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THE BIOSYNTHESIS OF POLYPHENOLS IN TISSUES WITH LOW PHENYLALANINE AMMONIA LYASE ACTIVITY*

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Abstract—Phenylalanine ammonia lyase (PAL) activity in the leaves of *Eucalyptus sideroxylon* and *E. sieberi* was determined at intervals throughout the year. Activity increased as leaves enlarged then declined as senescence approached. Some leaves lacked activity and there was variation within and particularly between seasons. The PAL species present was not affected by leaf polyphenols which lower the activity of the PAL species from barley. PAL activity was not detected in the zone between the sapwood and heartwood of *E. polyanthemos* and *Pinus radiata*. Pinosylvin synthase activity did not increase in the intermediate zone of *P. radiata*. Dehydroshikimate reductase could not be detected in leaves or woody tissues. Labelled phenylalanine was rapidly converted to polyphenols in *E. sideroxylon* leaves of low PAL activity but the sequence of polyphenols formed was different from that found when labelled glucose was fed. Evidence is presented supporting the view that neither phenylalanine nor its ammonia lyase were obligatory in the biosynthesis of eucalypt leaf or heartwood polyphenols.

INTRODUCTION

THE TRANSFORMATION of sapwood to heartwood is accompanied by a sharp increase in the amount of polyphenolic extractives. There is much evidence[1] to support the view that the heartwood extractives are formed at the boundary between these zones from translocated carbohydrate. However, most studies[2-5] have not revealed cytological evidence for an increase in metabolism although there is other evidence[6-9] of an increase or change in metabolism at this boundary. Difficulties in assessing experimental results could be partially due to seasonal changes in enzyme activity at the heartwood boundary and consequent sampling inconsistencies. On the other hand, the formation of polyphenols in such tissues may proceed slowly for many months in contrast to the behaviour in rapidly metabolizing zones adjacent to freshly damaged cells in potato tubers, fruit, etc. Also, the high content of polyphenols in the tissues could be responsible for the failure to detect metabolic activity in some cases, due to enzyme inhibition by some of these substances.

It is widely considered that phenylalanine is an obligatory intermediate in the biosynthesis of phenylpropanoid compounds. A parallel has been shown between the formation of these

* Part V in the series "The Formation of Polyphenols in Trees"; for Part IV see *Phytochem.* **7**, 13 (1968).
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[6] H. ZIEGLER, *Holz Roh- Werkstoff* **26**, 61 (1968).
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[9] H. H. BOSSHARD, *Wood Science Technol.* **2**, 1 (1968).

compounds and the activity of L-phenylalanine ammonia lyase (PAL; EC 4.3.1.5) in a number of cases. It has been found in high concentrations in those portions of tissues synthesizing lignin or polyphenols and almost or completely absent in those tissues that are not.[10-17] In one case, with strawberry leaves grown in a glasshouse,[14] there was considerable variation in PAL activity throughout the year. Labelled sucrose has been converted into phenylalanine and polyphenols at the heartwood boundary of *Prunus* wood[18a,b] but PAL activity in inner sapwood tissues was insignificant compared with that of cambial tissues.[3] Earlier workers failed to find PAL activity in eucalypt leaves with a high polyphenol content[19,20] although ^{14}C phenylalanine formed labelled polyphenols much more readily than did ^{14}C tryosine.[19]

The present work was undertaken to determine the activity of PAL and other enzymes in leaf and woody tissues and to study factors which could influence their activity. It also examines whether the PAL activity in mature tissues is sufficiently high for the formation of polyphenols by the accepted biosynthetic pathway and whether this enzyme or phenylalanine are obligatory in polyphenol biosynthesis.

RESULTS AND DISCUSSION

Variations in PAL Activity

In leaves. PAL activity was not detected in *Eucalyptus sideroxylon* leaves in earlier work[19] although phenylalanine ^{14}C was incorporated into polyphenols without appreciable breakdown into the precursors of sugars. In this present work examination over a 3-month period lack of activity in two batches of expanding and mature leaves but activity in three other batches of leaves (Table 1).

An examination of *E. sieberi* leaves (Table 1) over a 12-month period showed variations in PAL activity and absence in old mature leaves which were collected when new leaves were sprouting from the same twig (October 2). The enzyme activity in the acetone powder declined with increasing leaf weight and age. On this basis, activity was at its highest in enlarging tissues and comparable to the situation in buckwheat.[21] However, the calculated PAL activity in an average leaf first increased and then declined towards the end of the 12-month period (Table 1) when the leaves approached senescence.

The increase in PAL activity in eucalypt leaves could be the result of enzyme induction,[16,22-25] prevention of synthesis of a PAL inhibitory protein[26] or other inhibitors. The

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[24] M. ZUCKER, *Plant Physiol.* **43**, 365 (1968).
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decrease in PAL activity as the enlarged leaves became older could also be due to an increasing rate of degradation relative to synthesis or to inactivation.[27]

The leaf samples were taken from one tree of each species but PAL activity varied within a season and between two successive spring seasons (Table 1). These variations could be due to different amounts of deactivators or to varying formation of them under experimental conditions. Eucalypt leaves contain appreciable amounts of polyphenols. This class of

TABLE 1. AGE AND WEIGHT OF *Eucalyptus sideroxylon* AND *E. sieberi* LEAVES AND THEIR PAL ACTIVITY

Collection date	Fresh weight (g, average) of one leaf blade	Yield of acetone powder (% of fresh weight)	Units of PAL activity: Per 200 mg of acetone powder	Units of PAL activity: Total per leaf
E. sideroxylon				
23 Aug.	0·35	28	8·8	4·3
2 Oct.	0·49	32	0·0	0·0
2 Oct.	0·04	10	63·5	1·3
23 Oct.	0·10	23	0·0	0·0
23 Nov.	0·18	24	12·8	2·8
23 Jan.	0·02	22	0·5	0·01
E. sieberi				
7 Nov.	0·03	7	279·8	2·9
21 Nov.	0·14	9	229·0	14·4
28 Nov.	0·21	11	173·0	20·0
7 Dec.	0·30	17	94·8	24·2
21 Dec.	0·50	18	20·6	9·3
30 Dec.	0·52	21	36·4	19·9
16 Feb.	0·52	23	47·4	28·4
16 March	0·51	25	41·6	26·5
24 April	0·55	25	14·8	10·1
22 May	0·75	28	36·0	37·9
22 June	1·35	30	9·1	18·6
24 July	1·67	29	9·9	24·2
23 Aug.	1·68	32	5·1	13·8
2 Oct.	1·70	31	0·0	0·0
2 Oct.	0·04	7	99·2	1·4
23 Oct.	0·15	8	42·0	2·5
23 Nov.	0·50	10	10·2	2·6

compound can deactivate certain enzymes[28–31] and may be responsible for this variation. However, Polyclar AT, polyethylene glycol and *N*-methyl-2-pyrrolidone, which have been effective in minimizing the effects of polyphenols[28–31] in other cases, did not raise the activity

[27] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Botany* **44**, 341 (1966).
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[31] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *Phytochem.* **4**, 659 (1965).

of PAL isolated from *E. sieberi* leaves (Table 2). Also it is notable that the extractives of *E. sieberi* leaves, even after oxidation, did not affect the activity of PAL from this source (Table 2). These extractives, however, lowered the activity of PAL isolated from 12-day-old barley leaves from 37·8 to 28·3 units. Thus, the PAL species in *E. sieberi* leaves is more tolerant of eucalypt polyphenols than the PAL species in barley. There is evidence for species of PAL[32,33] that respond differently to polyphenols.[34] It therefore appears unlikely that polyphenols are responsible for the decrease of activity in mature eucalypt leaves or for the seasonal variations.

A possible explanation of the variation of PAL activity is that PAL may not be an obligatory enzyme for the formation of all phenolic compounds containing the C_6–C_3 moiety. It is noteworthy that PAL activity is highest in the enlarging leaves and may be more directly related to lignification (compare Ref. 10) rather than polyphenol biosynthesis.

TABLE 2. ACTIVITY OF PAL FROM *Eucalyptus sieberi* LEAVES AFTER DIFFERENT TREATMENTS

Sample collected	14 November	7 December	13 January
Buffer (pH 8·8) used in assay	0·05 M tris-HCl	0·1 M borate	0·1 M borate
Activity* of acetone powder:			
1. Without pretreatment	136	94·8	11·2
2. After addition to acetone of:			
(*a*) Polyethylene glycol 400	135	107·7	—
(*b*) N Methyl-2-pyrrolidone	142	83·4	—
(*c*) Polyclar AT	141†	97·8†	—
(*d*) 2-Mercaptoethanol (0·25 M)	139	—	—
(*e*) Sodium ascorbate (0·25 M)	138	—	—
3. After addition to acetone powder of extracts from *E. sieberi* leaves			
(i) water extract			11·7
(ii) 0·1 M borate buffer extract			10·6

* PAL units per 200 mg.
† Value calculated to allow for the addition of insoluble powder.

In woody sections. The acetone powders from the woody tissues were prepared by techniques (see Experimental) that minimized possible changes caused by heat, oxidation and contamination with polyphenols. Nevertheless PAL activity was not detectable in *Pinus radiata* (Table 3) and was variable in one cross-section of *E. polyanthemos*. Another cross-section of the latter species showed the sharp and continuous decrease in PAL activity from the cambial zone to heartwood as was found with *Cryptomeria* and *Chamaecyparis* sp.[35] In no case was activity observed in the intermediate zone adjacent to the heartwood.

The Relevance of PAL Activity to the Biosynthesis of Heartwood Polyphenols

Neither in this or other reported work has a significant, relative increase in PAL activity been observed in the intermediate zone even though it is adjacent to heartwoods containing

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[33] E. A. HAVIR and K. R. HANSON, *Biochem.* **7**, 1896 (1968).
[34] T. MINAMIKAWA and I. URITANI, *J. Biochem. Tokyo* **58**, 53 (1965).
[35] T. HIGUCHI and K. FUKAZAWA, *J. Japan Wood Res. Soc.* **12**, 135 (1966).

appreciable amounts of polyphenols. There are several possible reasons for this low activity. It may be due to seasonal variation of PAL activity and the choice of the wrong times for sampling. On the other hand, PAL may never become highly active but, nevertheless, together with other enzymes, slowly catalyses the formation of polyphenols which over a period of months accumulate in appreciable quantities. If this is so, then the common methods of analyses for enzyme activity are inappropriate because they are for tissues with high activity.

However, in view of the variation of PAL activity mentioned in the preceding sections, it is possible that phenylalanine may not be an obligatory intermediate in heartwood polyphenol formation. It is not a major amino acid in sapwood or heartwood and its amount

TABLE 3. ENZYME ACTIVITY IN CROSS-SECTIONS OF *Eucalyptus polyanthemos* AND *Pinus radiata*

	Yield* (%)	Nitrogen content (%)	Activity of PAL†	PS‡	DHSR‡
A. Tris-HCl buffer (pH 8·2) extracts of acetone powder from					
1. *E. polyanthemos*, collected 4 Dec.					
Outer sapwood	1·01	5·97	0·0		0
Middle sapwood	1·16	5·52	2·95		0
Intermediate zone	1·45	4·49	0·0		0
Outer heartwood	2·97	2·76	0·15		0
2. *P. radiata*, collected 4 Feb.					
Outer sapwood	1·80	8·65	0·0	+	tr
Middle sapwood	1·46	8·50	0·0	+	tr
Intermediate zone	1·84	8·05	0·0	+	tr
Outer heartwood	1·65	6·49	0·0	+	tr
B. Phosphate buffer (pH 7·4) extracts of acetone powder from					
1. *E. polyanthemos*, collected 13 Nov.					
Outer sapwood	0·92		1·35		0
Middle sapwood	0·66		0·18		tr
Intermediate zone	0·86		0·00		tr

* From dried acetone-extracted wood.
† Units/g buffer extract.
‡ PS = pinosylvin synthase, DHSR = 5-dehydroshikimate reductase.

does not vary appreciably across the stem of a tree.[36,37] If this amino acid (and its ammonia lyase) was obligatory then the formation of a heartwood containing 29 per cent extractives of the flavonoid type would produce also 1·7 per cent ammonia which at some stage should be, but was not, detected (compare Ref. 38). Furthermore, Bate-Smith and Swain[39] have proposed the direct formation of the *ortho*-dihydroxy substituted flavonoids from a monohydroxy-prephenic acid. Recent evidence[40] indicated flavonoids with vicinal trihydroxy

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substituted side-rings originated directly from shikimic acid. In view of the above observations it appears possible that pathways not involving amino acids and deamination can play a part in polyphenol biosynthesis in the intermediate zone.

Estimation of Other Enzymes

Because PAL may be involved in other reactions besides polyphenol formation, a search was made for an enzyme that acted near the end of the polyphenol pathway. This was found in the form of pinosylvin synthase (EC 4.2.1)[41] and it is expected that its activity would be more directly related to the amount of stilbenes present. The buffer extracts (Table 3) were examined and the synthase activity was estimated to be the same in all tissues of *P. radiata* whereas pinosylvin is present in appreciable quantities only in the heartwood. The only cinnamoyl triacetic acid available as substrate for these determinations had begun to change and was no longer suitable for quantitative estimations.

The pathway from carbohydrate to aromatic compounds probably passes through 5-dehydroshikimic to shikimic acids[42] and 5-dehydroshikimate reductase (DHSR; EC 1.1.1.25) has been extracted from different sources.[43–46] Despite this occurrence elsewhere the DHSR activity was negligible or absent in the extracts of the woody tissues (Table 3) and in three batches of young and mature leaves of *E. sieberi.*

In none of the cross-sections examined was there any increase in the activity of PAL, pinosylvin synthase or DHSR in the zone adjacent to heartwoods containing large amounts of polyphenol.

Polyphenol Biosynthesis in Old Tissues

It is necessary to know the extent, and how rapidly, polyphenols can be synthesized in tissues of low PAL activity. In view of the experimental difficulties encountered in studying the intermediate zone of woody tissues, relevant information was obtained from mature leaves with low enzyme activity.

Leaves appear to be a suitable comparative tissue. When they change from the small expanding to the fully differentiated and mature form, the importance of the Embden–Meyerhof–Parnas (EMP) glycolytic pathway is reduced.[47,48] A similar change in the ratio of the EMP and pentose phosphate (PP) pathways across the sapwood to the intermediate zone has been found.[3] Reduced water uptake diminishes the participation of the EMP pathway[49,50] in leaves and in many trees there is a sharp decrease of water at the heartwood boundary which may have a similar effect on metabolism. Mature leaves were used as the degree of incorporation of precursors into lignin would be small, whereas polyphenol synthesis could continue to be functional.

Conversion of Labelled Phenylalanine and Glucose to Polyphenols

The rate of incorporation of labelled compounds was studied using branches of dwarfed leaves from cultivated bonsai (dwarfed trees) of *E. sideroxylon.* The PAL activity in mature

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[44] T. HIGUCHI and M. SHIMADA, *Plant Cell Physiol.* **8**, 61 (1967).
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[50] H. BEEVERS and M. GIBBS, *Plant Physiol.* **29**, 322 (1954).

leaves was low or not detectable. The leaves were less than one-tenth of the weight of normal leaves and the composition of polyphenols was very similar to that in leaves from the fully grown parent. The main phenolic components were catechin, engelitin (dihydrokaempferol 3-rhamnoside), quercitrin (quercetin 3-rhamnoside), nicotiflorin (kaempferol 3-rutinoside), piceid (3,4′,5-trihydroxystilbene 3-glucoside), rhapontin (3,3′,5-trihydroxy-4′-methoxystilbene 3-glucoside), astringin (3,3′,4′,5-tetrahydroxystilbene 3-glucoside), *p*-coumarylquinic and chlorogenic acids.[51,52] Leaves were removed at intervals after feeding and extracts from them were chromatographed two-dimensionally. The rate of incorporation was assessed from radioautograms of the chromatograms.

When L-phenylalanine-U-^{14}C was fed to mature bonsai leaves it entered a pathway to polyphenols with little breakdown.[19] The order of appearance of labelled components is given in Table 4. For the first 6 hr component "X" (R_f 0·95–0·85 in BAW, 0·0 in HA)

TABLE 4. THE ORDER OF FORMATION OF POLYPHENOLS AFTER FEEDING PHENYLALANINE U-^{14}C TO *Eucalyptus sidroxylon* BONSAI LEAVES*

Metabolic period		Compounds†											
Sunlight (hr)	Darkness (hr)	PA	X	pC esters	Chl	Cat	S	Y	Rha	Pic	Ast	Eng	F'ol
1	—	4‡	5										
3	—	4	5	2	t								
6	—	5	5	5	2	1	5	3					
6	16	2	3	5	3	5	2	5	1	t			
10	16	1	4	5	2	3	2	4	2	1			
14·5	16	t	3	5	2	1	1	4	5	2	1		
14·5	31·5	t	4	5	4	1	2	4	5	5	3		
18·5	31·5	t	3	5	5	1	2	5	5	5	3	3	2
20·5	31·5	t	2	5	5	1	t	5	5	5	5	5	2

* Exp. 1. Only illustrative time intervals are shown.

† PA = phenylalanine, X = unknown R_f 0·8–1·0 in BAW, R_f 0·0 6 per cent HA, pC esters = isomers of *p*-coumarylquinic acid and *p*-coumaryl shikimic acid,[51] Chl = isomers of chlorogenic acid, Cat = catechin, S = unknown R_f 0·65–0·9 in BAW, R_f 0·88 in 6HA, Y = unknown R_f 0·5–0·8 in BAW, R_f 0·06 HA, Rha = rhapontin, Pic = piceid, Ast = astringin, Eng = engelitin, F'ol = flavonol glycosides.

‡ The numbers represent relative amounts of activity in any one time period, 5 is given to the compound of maximum activity and others are related to it, t = trace.

occupied a much larger spot size than the chlorophyll and oily components which have similar R_f values but much lower activity (Table 5). The spot size reduced to that of the oily components during the first period of darkness. After 4·5 hr of sunlight a component "Y" (R_f 0·4–0·8 in BAW, 0·0 in HA) became strongly labelled and thereafter it remained a strong component of the radioautograms. Chlorogenic and *p*-coumarylquinic acids also became active in this period and remained strongly active throughout the experiment in contrast to catechin, the next compound to be labelled. Chlorogenic acid could result from hydroxylation of the *p*-coumarylquinic acid[53,54] and it has been formed in 1–4 hr from cinnamic acid or phenylalanine in other plant tissues.[54,55] An unidentified spot, S (R_f 0·65–0·90

[51] W. E. HILLIS and K. ISOI, *Phytochem.* **4**, 541 (1965).
[52] M. HASEGAWA and W. E. HILLIS, *Botan. Mag. Tokyo* **79**, 940 (1966).
[53] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).
[54] W. STECK, *Phytochem.* **7**, 1711 (1968).
[55] A. O. TAYLOR and M. ZUCKER, *Plant Physiol.* **41**, 1350 (1966).

in BAW, 0·88 in HA), appeared during this period. The stilbenes were the next recognizable compounds to be labelled. Rhapontin had a stronger intensity than piceid, although astringin was labelled after piceid. Subsequently engeletin and then the quercetin glucosides were labelled. The activity of phenylalanine fell to a marginal value after 26 hr and weak activity appeared in glucose after 22 hr. Activity appeared in areas not associated with the above spots after 24 hr. In another experiment there was very rapid incorporation of phenylalanine into polyphenols in leaves of low activity (Table 5).

The order of appearance of labelled compounds when glucose-U-^{14}C was fed to mature bonsai leaves differed markedly from that in Table 4. In the early stages, a strongly radioactive streak "A" (R_f 0·0–0·7 in BAW and 0·9 in 6 per cent acetic acid) appeared with activity at the positions occupied by glucose, sucrose, shikimic acid and phenylalanine.[56] Another active streak "B" appeared on the BAW axis, R_f 0·7–1·0, and this became very strong when

TABLE 5. THE ACTIVITY OF POLYPHENOLS AFTER RAPID FEEDING OF PHENYLALANINE-U-^{14}C TO *Eucalyptus sideroxylon* BONSAI LEAVES*

Leaf extracts	Counts (× 1000) of compounds†										
	PA	X	cis-pC	trans-pC	pC esters	Chl	Cat	Y	Stilb	Su	I
Methanol extract:‡											
Fraction soluble in											
Ether	0	26	23	23	0	0	12	12	3	0	4
Ethyl acetate	0	0	0	0	38	22	12§	8	17	0	15
Aqueous residue	22§	0	2	0	63	31	5	1	3	3	29
Alkali extract of Methanol-extracted leaves	0	0	3	2	0	0	0	0	0	0	14

* Exp. 4. Metabolic period: 1·5 hr. Control leaves had PAL activity of 0·5 units per 200 mg of acetone powder or 0·01 units per leaf. Counts were obtained from two-dimensional chromatograms.

† Same as Table 4 and in addition cis pC = *cis p*-coumaric acid, trans pC = *trans p*-coumaric acid; Stilb = stilbene glycosides R_f 0·3–0·6 in BAW, 0·05 in 6HA; Su = sugars; I, 0·0–0·3 in BAW, 0·0–1·0 in 6HA but excluding stilbenes and sugars.

‡ The concentrated extract was poured into water and successively extracted with petrol (which removed material with R_f 0·85–1·0 in BAW, 0·0 in 6HA and activity 3000 counts) ether and ethyl acetate.

§ Incomplete resolution from adjacent compounds.

the polyphenols were clearly labelled. The maximum intensity of this streak had a lower R_f value than the accompanying chlorophyll and oily portion and the streak differed from component "X" in that it did not decrease in darkness but lengthened with time. Labelled catechin was the first polyphenol to appear (within 3 hr) followed by rhapontin and piceid, then quercitrin and engelitin followed by *p*-coumarylquinic then chlorogenic acid.[56] The early appearance of activity in catechin indicates that the activity in the other components is not solely due to the glucose moiety. After a metabolic period of 48 hr, the relative amount of activity was distributed as follows: streak A, score 5; streak B, 4; *p*-coumaric acid esters, t; chlorogenic acids, t; catechin, 1; rhapontin, 1; piceid, 1; engelitin, 1; quercitrin, t; where 5 represents the greatest intensity of activity and t represents trace.

It is evident that aged leaves with negligible PAL activity can transform phenylalanine or glucose to polyphenols readily. Consequently, the absence of detectable activity in the

[56] W. E. HILLIS and K. ISOI, unpublished data.

zone intermediate between sapwood and heartwood does not necessarily preclude these tissues forming polyphenols over a period of time.

Biosynthesis of Polyphenols in E. sideroxylon *Leaves*

In the preceding section it was reported that the order in which the labelled compounds appeared when glucose-^{14}C was fed to the leaves differed from that when phenylalanine-^{14}C was used. One of the distinctive differences was the early appearance of *p*-coumarylquinic acid followed by chlorogenic acid when phenylalanine (Table 4), but not glucose, was fed. When glucose was fed, catechin and the glycosides of stilbenes and flavonoids were formed before the above acids.

With the techniques used the ratio of incorporation and turnover of the precursors cannot be determined. The question arises whether the above cinnamic acid derivatives are normal intermediates in the biosynthesis of flavonoids and stilbenes. They may have accumulated because the small pool size of phenylalanine in *E. sideroxylon* had been exceeded during feeding with phenylalanine-^{14}C. It can be seen (Table 5) that when there is a quick uptake of this compound most of the activity detected was in the cinnamic acid derivatives within 90 min of administration. Furthermore, the activity in the alcohol-insoluble cinnamic acids was less than one-tenth that in *p*-coumarylquinic and chlorogenic acids. (In wheat, 24 hr after feeding phenylalanine-^{14}C, the ratio of activity was 3:1 in the insoluble and soluble esters).[57,58] Also the *cis*- and *trans*-isomers of *p*-coumaric acid were formed in appreciable amounts (about 15 per cent of extractable activity). It is noteworthy that *p*-coumaric acid was the only active component released from the alcohol-insoluble leaf residue by alkali. After 4·5 and 6 hr (Expt. 3, Experimental) the relative amount of free *p*-coumaric acids was much less than after the 1·5 hr metabolic period.

Tracer and enzyme studies have implicated cinnamic acid derivatives as key intermediates in the biosynthesis of lignin and flavonoid compounds.[59,60] The part played by the soluble esters, such as chlorogenic acid, is uncertain at this stage.[54,61,62] El-Basyouni and Neish[63] have concluded that the intermediates en route to flavonoids are the cinnamic acid esters that are insoluble in acetone or alcohol and not the soluble esters or free acids. The metabolically important cinnamoyl esters are then hydroxylated to form the hydroxycinnamoyl esters required for the biosynthesis of other polyphenols. However, while these esters have been found in several plant tissues[57,58,63,64] they are absent in others.[61,62] Consequently, it cannot be concluded that the soluble esters and the free *p*-coumaric acid found in this work are in the obligatory pathway for the biosynthesis of flavonoids and stilbenes.

When phenylalanine-^{14}C was fed, component "X" quickly formed (Table 4). The accompanying oils and chlorophyll had little activity (Table 5). That X forms rapidly after feeding phenylalanine and before the appearance of any other compound (Table 4) suggests that it is a complex incorporating phenylalanine or a cinnamic acid derivative. When a concentrate of labelled compound X isolated from *E. sideroxylon* leaves (ether fraction,

[57] SAID Z. EL-BASYOUNI, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* **3**, 627 (1964).
[58] A. FUCHS, R. ROHRINGER and D. J. SAMBORSKI, *Can. J. Botany* **45**, 2137 (1967).
[59] A. C. NEISH, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 295, Academic Press, London (1964).
[60] A. C. NEISH, in *Formation of Wood in Forest Trees* (edited by M. H. ZIMMERMANN), p. 219, Academic Press, New York (1964).
[61] A. O. TAYLOR and M. ZUCKER, *Plant Physiol.* **41**, 1350 (1966).
[62] A. O. TAYLOR, *Phytochem.* **7**, 63 (1968).
[63] SAID Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).
[64] SAID Z. EL-BASYOUNI and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **42**, 203 (1964).

Table 5) was incubated with unpurified acetone powder from the same species, the ratio of *cis p*-coumaric acid increased considerably. After the completion of this work, it was found that Zaprometov and Bukhlaeva[65] had also isolated a similar substance after feeding tea shoots with small amounts of phenylalanine. Their compound, which was assumed to be acidic in nature, did not accumulate after injection of shikimic acid or sugars.

CONCLUSIONS

The PAL activity in the leaves of *Eucalyptus sideroxylon* and *E. sieberi* increased as the leaves enlarged and then declined as senescence approached. The activity varied considerably within and particularly between seasons. PAL activity was not detected in the zone between sapwood and heartwood of *E. polyanthemos* and *Pinus radiata* and no direct relationship between PAL activity and polyphenol content of leaves and heartwood was seen. When labelled phenylalanine-^{14}C was fed to non-expanding leaves it formed largely cinnamic acid derivatives and the order in which the polyphenols were formed was different to that when glucose-^{14}C was the substrate. With glucose the cinnamic acid derivatives were formed after the flavonoids and stilbenes. The involvement in flavonoid biosynthesis of the cinnamic acid derivatives detected in this work has not yet been proven. From a consideration of the above and other evidence we conclude that further attention should be given to the possibility that in mature eucalypt and pine tissues phenylalanine and its ammonia lyase are not obligatory for the biosynthesis of flavonoids and stilbenes. It is noteworthy Zaprometov and Bukhlaeva[65] also considered that there is an alternative pathway to flavonoids that does not involve phenylalanine. Furthermore they have shown that shikimic acid was utilized much better than L-phenylalanine in the formation of catechins in tea shoots.

EXPERIMENTAL

Materials

The leaf samples examined at intervals for PAL activity were obtained from 13-year-old trees of *Eucalyptus sieberi* L. Johnson and *E. sideroxylon* A. Cunn. ex Woolls (chemovar. stilbenoid, chemosubvar. dihydrokaempferol, chemoform. S1).[19,66] The bonsai leaves of *E. sideroxylon* were obtained from 2–7-year bonsai plants 8–40 cm high, grown with a confined root system in a glasshouse. The weight of bonsai leaves ranged from 10–65 mg.

The *E. polyanthemos* Schau cross-sections were collected from localities in the Broadford and Alexandra districts about 80 miles from Melbourne. The diameter of the logs were 30 cm (collected 13 November) and 30 cm (collected 4 December). The cross-section of *Pinus radiata* D. Don collected from the Mt. Macedon plantation (4 February) had forty-eight growth rings and was 43 cm dia. The logs were wrapped in plastic immediately after felling and within a few hours cooled at 0–4° for 16–18 hr. Disks, 11·5 cm wide, were cut and without delay were peeled on a lathe. Veneers of 0·1 mm (for pine) and 0·15–0·25 mm (for eucalypt) thickness were prepared and the selected zones immersed in cold (about −20°) acetone within 5 sec of peeling.

The intermediate zone of *E. polyanthemos* was 40 mm wide, whiter in appearance than the sapwood and it enveloped the strong red-brown-coloured heartwood. The inner portion of the zone darkened considerably on air-drying, suggesting the presence of oxidative enzymes. The samples of *E. polyanthemos* intermediate zone were less contaminated by adjacent tissues than those from *P. radiata* where the intermediate zone was less distinct.

Determination of Phenylalanine Ammonia Lyase Activity

In the study of changes in PAL activity (Table 2), the third and fourth leaves only were taken from each twig. Fresh leaf blades (10 g) were chopped to a small size and soaked in three changes of cold acetone (120 ml, 2–4°) for 10 min and then disintegrated in cold acetone (70 ml, −30° to −40°) in a M.S.E. homogenizer for 6 min. The homogenate was suction-filtered, washed with 50 ml of cold acetone, dried in vacuum and

[65] M. N. ZAPROMETOV and V. YA. BUKHLAEVA, *Biochemistry (USSR)* **33**, 317 (1968).
[66] W. E. HILLIS, *Phytochem.* **5**, 541 (1966).

stored in a sealed glass container at 2–4°. A tissue-disintegrating machine[41] produced an acetone powder (26 per cent yield) from young leaves (3–7 cm, 0·14 g average weight) of *E. sieberi* of a more colourless appearance and higher PAL activity (11·2 units) than that obtained with a M.S.E. homogenizer (26 per cent yield and 9·6 units activity). Except for the bonsai leaves, the powder from the leaves (Table 1) was produced with the homogenizer before the development of the machine.

In studies of the effect of polyphenol absorbents, Polyclar AT (2·5 g), polyethylene glycol (mol. wt. 400) (0·5 g), *N*-methylpyrrolidone (0·5 g), sodium ascorbate (0·25 M) and glutathione (10^{-4} M) were added for each 10 g of fresh leaves. The leaves were disintegrated in the M.S.E. homogenizer and activity of the acetone powders obtained with Polyclar were corrected for the addition of the latter. Acetone containing 0·25 M mercaptoethanol was used in all other preparations of acetone powders because of reported effectiveness of the thiol in combining with quinones and improving the efficiency of the extraction of enzymes.[67]

The wood veneers were immersed for 15 min in three changes of acetone (about ten times volume of material, 2–4°) containing 0·25 M mercaptoethanol and then disintegrated by the tissue-disintegrating machine, washed with cold acetone and dried. Satisfactorily pulverized powders were obtained from eucalypt veneers but less satisfactory results were obtained from the pine.

The acetone powder from the wood veneers was extracted with 0·05 M tris-HCl buffer (pH 8·2) containing mercaptoethanol, or 0·1 M sodium phosphate buffer (pH 7·4) (10 ml/1 g of acetone powder) by stirring for 1 hr at 2–4°, the extract squeezed through a nylon cloth and centrifuged at 27,000 *g* for 20 min. The supernatant was dialysed (tubing size 36/32, Visking Co. U.S.A.) against two changes of distilled water (20 times volume) for 24 hr and freeze-dried.

The PAL activity in the leaves was assayed essentially by the method of Koukol and Conn.[68] In the examination of the enzyme from woody tissues, the ether extract from the reaction mixture was developed on TLC plates (silica-gel G.F. 254) with solvent benzene–dioxane–acetic acid (90:25:4) and the band containing *trans*-cinnamic acid was quickly scraped away, eluted and the acid estimated as before. A unit is expressed as the amount of enzyme which catalyses the formation of 1 μg of *trans*-cinnamic acid formed in 1 hr.

The leaf extracts of *E. sieberi* leaves added to acetone powders were prepared as follows: (*a*) a concentrated acetone extract from 5 g of old leaves (blade size 15 cm) were added to water (50 ml) and the water-soluble portion collected, (*b*) old leaves (5 g) were extracted with 0·1 M borate buffer solution (50 ml) and the extract was heated at 100° for 30 min, cooled and filtered. Aliquots of 2·5 ml of extracts (*a*) and (*b*) containing about 2·5 per cent polyphenols were added to acetone powders (0·2 g) from *E. sieberi* or barley leaves.

Determination of the Activity of other Enzymes

Pinosylvin synthase was determined by the published method.[41]

For the determination of 5-dehydroshikimate reductase the following procedure was used. Fresh leaves (about 10 cm long; 20 g) mixed with Polyclar AT (12 g), were homogenized in 10 times volume of 0·1 M sodium phosphate buffer (pH 7·4) by M.S.E. homogenizer for 6 min, squeezed through a nylon cloth, centrifuged at 3200 rev/min for 1 hr, the supernatant was dialysed against two changes of distilled water for 24 hr and freeze-dried with a yield of 331·5 mg. The enzyme preparation (2–5 mg) from leaves or wood was assayed by the method of Sanderson[43] except that 0·1 M glycine–NaOH buffer, pH 9·0, was used. (5-Dehydroshikimic acid decomposes rapidly in solutions above pH 9·0.[69]) The increase in extinction at 340 nm was measured at intervals for 1 hr.

Administration of Glucose-U-^{14}C and Phenylalanine-U-^{14}C to E. sideroxylon *Shoots*

All feeding experiments were done with shoots taken from bonsai *E. sideroxylon* grown in a glasshouse.

Exp. 1. Two shoots were selected, one with sixteen and the other twenty-four mature leaves, with a total leaf weight of 0·979 g and stems of 0·146 g. The leaf weights ranged from 12–35 mg. The shoots were immersed in a solution (0·08 ml) of L-phenylalanine-U-^{14}C (0·576 mg; 23·6 μc) in a glasshouse with air temp. about 35°. The solution was taken up in 1 hr and was followed with H_2O. At intervals (1–2 hr during daylight) the bottom pair of leaves were removed and the stem trimmed and reimmersed in water. The subsequent daylight temperatures ranged from 32–22° and the night (darkness) temperatures 22–15°. The leaves were dropped into boiling MeOH, concentrated to 1 ml and an aliquot (approximately equal to 4·6 mg leaf tissue) added to a two-dimensional chromatogram which was developed first in butanol:acetic acid:water (4:1:5; BAW) and then 6 per cent acetic acid (HA).

Exp. 2. One shoot with a number of small axial as well as the usual bonsai leaves (total leaf weight 0·769 g and stem 0·052 g) was selected. The leaf weights ranged from 4–11 mg. The stem was placed in a solution (0·08 ml) of D-glucose-U-^{14}C (1·23 mg; 25 μc) in a glasshouse with air temp. about 25°. The solution

[67] A. KLEINHOFS, F. A. HASKINS and H. J. GORZ, *Phytochem.* **6**, 1313 (1967).
[68] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[69] S. YOSHIDA, in *Colourimetric Methods in the Biochemical Field* (edited by T. SEKINE), Vol. 3, p. 106, Nankodo, Tokyo (1961).

was taken up in 2·5 hr, and H_2O then added. The leaves were removed at intervals (30 min–2 hr) from the bottom of the shoot during daylight for the next 60 hr, weighed, dropped into boiling MeOH and an aliquot equal to 3–5 mg of leaf tissue was added to two-dimensional chromatograms and resolved with BAW and HA.

Exp. 3. Two shoots (each with six mature leaves) were fed in late February with a solution (0·05 ml) of L-phenylalanine-U-^{14}C (0·062 mg; 2·5 μc) in a glasshouse on a cloudy day, temp. 13–15°. The solution was taken up in 10 min and followed with H_2O. The leaves (316 mg) from one shoot were taken after 4·5 hr and those (282 mg) from the other after 5·75 hr, cut up, and dropped into boiling MeOH.

Exp. 4. Two shoots with ten mature leaves were fed in early March with a solution (0·05 ml) of L-phenylalanine-U-^{14}C (0·062 mg; 2·5 μc) in a glasshouse on a sunny day, temp. 25°. The solution was taken up in 8 min and followed with H_2O and after 1·5 hr the leaves (397 mg) and stems (7·5 mg) were cut up and dropped into boiling MeOH.

Examination of Extracts from Labelled Leaves

The two-dimensional chromatograms from Experiments 1 and 2 were air-dried for several days, covered with "Kodirex" Medical X-ray Film "No Screen" and exposed for 17 months (Exp. 1) and 210 days (Exp. 2). The exposure time for some of the chromatograms in Experiment 1 was greater than optimum and in the latter shorter than optimum.

The cut up leaves from Experiments 3 and 4 were extracted repeatedly with cold methanol and these and the hot methanol extracts were concentrated, dropped into H_2O, extracted with petrol (40–60°), ether and ethyl acetate. The remaining aqueous liquor and the extracts were evaporated to dryness, weighed, dissolved in MeOH and a measured volume (see Table 6), chromatographed two-dimensionally in BAW and 6 per cent acetic acid. In some cases, benzene:acetic acid:water (BzAW 6:7:3) and then 2 per cent formic acid were used also for the identification of *p*-coumaric acid.

The extracted leaves (0·232 g) from Experiment 4 were treated with N NaOH (6 ml) on a steam bath for 1 hr under N_2. The liquor was acidified (HCl), extracted with ether and the extract dried (0·048 g) and chromatographed two-dimensionally in BzAW and 2 per cent formic acid.

The two-dimensional chromatograms from Expts. 3 and 4 were cut into strips and scanned in a Packard Model 7201 Radiochromatogram Scanner using 2·5 mm slit width, and strip speed 5 mm/min. The areas under the curves were measured by planimeter and adjusted by the appropriate linear range during scanning. In most cases, the peaks of maximum activity corresponded very well with the R_f values of the observed spots. The identifications listed in Table 5 are tentative. The proximity of a few compounds to each other prevented accurate assessment of the activity for each spot in certain instances.

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