence or the absence of the dehydration of PLA or HPLA. It remains an open question then whether PLA and/or HPLA are incorporated into lignin via the keto-acids, PPA and HPPA, and the amino acids, phenylalanine and tyrosine (Routes 1A and 2A, Figure 12), or whether they are converted directly to cinnamic acid and p-hydroxycinnamic acid (Routes 1B and 2B, Figure 12). An attempt is made to answer this question in the experiments that follow with respect to HPLA by making use of HPLA tritium labelled at the β-carbon. Figure 13 outlines the alternative incorporation patterns into p-hydroxy-

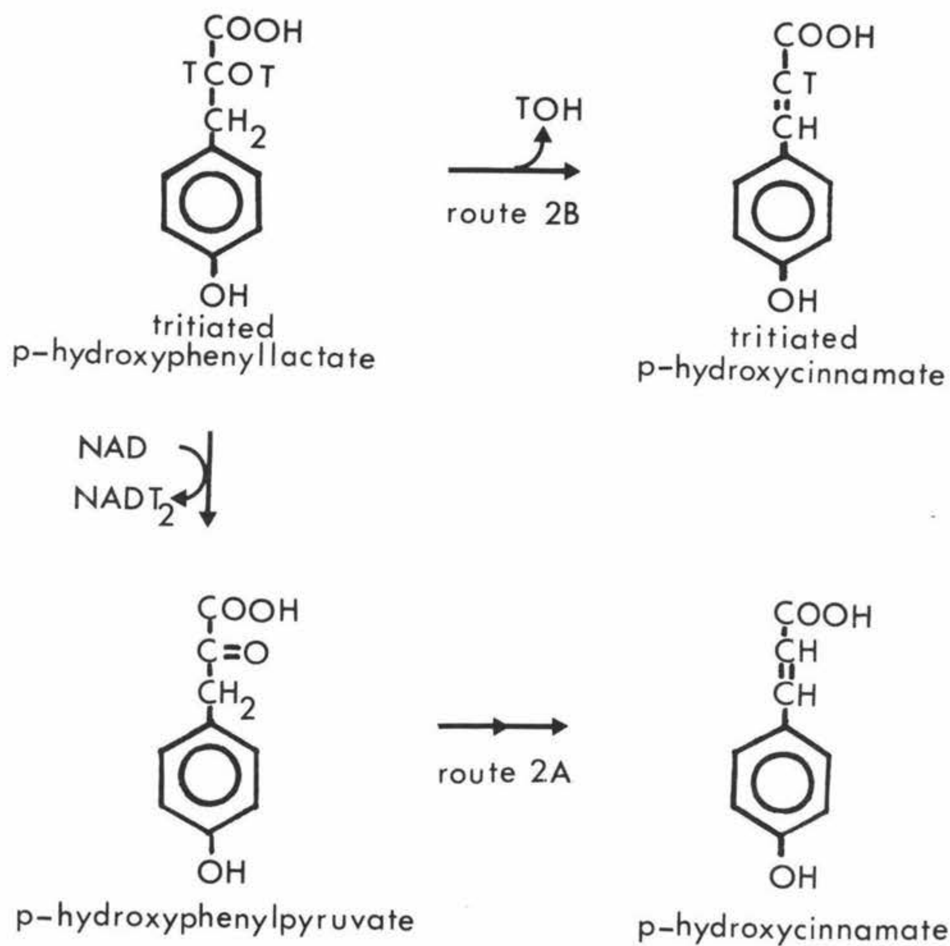


FIGURE 13. The possible routes for the conversion of tritiated p-hydroxyphenyllactic acid to p-hydroxycinnamic acid. (T = tritium)

cinnamic acid to be expected if either route 2A or 2B is used. The pattern would not change during the conversion of p-hydroxycinnamate to ferulate. Oxidation of ^{3}H-HPLA would remove all the label and dehydration only 50%. Therefore the detection of radioactivity in the cinnamic acids would support the direct conversion of HPLA to p-hydroxycinnamic acid. The administration of ^{14}C-HPLA also is a check that HPLA is being metabolized by the plant. The label from ^{14}C-HPLA is expected to be retained if either route is taken.

II.3 MATERIALS AND METHODS

II.3.1 CULTIVATION OF THE PLANTS

As in Part I, Section 4.1.

II.3.2 SOURCE OF THE ISOTOPES USED

Uniformly labelled L-^{14}C-tyrosine was obtained from the Radiochemical Centre, Amersham, England. ^{3}H-hydroxyphenyllactate (^{3}H-HPLA) was prepared by the reduction of hydroxyphenylpyruvic acid (HPPA) by $NaB^{3}H_4$. The $NaB^{3}H_4$ was obtained in powder form from New England Nuclear, 575 Albany St., Boston, Mass., U.S.A. ^{14}C-hydroxyphenyllactic acid (^{14}C-HPLA) was prepared by the reduction of ^{14}C-hydroxyphenylpyruvic acid (^{14}C-HPPA) by $NaBH_4$ also, the ^{14}C-HPPA was labelled at the ß-carbon and was kindly donated by Dr B.A. Bohm, Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada. For the details of preparation, purification and identification of ^{3}H-HPLA and ^{14}C-HPLA see Appendix 1.

II.3.3 ADMINISTRATION OF THE COMPOUNDS

Experiment 1

1 ml of L-^{14}C-tyrosine (containing 0.84µCi, S.A. = 483 mCi/mmole) was administered to wheat plants aged 10, 20, 40 and 60 days through the cut ends of the stems for 2, 4, 6, 12 and 24 hours. The compound was fed as an aqueous solution and when all the solution had been taken up (this took approximately 2 hours, except in the case of 10 day plants) distilled water was supplied to the plants.

Experiment 2

^{3}H-HPLA and ^{14}C-HPLA were first neutralized with dilute $NaHCO_3$ solution to prevent wilting and administered to 10 and 40 day-old plants for 2 hours.

Experiment 3

^{3}H-HPLA and ^{14}C-HPLA were administered after neutralization to 20 and 60 day-old plants for 2, 6 and 12 hours.

Experiment 4

High specific activity ^{3}H-HPLA was administered to 10 and 40 day-old plants for 2 and 4 hours.

In all experiments the plants were placed under continuous light (cool white fluorescent tubes at an intensity of approximately 35000 lux) for the duration of isotope incorporation time.

II.3.4 EXTRACTION AND PURIFICATION OF THE CINNAMIC ACIDS

1. Ethanol-soluble cinnamic acids

The isolation of the cinnamic acids was by an adaptation of the method used by D.R. McCalla and A.C. Neish (97) to extract amino acids from plant material. After the time allowed for isotope incorporation the plants were cut up and placed in hot 80% ethanol. After filtration the plant material was re-extracted 3 to 4 times with ethanol and the filtrates combined. The residue was set aside for the isolation of insoluble ferulic acid and the ethanol-soluble fraction evaporated to dryness on a rotary evaporator. A small amount of hot water was then added with some Celite Analytical Filter Aid to make a paste. The volume was made up to approximately 80 ml with more hot water, the mixture filtered and the residue washed with a further 20 ml of hot water. As the majority of the cinnamic acids do not occur in the free form in wheat (68), base and acid treatment is required to hydrolyse glycoside and ester linkages. Sufficient (4gm) NaOH pellets were added to the aqueous extract to make the solution 1N and, after boiling for 15 minutes, the pH of the solution was adjusted to less than pH 2 with conc. HCl and boiled again for 15 minutes. The solution was allowed to cool and then extracted continuously with

diethyl ether for 8 hours. A preliminary experiment using ^{14}C-ferulic acid showed that approximately 90% of the label incorporated into the ethanol-soluble fraction was recoverable by this procedure. The ether extract was evaporated to dryness on a rotary evaporator and the residue taken up into 1 ml 80% ethanol. In each case 0.1 ml was counted. Thinlayer chromatography on cellulose (MN 300G, with binder) was carried out to gain an approximate idea of the quantity of ferulic acid present as in some cases, where the concentration was low as to be invisible under u.v. light, cold ferulate was added prior to paper chromatography so that even small traces of activity could be recovered. A known aliquot of the extract was then spotted onto Whatman no.3 chromatography paper and developed in one direction with the organic phase of benzene:acetic acid:water (10:7:3, v/v) and in the second direction with 2% acetic acid. Ferulic acid (and p-hydroxycinnamic acid when necessary) was identified by fluorescence under u.v. light and checked spectrophotometrically on an Hitachi recording spectrophotometer after elution of the spot with 50% ethanol overnight.

2. Ethanol-insoluble ferulic acid

The procedure of S.Z. El-Basyouni, A.C. Neish and G.H.N. Towers (37) was used for the isolation of ethanol-insoluble ferulic acid in Experiments 1, 2 and 4. The ethanol-extracted plant residue was air-dried and 300 mg suspended in 20 ml 1N NaOH at 30°C for 4 hours. The suspension was then acidified with conc. HCl, heated for 15 min and extracted continuously with ether for 20 hours. The ether was evaporated off as before and the residue taken up in 1 ml 80% ethanol. 0.1 ml was counted and a known aliquot chromatographed. Samples from Experiments 1 and 2 were chromatographed as above and samples from Experiment 4 were chromatographed in the solvent system used by H.A. Stafford (145),

the organic phase of benzene:acetic acid:water (40:12:2, v/v) and n-butanol:ammonium-hydroxide:water (40:5:5, v/v). Further chromatography of both ethanol-soluble and ethanol-insoluble ferulic acid from Experiment 4 was carried out in 2% acetic acid. Sample identification under u.v. light was checked spectrophotometrically.

Duplicates were prepared for each sample for the isolation of ethanol-insoluble ferulate and for the isolation of the ethanol-soluble acids each sample was divided into two and taken separately through the extraction procedure after the initial ethanol extraction.

TABLE I R_f VALUES

SOLVENT SYSTEM	TYROSINE	p-OHCA	FERULATE	HPLA
Benzene:acetic acid:water (10:7:3 v/v)	0	0.20-0.25	0.70	0.08-0.10
Benzene:acetic acid:water (40:10:2 v/v	0	0.25	0.61	0
2% acetic acid	0.88	0.35,0.66*	0.31,0.61*	0.85
n-butanol:NH_4OH:water (40:5:5 v/v)	0.17	0.14	0.07	0.17

*trans and cis isomers for p-hydroxycinnamate and ferulate in 2% acetic acid are recorded.

3. Re-crystallization of p-hydroxycinnamic acid

The solution containing ethanol-soluble p-hydroxycinnamic acid eluted from the chromatogram in Experiment 2 was divided into two parts. The radioactivity in one part was determined and the other purified by re-crystallization with pure p-hydroxycinnamic acid. The sample was taken to dryness, 2 ml 50% ethanol added, and pure p-hydroxycinnamic acid added until no more dissolved. The solution was left overnight in the refrigerator to cool. It

was then filtered and the crystals dried at 90°C. 10 mg was then weighed out into a vial and the activity determined. The remaining crystals were re-dissolved in 50% ethanol and the process repeated until the specific activity became constant.

II.3.5 MEASUREMENT OF RADIOACTIVITY

Radioactivity measurements were made with a Packard Tri-Carb Model 3320 Liquid Scintillation Spectrometer. 10 ml of scintillation fluid (containing PPO, POPOP, toluene and Triton-X100) was added to both ^{3}H and ^{14}C samples and each sample was counted for 10 minutes or longer. Counts were adjusted for efficiency of counting (which was generally above 65% for ^{14}C and as low as 35% for ^{3}H) and corrected for background radiation in each case. Activity is expressed as a percentage of the activity absorbed by the plants in Figures 14 and 15 and as actual c.p.m. as well as a percentage of the activity absorbed in Table II.

II.4 RESULTS

Experiment 1

Preliminary experiments were carried out using L-(^{14}C-U)-tyrosine to determine the time at which precursor incorporation into ferulate was greatest. The assumption is made that this is also likely to be the time at which the plant is using HPLA in the biosynthesis of lignin as presumably HPLA is derived from tyrosine as well as prephenic acid. It is possible that HPLA dehydration, if it does occur, does not reach a maximum at the same time as tyrosine incorporation into ferulate and in Experiment 3 plants were allowed to metabolize HPLA for a range of time intervals.

The results of ^{14}C-tyrosine incorporation into ethanol-soluble ferulate for wheat aged 10, 40 and 60 days are presented in Fig. 14. Plants older than 10 days contained undetectable (by chromatography) amounts of ethanol-soluble ferulate and in order to measure traces of activity a small ammount of non-radioactive ferulate was added to each sample spot immediately before chromatography. After 4 hours there was an apparent increase in the incorporation of label into ferulate in 10 day plants and the time period for incorporation was extended to see if the pattern was repeated for older plants but in each case the activity present in ferulate was not significantly higher than background. The maximum activity in ferulate for 10 day plants was observed at 2 hours. To obtain some assessment of the flow of activity through to the cinnamic acids from tyrosine in 20 day plants the activity in p-hydroxycinnamate was recorded (see Figure 14) and also found to be highest at 2 hours. As the activity in the aqueous extract was high in each case the bulk of the ^{14}C-tyrosine is thought to have remained unmetabolized.

As the activity incorporated into the ethanol-soluble

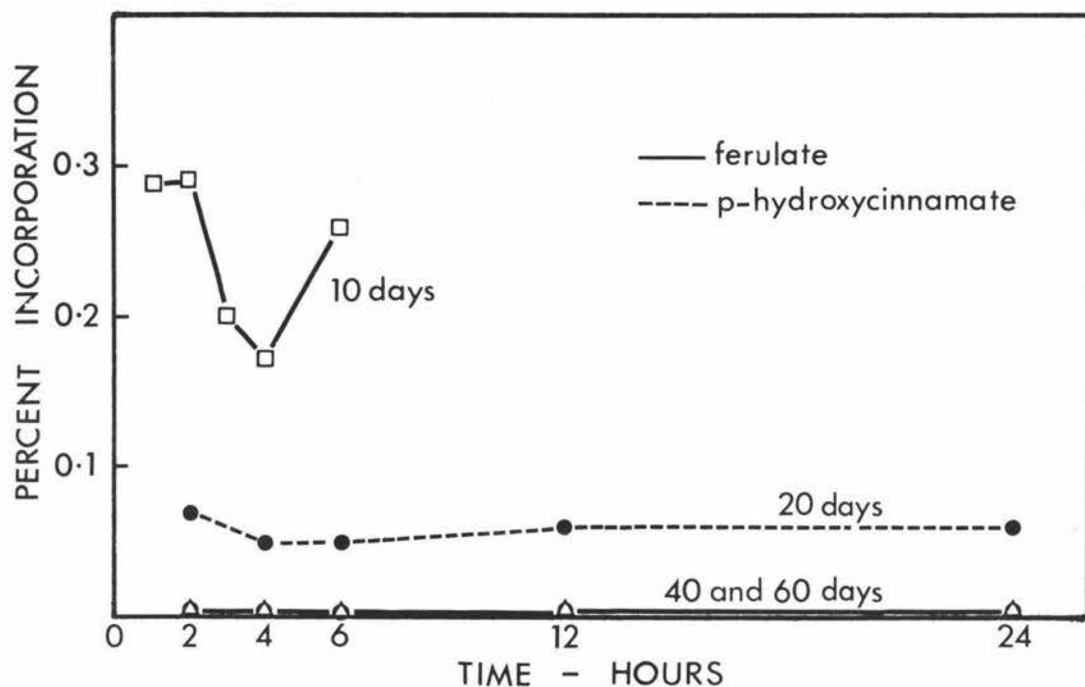


FIGURE 14. The incorporation of ^{14}C-tyrosine with time into ethanol-soluble ferulate or p-hydroxycinnamate for wheat plants aged 10, 20, 40 and 60 days.

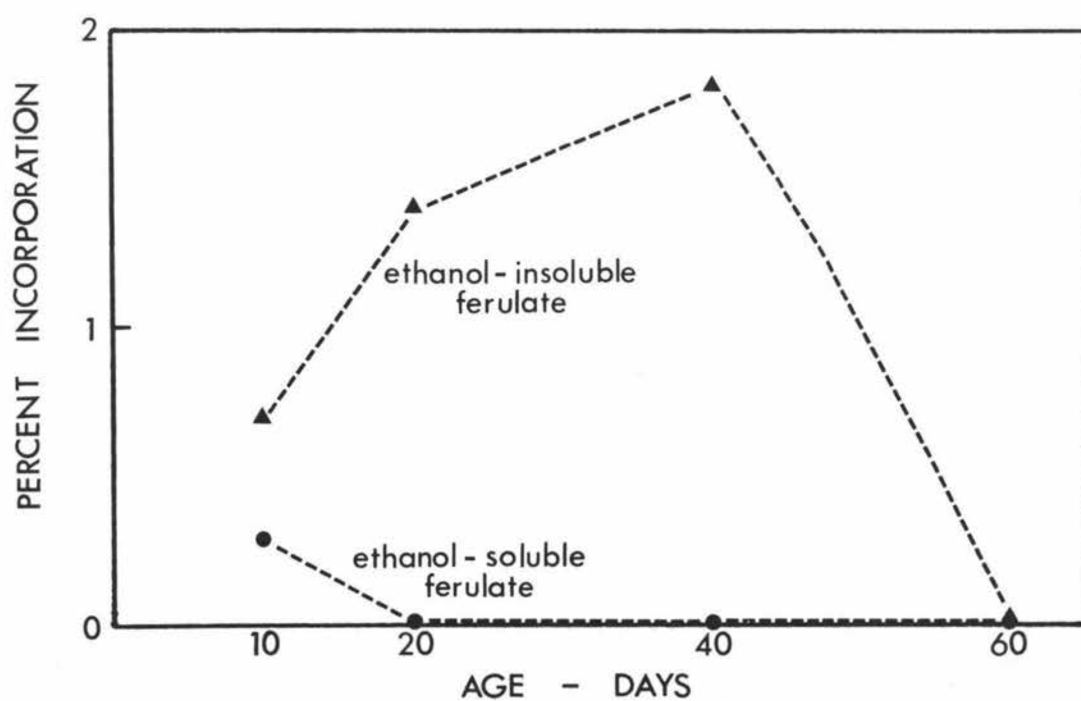


FIGURE 15. Change in activity in ethanol-soluble and ethanol-insoluble ferulate with age after 2 hours incorporation of ^{14}C-tyrosine.

acids was generally low, ethanol-insoluble ferulate was isolated from the 2-hour samples for each age (10, 20, 40 and 60 days) and the activity measured.

Each value shown in Figures 14 and 15 is the activity in the total amount of ferulate (or p-hydroxycinnamate) for each sample expressed as percentage of the activity taken up by the plants. Variation was in all cases within 10% of the mean value for duplicate samples.

Experiment 2

On the basis of the information obtained in Experiment 1 ^{3}H-HPLA and ^{14}C-HPLA were administered to 10 and 40 day-old plants for 2 hours only. No detectable activity was present in ethanol-soluble or ethanol-insoluble ferulic acid for either age. Low activity (approximately 5X background) was however recorded in ethanol-soluble p-hydroxycinnamic acid isolated from the 10 day-old plants from both the ^{3}H-HPLA and ^{14}C-HPLA samples. Upon re-crystallization with authentic p-hydroxycinnamic acid the activity disappeared and is thought to have resulted from slight contamination from unmetabolized substrate. Counts in the ether extract were high because of the unmetabolized HPLA present. (For 10 day wheat, 4.2% of the activity absorbed by the plants was present in the ether extract of the ^{3}H-HPLA sample and 5.6% for the ^{14}C-HPLA sample; for 40 day wheat 4.4% for the ^{3}H-HPLA sample and 2.9% for the ^{14}C-HPLA sample.) The relatively high activity recorded in the aqueous extract in ^{14}C-HPLA samples suggests that this was possibly converted to tyrosine. In the case of ^{3}H-HPLA, label would presumably be transferred to NAD and distributed around the plant. The R_f values for HPLA and p-hydroxycinnamic acid, while very different in 2% acetic acid are closer in the first dimension of chromatography, benzene:acetic acid:water (10:7:3) (see Table I)

and even slight "tailing" of the HPLA would have been sufficient to contribute the activity presumed present in the p-hydroxycinnamate.

Alternatively the activity present in the p-hydroxycinnamate may have been diluted by the non-radioactive material added for re-crystallization and this possibility is investigated for ^{3}H-HPLA by using precursor with a high specific activity (see Appendix 1) in Experiment 4.

Experiment 3

To account for the possibility that maximum HPLA metabolism may have been occurring at a time later than 2 hours ^{3}H-HPLA and ^{14}C-HPLA were administered to 20 and 60 day-old plants for 2, 6 and 12 hours. Activity in ferulate was also undetected in these experiments.

Experiment 4

After failing to detect the incorporation of activity into either ethanol-soluble or ethanol-insoluble ferulate in Experiments 2 and 3 a sample of high specific activity ^{3}H-HPLA was prepared (Appendix 1) and administered to 10 and 40 day-old wheat plants for 2 and 4 hours. The results of this experiment are set out in Table II. A comparison of Columns A and B with C and D shows the decline of activity in ferulate upon further purification. From $5 \times 10^{-4}\%$ to $8 \times 10^{-4}\%$ only of the activity absorbed by the plants is incorporated into the ferulate and is not thought to be significant. Values for duplicate samples are not consistent with each other and this could indicate that the few counts present are most probably contamination.

TABLE II The activity in ethanol-soluble and ethanol-insoluble ferulate after the uptake of ^{3}H-HPLA by wheat shoots

			ACTIVITY IN FERULATE AFTER INITIAL CHROMATOGRAPHY*				ACTIVITY IN FERULATE AFTER FURTHER CHROMATOGRAPHY IN 2% ACETATE*			
AGE	UPTAKE TIME	^{3}H-HPLA TAKEN UP	ETHANOL-SOLUBLE		ETHANOL-INSOLUBLE		ETHANOL-SOLUBLE		ETHANOL-INSOLUBLE	
(DAYS)	(HOURS)	(µCi)	c.p.m.(A)	dilution	c.p.m (B)	dilution	c.p.m. (C)	dilution	c.p.m. (D)	dilution
10	2	46.5	49850	0.057*	2145	0.0046	500	0.0005	294	0.0005
			8900		2642		66		190	
10	4	51.3	15500	0.033	5446	0.0072	273	0.0005	752	0.0007
			22580		2774		320		27	
40	2	51.7	37920	0.048	7383	0.0170	393	0.0006	373	0.0008
			17460		12154		286		539	
40	4	51.7	21960	0.033	8586	0.0127	240	0.0005	497	0.0008
			15700		6014		353		373	

*The values for duplicate samples are included in columns (A), (B), (C), and (D). The sum of each pair of duplicates is expressed as a percentage of the activity taken up by the plant in the columns immediately following (A), (B), (C) and (D).

II.5 DISCUSSION

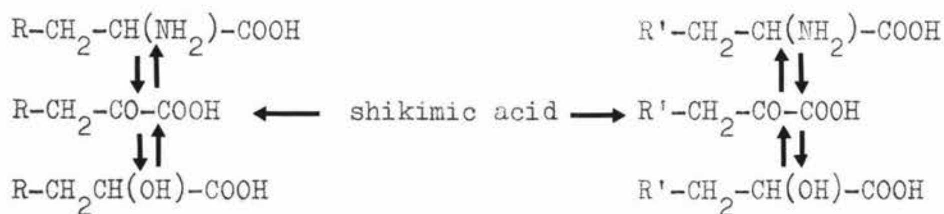
The results of these experiments suggest that it is unlikely that the direct conversion of p-hydroxyphenyllactic acid to p-hydroxycinnamic acid takes place in wheat. This study is not however definitive as no activity from ^{14}C-HPLA was detected in either p-hydroxycinnamate or ferulate. The high dilution values observed in some cases for phenylactate and hydroxyphenyllactate incorporation into lignin (20) and lignin precursors (98) make the use of a highly radioactive precursor necessary and the failure to detect label in the cinnamic acids is thought to be due to the low specific activity (0.0102μCi/μmole) of the ^{14}C-HPLA used. Increasing the specific activity of the ^{3}H-HPLA from 0.091 to 6.878μCi/μmole could therefore be expected to demonstrate the direct conversion of even minor amounts through to the cinnamic acids and so to lignin. The activity of the ^{3}H-HPLA used in Experiment 4 is approximately 120X that of HPLA administered to wheat by S.A. Brown, D. Wright and A.C. Neish (20) when they found a significant quantity of p-HPLA was incorporated into the lignin aldehydes, p-hydroxybenzaldehyde, vanillin and syringaldehyde.

Alternatively the HPLA may not have been metabolized by the plants but is more likely that the bulk of it was converted to tyrosine and subsequently protein (in the case of ^{14}C-HPLA) or transferred to nicotinamide adenine dimucleotide (NAD) (in the case of ^{3}H-HPLA) and distributed throughout the plant.

10 and 40 days were chosen in the final experiment as the most critical time at which the regulation of lignin biosynthesis may have been taking place. 10 days because this is the time at which L-tyrosine ammonia-lyase activity is known to be high (171) and 40 days because this is the time of rapid lignification (see Part I of this study and J.E. Stone, 1951 (154)). While it is

still possible that HPLA may, at some other time during the development of the plant, contribute to the "pool" of lignin precursors via direct conversion to p-hydroxycinnamic acid, this contribution is not likely to be of regulatory significance.

O.L. Gamborg and A.C. Neish, 1959 (50) suggested that HPLA was in fact part of the tyrosine "pool" in wheat and buckwheat plants (similarly PLA is part of the phenylalanine "pool", see below) and so may be incorporated into either protein or cinnamic acids. It may be that the separate regulation of the pathways of protein and phenolic biosynthesis is dependent upon the spatial separation of precursors. The possible spatial separation of precursor "pools" occurring in flavonoid biosynthesis after the formation of phenylalanine in Pisum sativum has been suggested by D.B. Harper et al (71) and it is probable that spatial regulation of some kind is required to maintain the balance between the more comprehensive plant processes of protein synthesis and lignification.



R = phenyl and R' = p-hydroxyphenyl

FIGURE 16 The role of HPLA and PLA as "pool" metabolites contributing to the origin of tyrosine and phenylalanine (O.L. Gamborg and A.C. Neish, 1959).

The possibility that the ethanol-soluble and ethanol-insoluble derivatives of ferulate may each be intermediates in the biosynthesis of lignin was accounted for in these experiments by isolating both. The radioactivity incorporated into the ethanol-

soluble ferulate after 2 hours follows the pattern of TAL activity with increasing age observed for wheat by M. Young, 1966 (171) and a comparison of Figure 15 with Figure 2 shows this clearly. It appears that only in very young wheat plants are large quantities of ethanol-soluble ferulate present. It was thought initially that the presence of only trace amounts of ethanol-soluble ferulate and the absence of any activity at 40 days may have been due to the rapid turnover of this substance at this age. This is reasonable in view of the rapid lignification at this time. However an experiment to evaluate this hypothesis in which the incorporation times of L-^{14}C-tyrosine were very brief (30, 60 and 120 minutes) in fact gave no indication of a rapid turnover, no activity being detected in the ethanol-soluble ferulate. These results are not in agreement with those of S.A. Brown _et al_ (20) who found that after an incorporation time of 24 hours ferulate is formed from tyrosine in wheat plants that are about to flower. Differences in growing conditions, for example, photoperiod, may account for differences in the pattern of incorporation at specific times during the life of the plant.

10, 20 and 40 day plants each incorporated a much larger percentage of the administered L-^{14}C-tyrosine into the ethanol-insoluble ferulate derivatives. The change with increasing age in the quantity of activity incorporated into the insoluble ferulate derivatives supports their role as the natural intermediates in lignification. The values for 10 day plants correspond to values S.Z. El-Basyouni, A.C. Neish and G.H.N. Towers (37) obtained for 8 day-old wheat plants. After an incorporation time of 24 hours for 8 day-old plants (shorter time-periods were not investigated) 2.6% of the ^{14}C-tyrosine administered was found in the insoluble ferulate derivatives and 0.28% in the soluble ferulate. This

compares with 0.7% in insoluble ferulate and 0.3% in soluble ferulate after 2 hours in Experiment 1. The complete absence of activity in the insoluble ferulate esters at 60 days shows that either the plant is not making use of tyrosine in lignification, or that the label from tyrosine takes longer than 2 hours to become incorporated into precursors, at this age. Large quantities of ethanol-insoluble ferulate derivatives were still present in 60 day plants (an intensely fluorescent spot was visible after chromatography of the ether-extracted alkaline hydrolysate) and it may be that the concentration at this age is high enough to exert some inhibitory (regulatory?) influence over the formation of more lignin precursors.

There is the suggestion by El-Basyouni _et al_ and later by H.A. Stafford (150) that at no time in the natural situation are lignin precursors, once bound to an active enzyme complex at the tyrosine and phenylalanine stage, released in an ethanol-soluble form. This would explain the failure to detect ethanol-soluble derivatives of ferulate at ages greater than 10 days in these experiments. It is possible that the large quantities of ferulate obtained by alkaline hydrolysis of the ethanol-insoluble fraction are not intermediates in lignification. Compounds such as the ferulic ester of xylan, for example, may be present and need not be regarded as intermediates (42).

The uncertainty as to the nature of grass lignins (14) provides a stumbling block to speculation on the nature of control mechanisms both before and after the formation of the cinnamic acid esters. The extent of transformations in the cell wall itself of the lignin monomers is not known. There is the suggestion by H.A. Stafford (145) that p-hydroxycinnamic acid, once bound to the wall, may be converted to either ferulyl or

sinapyl derivatives and the possibilities of the conversion of sinapate to 5-hydroxyferulate (81) and p-hydroxycinnamate (37) and also to a lesser degree, the conversion of ferulate to p-hydroxycinnamate (37) are known to exist. Kinetic studies on the enzymes that are involved in the reduction of p-hydroxycinnamate, ferulate and sinapate to the corresponding alcohols prior to incorporation into lignin may provide some of the answers and the study of the taxonomic distribution of these enzymes (102) may be the first step in an investigation of their role in the regulation of lignification.

II.6 CONCLUSION

1. It is unlikely that the direct conversion of p-hydroxyphenyllactic acid to p-hydroxycinnamic acid takes place in 10 and 40 day-old wheat plants. Since these ages are critical for the regulation of phenolic biosynthesis it is therefore highly unlikely that this conversion is of significance in regulation.

PART III AN OUTLOOK ON THE CONTROL OF LIGNIFICATION

The growth, development and differentiation of any organism is seen as the result of continuous gene expression modified by interaction with the environment (86, 151, 12). Between the external environment and the genome of the individual cell in the plant, as in other organisms, there may be many opportunities for the regulation of any one metabolic process. An understanding of a problem as complex as the control of lignification must therefore be on several levels simultaneously. While individual cells, discrete tissues and organs are levels of organization that may usefully be considered separately, the role of each within the intact plant is important overall.

Any mechanism for controlling lignification would have to account for a number of observations. Firstly lignin composition may differ according to cell type (166) and species (131); it is affected by genetic lesions and maize mutants containing abnormal lignin are known to exist (93). Secondly, lignification may be influenced both by environmental conditions external to the plant and the extracellular physiological environment. For example, light may stimulate the development of differentiated tissues (142) and lignification may be prematurely induced in cultured plant tissue by altering the composition of the medium (44, 88, 67). Thirdly, growth and lignification are in most cases incompatible (140, 165). Fourthly, lignification is on a cell by cell basis, a completely lignified cell often being situated next to an unlignified one (161). And finally, in any one cell lignin deposition starts at a point in the wall furthest from the cytoplasm - the middle lamella - and may not always follow the same pattern (161, 74). Compare for example, spiral, annular or reticulate xylem wall thickening with the largely homogeneous lignification of fibre cell walls. How precursors are

prevented from polymerizing until they reach the middle lamella or alternative sites of deposition, and how they are transported across the plasmalemma and through the cell wall are additional unanswered questions.

The information presented in this study has been obtained from inquiry at three levels - the development of the whole wheat plant, the lignification of stem tissues and the metabolism of a possible intermediate in the biosynthesis of lignin. The following discussion will be of the problems associated with identifying regulators of lignification at each of these three levels.

An examination of the whole plant has revealed events during development that are likely to be related to each other and information about one process may be of use in thinking about another. More specifically, the way in which plant hormones regulate flowering and maturation may be similar to the hormonal control of lignification.

Gibberellic acid, which is known to induce "bolting" and flowering in some long day plants (84), has also been reported to promote lignification (54, 55, 112). In addition, the suggestion has been made that gibberellic acid may play a part in the induction of L-phenylalanine ammonia-lyase and that this may be related to an increase in lignin formation in dwarf pea (25). However, this is likely to be an indirect effect because of the time lag (16 to 18 days) between application of the hormone and increase in enzyme activity.

The role of auxin in lignin biosynthesis is unclear. The well known participation of auxin in promoting cell enlargement and meristematic activity seemingly conflicts with the observation that it is also a promoter of xylem differentiation which requires an increase in wall thickening and a repression of cell enlargement.

It is possible that the "products" of meristematic activity more directly influence cell differentiation than does auxin itself (142). The action of auxin on lignification is not always consistent. While the inhibition of lignification and other peroxidations by auxin in model systems has been observed (139), root vascular cells respond very early in development to the application of auxin by producing peroxidase and becoming active in lignin formation (89). The change from a reducing to an oxidising extracellular environment may in itself be sufficient to maintain the balance between growth and lignification (140). The primary action of growth regulators on lignin formation may, therefore, be through a hastening of growth processes.

The enhancement of lignification in the presence of kinetin is thought to result from a stimulation of carbohydrate metabolism, in particular the production of shikimic acid (8), which then activates phenylpropanoid metabolism. It has also been suggested that cytokinins promote the adherence of cells to each other (67) - a necessary preliminary to further wall development.

Light is known to influence both flowering and secondary metabolism in many plants but whether the effect on lignification is by supplying carbohydrate for incorporation into lignin precursors or by the phytochrome-mediated induction of ammonia-lyase activity is uncertain. The presence of a light-induced, phytochrome-mediated increase in PAL has so far been reported for one monocotyledon only, barley, and is in this instance correlated with flavonoid production (99).

The interaction between the environment (e.g. changing day length), "bolting", flowering and lignification at all stages in the development of wheat needs to be defined more closely. The temporal separation of these processes as well as their differential

sensitivity to the environment will be an important base for establishing causal relationships. The main problem in this field, as in almost every aspect of the coordination of whole plant development, is the paucity of information.

A study of the problem at the tissue level revealed that cells distributed differently within the stem, and fulfilling diverse functions, lignify at different times during development and contain lignin with differences in composition. This is an indication that either the stimulus inducing lignification changes during development, or that the cells of specific tissues translate similar stimuli differently. It is probable that both occur to some extent. The later differentiation of sclerenchyma compared with xylem vessels, and the coincidence of this with extensive changes in the whole plant, is compatible with the idea that the extracellular environment is chiefly responsible for bringing about lignin formation. However, the simultaneous formation of xylem vessels and fibres and the lignification of the former only in decapitated Xanthium (136) suggests that the difference may lie in the capacity or potential of different tissues to respond to like stimuli. The degree to which cells within a tissue communicate during wall development is also unknown. Presumably the alignment of pits during xylem wall thickening to maintain an efficient transport system, for example would require some form of intercellular communication, possibly via the plasmodesmata. While gross morphological differences exist between vessels and tracheids and fibres, biochemical differences, so far as is known, are restricted to the presence or absence of syringyl and p-hydroxycinnamyl units (131).

Studies on the enzymology of separate tissues are likely to yield more useful information than studies on whole plant extracts at this stage and a priority in this field is the devel-

opment of adequate techniques for dealing with homogeneous tissues. The technical difficulties associated with the isolation of specific tissues while maintaining their unique characteristics are no less than the difficulties associated with the in vitro cultivation of plant cells, a somewhat artificial situation that may lack relevance to the process of lignification as it occurs in the intact plant.

At the metabolic level it is unlikely that p-hydroxyphenyllactic acid is dehydrated directly to p-hydroxycinnamic acid and neither does this compound appear to play an important part in the biosynthesis of precursors at the critical times of lignification in wheat shoots. It is possible that the treatment of the whole shoot as if it were homogeneous may have masked the slight conversion of p-hydroxyphenyllactic acid to p-hydroxycinnamic acid in any particular tissue and the detection of even minor amounts of activity would have justified working with this problem at the tissue level.

In conclusion it is probable that the ammonia-lyases do have some influence over the supply of precursors for secondary matabolism in general, but it is unlikely that the finer control of lignification operates at this stage in the pathway. An investigation of the relationship between the activity of the enxymes involved in the final stages of biosynthesis (e.g. the aromatic alcohol oxidoreductases) and lignin composition may provide some answers. Time-studies on the activity of these enzymes similar to those carried out by M. Young for PAL and TAL (171) and A.D.M. Glass and B.A. Bohm (53) for caffeic acid O-methyl transferase would indicate their significance, if any, during and immediately prior to the time of rapid lignification in wheat.

APPENDIX 1

THE PREPARATION AND PURIFICATION OF ^{3}H-HPLA AND ^{14}C-HPLA

1. ^{3}H-HPLA

20 mg HPPA was dissolved in 1 ml ethyl alcohol and 5 mg $NaB^{3}H_4$ added. Approximately 5 mg of non-radioactive HPLA was added at the completion of the reaction to assist in identification. The reaction mixture was acidified with 1N HCl, 50 ml of distilled water added and continuously extracted for 4 hours with 150 ml of diethyl ether. The ether was evaporated, 1 ml of ethanol added to the residue and the solution applied in a narrow band to Whatman no. 3 chromatography paper. A small amount of pure HPLA was run as a standard alongside this band on the same paper. After chromatography in 2% acetic acid (R_f HPLA, 0.85) the side-strip containing the standard HPLA was cut away and sprayed firstly with diazotized p-nitroaniline (0.3% p-nitroaniline in 8% HCl (w/v):20%(w/v) sodium acetate:5%(w/v) sodium nitrite, 5:10:15 (v/v)) and secondly with 0.5N NaOH. HPLA is a mauve colour in this spray. This strip was placed beside the remainder of the chromatogram to find the position of the prepared ^{3}H-HPLA. Also a narrow strip, 5 mm wide and the length of the chromatogram, was cut into segments, the segments placed with 0.5 ml ethanol in a vial, scintillation fluid added and the activity in each measured. The segment of paper with the highest activity corresponded to an R_f of 0.85 and was taken to be ^{3}H-HPLA (see Figure 17A). The band of high activity was cut out and eluted for 20 hours with 70% ethanol simultaneously with a control strip from a blank chromatogram also run in 2% acetic acid. This solution was checked for contaminants by co-chromatography with authentic HPLA in toluene:acetic acid:water (4:1:5, v/v) (R_f 0.0)

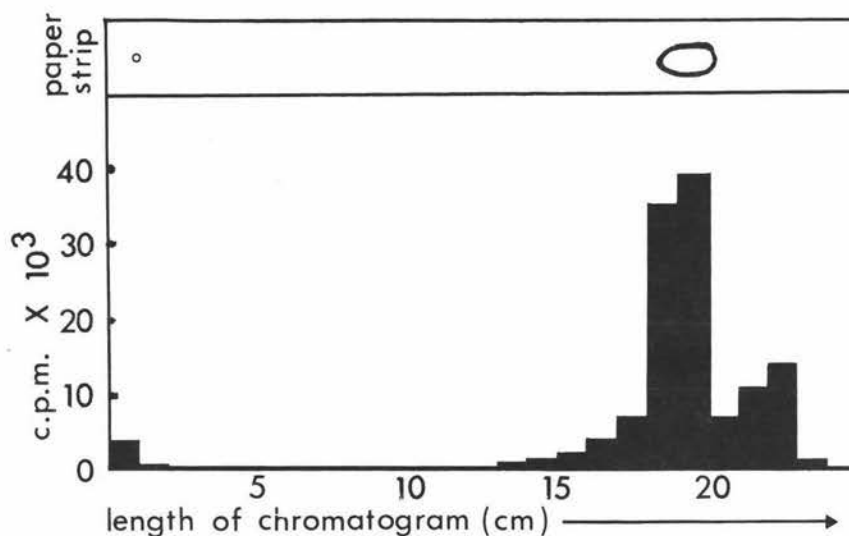


FIGURE 17. (A) Radioactivity in segments cut from a narrow strip after chromatography of the reaction mixture in 2% acetic acid. The HPLA standard is shown above.

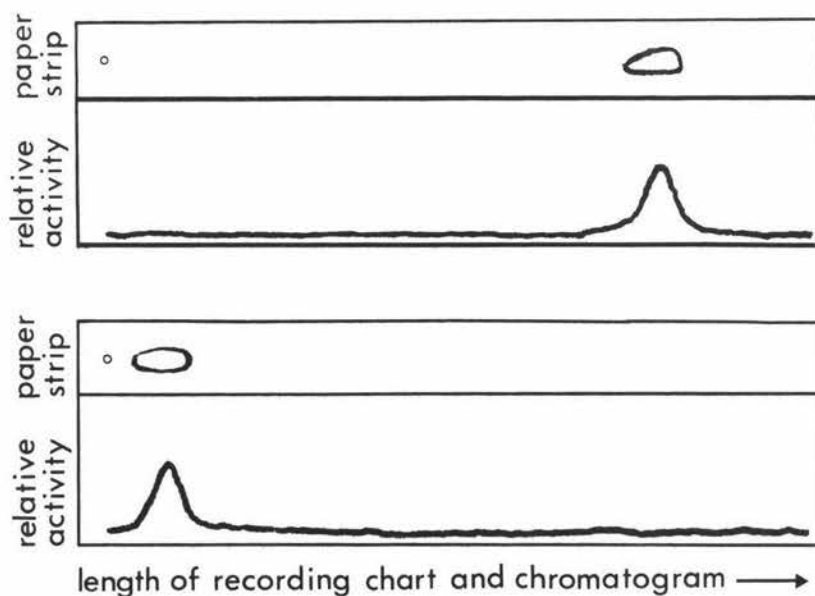


FIGURE 17. (B) Chromatography of an aliquot of purified ^{3}H-HPLA in 2% acetic acid and the scan of this strip for radioactivity.

FIGURE 17. (C) Chromatography of an aliquot of purified ^{3}H-HPLA in benzene:acetic acid:water (10:7:3) and the scan of this strip for radio-activity.

and benzene:acetic acid:water (10:7:3, v/v) (R_f 0.08 -0.10) as well as 2% acetic acid and after chromatography strips were scanned for radioactivity by a radiochromatogram scanner (Packard Model 7200) to check that the activity and the mauve spot revealed after spraying were coincidental (see Figures 17B and 17C).

The solution was made up to 100 ml and quantified spectrophotometrically on an Hitachi recording spectrophotometer. The absorbance spectrum for the prepared ^{3}H-HPLA corresponded to the spectrum for pure HPLA, the maximum at 279 nm indicating the concentration of the solution to be 170 μg/ml. The specific activity was shown to be .091 μCi/μmole. The solution was concentrated to a volume of 10 ml and 1 ml aliquots of this were taken to dryness on a rotary evaporator and re-dissolved in dil. $NaHCO_3$ immediately prior to administration to the plants in Experiments 2 and 3.

This procedure was repeated for the preparation of the ^{3}H-HPLA used in Experiment 4. The total activity of the $NaB^{3}H_4$ used for this was 25 mCi. After purification, the specific activity of the prepared ^{3}H-HPLA was 6.878 μCi/μmole. For each sample in Experiment 4 one tenth of the total ^{3}H-HPLA prepared was administered to the plants and in each case contained approximately 52.7 μCi. compared with approximately 0.85 μCi per sample in Experiments 2 and 3.

2. ^{14}C-HPLA

This was prepared as for ^{3}H-HPLA except that the substrates for the reaction were ^{14}C-HPLA labelled at the β-carbon and $NaBH_4$. The final specific activity was low, 0.0102 μCi/μmole and approximately 0.18 μCi. were included in each of the samples in Experiments 2 and 3.

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