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# PROANTHOCYANIDINS AND THE LIGNIN CONNECTION

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**Key Word Index**– lignin, proanthocyanidins, condensed tannins, structural vs defense roles.

**Abstract**—Since both lignin and proanthocyanidins (including condensed tannins) are frequently found together in woody plants, a common structural role has sometimes been postulated. These two phenolic polymers (or oligomers) are compared in terms of their stereochemistry, absorption spectra, biosynthetic pathways, intracellular and tissue localization, solubility, concentration and association with carbohydrates. The present evidence indicates that a common function in defense is more plausible than in structural support. The need for new histochemical techniques and the possibility of secondary changes in chemical composition and in localization of proanthocyanidins as cells die in normal development are discussed.

## INTRODUCTION

The presence of insoluble as well as soluble PAs and recent evidence that the insoluble forms may sometimes be complexed with carbohydrates has led to a consideration of the analogies between PAs* and lignin, especially in terms of a structural function. [1]. This is similar to an older view of Bate-Smith that there might be a relationship between these substances in the development of vascular plants since they are so often found together in woody plants [2, 3]. A structural role, based on a postulated localization in the cell walls of plants, has also been discussed recently by Zucker [4]. However, while both PAs and lignins are phenolic polymers (or oligomers) formed by condensation of highly reactive intermediates in processes that can be mimicked non-enzymically [1], there are some important distinctions that need to be considered, especially as related to any function in support [Table 1]. The role of both lignin and PAs in defense has previously been discussed by Swain [5]. While he included other phenolics such as the less widely distributed hydrolysable tannins, the present review will consider only the oligomeric PAs (condensed tannins).

### *Taxonomic distribution*

Lignin is found in all vascular plants, except in a few species that have secondarily evolved into an aquatic environment such as *Elodea*. PAs are less widely distributed, but are probably universal in the major groups of gymnosperms and widespread in woody angiosperms [5–7]. PAs have not been identified in primitive vascular groups such as Psilopsida and Lycopsida and in many herbaceous angiosperms; all of these contain lignin in some cells.

### *Heterogeneity of lignin and PAs*

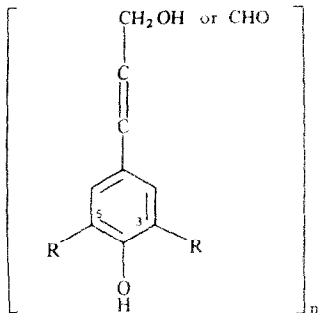
Both polymers are made up of units derived from different immediate precursors. The chemistry of lignins, polymers of $C_6$–$C_3$ units mainly with terminal alcohol but some aldehyde groups [8–10], and that of PAs ($C_6$–$C_3$–$C_6$ flavanoid units) [11–14] has been well reviewed (Fig. 1). All vascular land plants contain lignins with a guaiacyl type nucleus; gymnosperms contain predominantly only this grouping while angiosperm dicots contain the syringyl nucleus in addition, and monocots contain both this and a *p*-hydroxyphenyl nucleus (**1**) [15, 16]. These basic units, arising from phenoxy radicals, are linked head-to-head, tail-to-tail, and head-to-tail (the most dominant type). The various levels of heterogeneity in lignins (or inhomogeneity) are discussed by Monties [9]. No residual stereochemistry has been detected in any of the isolated lignins, but stereospecific linkages at the $\beta$-carbons are found in the related dimeric lignans [9, 17].

The basic unit of PAs $(C_6$–$C_3$–$C_6)_n$ contains an aromatic A-ring, commonly with a phloroglucinol hydroxylation pattern (5,7-OH), and an aromatic B-ring that is either diphenolic (3′,4′-OH) or triphenolic (3′,4′,5′-OH). A monophenolic B-ring with 4′-OH is relatively uncommon (**2**). The substituents of the central heterocyclic ring form either 2,3-*cis* or 2,3-*trans* stereoisomers. Condensation to form oligomeric PAs involves the addition of a 3,4-diol, or its carbocation or quinone methide, to a flavan-3-ol or to an existing chain to form the 'upper' units. Interflavanoid bonds are generally between carbons 4 and 8 to form linear chains but branching can occur due to 4→6 linkages. The only free phenolic hydroxyl group in lignin is in the 4-position since any hydroxyls present at the 3- and 5-positions are methylated [9], while such methoxyl

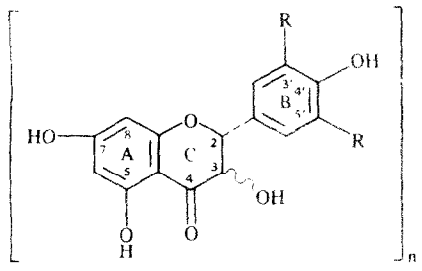
*The term proanthocyanidin (PAs), consisting of procyanidins and prodelphinidins, has been used for the following older terms: condensed tannins, catechol tannins and leucoanthocyanidins. The latter term should now be restricted to the monomeric flavan-3,4-diols.

Table 1: Summary of major aspects of a comparison between lignin and PAs

| | Lignin | PAs |
|---|---|---|
| Chemistry | $(C_6\text{-}C_3)_n$ | $(C_6\text{-}C_3\text{-}C_6)_n$ |
| Stereochemistry | Lost | Retained |
| UV peaks | 270–280 nm | 270–280 nm |
| Biosynthetic pathway | Phenylpropane-CoA | Malonate-CoA:phenylpropane-CoA |
| Intracellular localization | Walls | Vacuole |
| Tissue distribution | Xylem (vessels, tracheids, fiber), phloem fibers, parenchyma of cortex and stele, periderm | Parenchyma of periderm cortex and stele, endodermis, cambial layer |
| % dry weight tissue | Up to 36% | Up to 30% |
| % dry weight walls | Up to 85% | 0% in living cells |
| % dry weight Hemlock: [40] | | |
| Wood | 29.8 | 0.2 |
| Bark | 15.2 | 12.7 |
| CHO linkage | Linkage unknown | Mainly non-glycosidic |
| Solubility | Insoluble in aqueous methanol or acetone | Majority soluble in aqueous methanol or acetone in living cells |
| Function | Support, defense | Defense |




**1** R = H or OMe




**2** R = H or OH

Fig. 1. The basic units of lignin (**1**) and proanthocyanidin (**2**) polymers or oligomers.

groups are rare in PAs [11]. The interesting biosynthetic differences in both hydroxylation and methylation patterns in the PAs and lignins of gymnosperms and angiosperms need further investigation.

*Biosynthesis*

While the nine carbons of the lignin basic unit and those of the B-plus C-rings of PAs are synthesized from cinnamic acid derivatives arising from the shikimic and phenylpropane pathways, the six carbons of the A-ring of PAs require in addition the malonate-CoA pathway [10, 11, 18]. Further biosynthetic differences are that lignification occurs generally only after cell wall elongation has ceased [6], while PAs can be synthesized during the cell elongation phase as well [19, 20].

*Intracellular and tissue localization*

Intracellular and tissue localization of both polymers have been based mainly on *in situ* histochemical tests, with the use of light or electron microscopy. However, strictly specific stains for each product are not available. The histochemical stains for lignin are generally more specific than those for PAs. Identifications of lignin are sometimes based on UV microscopy, but since both lignin and PAs have absorption peaks between 270 and 280 nm, this would not differentiate between them if both are in the cell walls [19, 21]. Classical lignin histochemical stains are with phloroglucinol–HCl (for cinnamaldehyde groups), with Maule and $Cl_2$–$Na_2SO_4$ reagents (for syringyl groups) and the less specific safranin (for phenolic hydroxyl groups in general). None of these will differentiate between monomers and polymers [8, 15, 16, 21–25].

PA's have been identified histochemically by the pro-

duction of a bright red colour with either the nitroso [26–28] or dimethoxybenzaldehyde reagent [29, 30]. The nitroso reaction gives a red colour with *ortho*-diphenols, but brown ones with oligomeric triphenols. Monomeric mono- and triphenols give a yellow colour, but this is generally due to the NaOH alone [20]. The chemistry of this reaction needs to be re-studied with modern methods, but it is quite useful as a PA indicator, especially of *o*-diphenols found in procyanidins, when it has been shown by other analytical methods that the tissues contain predominantly PAs as the phenolic constituents. However, this reagent cannot be used to differentiate between PAs and chlorogenic acid. In the often cited papers of Reeves on the nitroso reagent [26, 27], PAs (called catechol tannins) were mistakenly considered to be the same as alkali induced polymers of chlorogenic acid.

Both potassium permanganate and iron (II) sulphate have been used to stain both lignin and PAs [24, 31], based apparently on the assumption that lignin is only in the cell walls, and PAs only in vacuoles. EM workers have generally used osmium tetroxide ($OsO_4$); sometimes caffeine was added to stabilize the localization [31–36]. Osmium tetroxide combined with Sudan black B has been claimed to permit the clearest distinction between liposomes and PA-containing vesicles and vacuoles [37]. Phloroglucinol–HCl and the nitroso stain are probably the best available histochemical stains to distinguish between lignin and PAs respectively, but since both can react with other compounds, even they should be used with caution. Histochemical tests, rather than differential centrifugation of extracts of cells in the appropriate osmotic medium to maintain the integrity of organelles, are the best for PA-containing cells because of the artifacts caused by the ease of complexing of carbohydrates and proteins by PAs during cell free isolation procedures.

Lignin is found only in cell walls, and the final biosynthetic steps catalysed by peroxidase producing mesomeric forms of a phenoxy radical occur at this site [18, 38]. PAs, on the other hand are synthesized in vesicles budded off from the endoplasmic reticulum, both during and after cell elongation. These vesicles coalesce and are ultimately incorporated into the large central vacuole in either a diffuse or aggregated state [32–34, 39]. A vacuolar localization was also clearly shown in several PA containing cell suspension cultures by the use of the nitroso reagent [20]. A secondarily derived wall localization for PAs in non-living cells, however, is plausible. This possibility will be discussed later.

*Tissue localization*

The distribution between tissues, and even within one cell type, of the two polymeric products can also be compared. PA deposits within cells of one morphological type are sometimes quite variable. For instance, in cortical or mesophyll cells, ones with high PA content may occur side by side with non-PA containing cells [20, 28, 31]. This is generally not true with lignified cells; they tend to be found in discrete cylinders (containing tracheids and vessels) or in clumps of contiguous cells (fibres or sclereids, although the latter sometimes appear as isolated cells). Lignin is most abundant in tracheids of the xylem or rays and sclerenchyma fibres in the xylem and cortex [23]. In general, more PA's are present in bark than in wood [40]. The chemistry of the walls of the endodermal layer containing suberin, found also in wound tissue, is still unclear [41]; both positive phloroglucinol–HCl and safranin stains are generally found in such walls, but this does not prove that the lignin polymer is present. High concentrations of nitroso and dimethylbenzaldehyde positive materials, interpreted as PAs, fill the entire central parts of the endodermal cells in young cotton seedlings [28–30]. PAs have also been reported in cambial cells [42], and PA containing cells are known to divide, producing either similar cells or non-PA containing cells [19, 20, 31, 35, 43].

In a recent study of the localization of PAs and lignin in cell cultures and needles of several plants [20 and unpublished data], the pattern of staining with the nitroso reaction for PAs and the phloroglucinol–HCl stain for lignin was quite different. In general, tissues and cell types that gave a red colour in the walls with the phloroglucinol–HCl reagent did not give a red nitroso-positive colour in the vacuoles and *vice versa*.

Neither lignin nor PAs are homogeneous polymers, and at least with lignin, there is evidence that different tissues may show different staining patterns. For instance, staining with the Maule reagent (for syringyl units) compared with phloroglucinol–HCl (for coniferylaldehyde groups), indicated that the components of typical tracheids and of sclerenchyma fibres differ in composition [9, 15, 16, 44]. Histochemical detection of different localizations of procyanidins and prodelphinidins will be more difficult. While the red colour of the nitroso stain is a very good indicator of procyanidins, the increased brown to black reaction with vacuoles containing high concentrations of prodelphinidins may be less specific.

*Carbohydrate linkages*

While most flavonoids are present as glycosides, flavan-3-ols and PAs soluble in aqueous methanol have been detected only as the aglycones until recently. One might argue that they are sufficiently water soluble to be retained inside the vacuole without the addition of sugar moieties. Recently, however, evidence of covalent linkages of carbohydrates with both monomeric flavan-3-ols and even higher oligomers has been demonstrated in solubilized forms [45, 46]. The universality of these linkages, however, is unknown, and in some cases, artifacts during extraction have not been ruled out. Lignin is also considered to be associated with the non-cellulosic components of the wall and in some cases the linkages appear to be covalent [18, 47].

*Insoluble PAs*

Lignin, well known for its insolubility, can be isolated only after treatments that alter some of the linkages. Variable amounts of PAs have also been reported to be insoluble in most organic reagents, but the degree of insolubility varies with the tissues being extracted. While Shen *et al.* [1] reported that over 80% of the PAs were insoluble in bark amd other plant parts, Foo and Porter have shown that some bark PAs can be almost entirely solubilized if 70% acetone is used instead of the usual 70% methanol [48]. We have reported that 16% of the total PAs in needles were insoluble in Douglas fir and 30% in six members of the Taxodiaceae [49, 50]. In cell cultures of gymnosperms, insoluble PA values ranged from 12 to 19% [19]. In some of these cases examined,

very little or none of the methanol insoluble residue could be subsequently solubilized with acetone. The insoluble forms in cell suspension cultures of Douglas fir cotyledons were not associated with the cell walls since protoplast preparations also contained the insoluble forms [51].

The problem of artifacts produced during extraction as a cause of the insolubility of PAs, however, must be considered (see ref. [4] for a review of some of the complexes formed). When cell cultures of Douglas fir that contained only vacuolar PAs were analysed after differential centrifugation of cellular organelles and components, PAs were found in every fraction isolated (walls, chloroplasts, mitochondria, microsomes); these were interpreted as artifacts of isolation [51].

One explanation for such artifacts of carboydrate- or protein-PA linkages during extraction is based on the fact that when tissues with relatively low amounts of PAs are assayed such as cotyledons or young needles of Douglas fir seedlings, 60–90% of the PAs are insoluble in methanol [49, 51]. As the seedling needles mature and the PA content increases, however, lower amounts of the total PAs are methanol insoluble. Since there is considerable specificity to the binding and subsequent precipitation of PAs with proteins and presumably with carbohydrates [48, 52], one might argue that the pool of proteins most easily precipitated is saturated in the case of mature tissues, leaving the bulk of the increased PAs in a soluble form. Another example of how artifacts are formed is shown in the behavior of the 70% methanol soluble fraction when chromatographed on paper or thin-layer-cellulose. Oligomers larger than trimers, originally soluble in aqueous methanol, did not migrate in the butanol–acetic acid water solvent, but formed a basal streak in the 5% acetic acid solvent; not all of these oligomers in the basal streak could be subsequently eluted in the same methanol solvent, presumably due to hydrogen bonding to the cellulose [50].

*Secondary changes: chemically modified PAs*

A distinction must be made between unmodified PAs and those modified by subsequent oxidations and cross-bondings within the cell. Unaltered PAs are colourless with long wave length absorption peaks between 270 and 280 nm, depending on the ratio of prodelphinidins to procyanidins [19, 53]. The higher oligomeric PAs from needles and from Douglas fir cell cultures, isolated from predominantly living cells, still showed the typical 270–280 nm peaks. PAs isolated from bark and wood, however, might fall into the category of PAs modified during normal development as mature tissues contain many non-living cells in which the cytoplasm has disintegrated. PAs might then come in contact with enzymes such as polyphenoloxidases and peroxidases, forming oxidized complexes. The poorly defined term, phlobaphenes, is sometimes used to describe highly oxidized and often coloured forms of PAs derived from bark or wood preparations. The varied secondary changes in extractives from wood have been recently summarized [40, 42]. The past history of bark and wood preparations was rarely discussed in published work; the method of drying or storage could radically alter the products. Seed coats are another source of modified PAs [5]. In addition to the developmental modifications mentioned above, other changes might occur upon infection by pathogens or insect attacks. These will be discussed later.

Bark and wood are non-technical terms. The bark preparations of tree trunks reported in the literature might consist of only the inner portion, containing both non-living (generally crushed) and living phloem cells, but might also include the predominantly non-living periderm and cortex (rhytidome or outer bark) [23]. PAs have been reported in periderm and rhytidome tissues [40, 53]. The phloem is a complex tissue consisting of sieve elements, fibers and parenchyma cells. The xylem (wood) is a complex tissue consisting of tracheids in gymnosperms, plus vessels in angiosperms, as well as fibres and parenchyma [22, 23]. The distribution of lignin in wood (xylem) has been recently reviewed [15]. Lignin was more concentrated in hemlock wood compared with bark, while just the opposite was true with PAs [40].

The vertical systems of xylem and phloem are transected by the horizontal system of rays, containing parenchyma cells filled with storage products and tannins [23, 43, 53]. Rays also include lignified cells [15, 16]. It is not clear whether both products are synthesized in the same cell. A similar possibility exists with endodermal cells. Young endodermal cells have been reported to contain nitroso positive compounds in vacuoles, presumably PA's [28, 30], while older endodermal cells gave positive lignin reactions [41].

*Secondary changes: modified locations of PAs*

Although there are no definitive reports of PAs localized in cell walls, Hillis implies that PA monomers and at least the smaller PA oligomers could penetrate the pores of some cell walls in heartwood (non-living cells) but not sapwood (living cells) when the tonoplast (vacuolar membrane) ruptures upon death of the cells [42]. However, few details were given and the problem of artifacts during extraction is unknown. Reddish-brown phenolics (oxidized PAs or phlobaphenes) have been observed histochemically not only in the lumina, but also adhering to the inner surface of the walls of parenchyma cells near fungal checks [54]. A secondary location of PAs in walls has been suggested in studies on barley seed development [55].

Another secondary change in location of PAs involves the formation of tyloses in which cytoplasmic outgrowths of ray parenchyma cells filled with PAs enter non-living vessel cells [23, 42, 43]. This would put PAs in lignified cells by transfer of vacuolar contents of the original ray storage cells containing PAs into the lignified vessels of the xylem. In cotton seedlings, histochemical tests with the dimethylbenzaldehde reagent showed isolated parenchyma cells full of red stain right next to colourless vessels [30]. This is what would be expected prior to the formation of tyloses.

There is a great need for new histochemical and other analytical techniques to determine the *in situ* localization and composition of both of these polymers in both living and non-living cells. Perhaps some of the solid-state $^{13}C$ and NMR techniques used with insect cuticles can be used effectively with both lignin and PAs [56].

*Functional analogies*

The physiological significance of phenolic compounds in plants has been reviewed recently by Rhodes [57]. Lignin is well known as having a supporting function. A

supporting function for PAs, however, even with carbohydrate connections as visualized by Zucker [4] and Shen *et al.* [1], would not be expected if the PAs remain in the large central vacuole in living cells. On the other hand, if PAs become adpressed to or infiltrate the cell wall upon degradation of the cells in either bark or wood tissue of a tree trunk as indicated above, they might serve this function. There is a definite need for more evidence of this possibility.

In contrast to such a supporting function, however, both modified and unmodified PAs could serve as generalized defenses against herbivores or invading parasites. The analogy with lignin would now be valid as this polymer is also considered to have a defense function [58]. While both polymers are relatively resistant to microbial degradation compared with other secondary products, the presence of methoxyl groups at position 3 rather than 3,4-hydroxyl groups endows lignin with greater resistance than PAs. Microbial degradation studies of PAs [59] and of lignin [60–62] can aid in understanding both the basic structure and means of resistance to pathogens. In favour of this common defense function, although the cause and effect relationships are not well defined, are the demonstrations that both lignin [10, 25] and PAs [53, 63, 64] appear in the resistance wood (reaction parenchyma zones) and in areas surrounding necrotic spots produced by insect or fungal damage. The so-called wound lignin may have a different composition than the lignin of xylem elements [58]. Lignosuberized cell walls have been demonstrated in wound tissue upon pathogen attack in both cortical parenchyma cells of primary growth tissue [65] and in periderm produced by secondary growth [66]. Growth of the mycelium was arrested in fleck spots in sugar pine associated with dense, brown deposits assumed to be PAs [64]. The fact that some insects and microbes successfully bypass some of these defenses does not lessen their possible effect towards other insects [67].

An intriguing additional function of soluble PAs, which might be related to a defense function, is the potential information available in the sequence of flavonoid units of the oligomeric chains of PAs, since frequently both 2,3-*trans* and 2,3-*cis* isomers as well as diphenolic and triphenolic B-rings are involved. A similar 'informational' function might also be associated with the order of lignin units. Recent evidence of the participation of plant phenolic compounds related to lignin and flavonoids as signals inducing the expression of *Agrobacterium* and *Rhizobium* genes in plants is intriguing [68–70]. Both defense and informational functions would imply that the biosynthetic sequence leading to these polymers is regulated enzymatically and is not formed just by a random assortment of units.

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