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THE BIOSYNTHESIS OF POLYPHENOLS IN *EUCALYPTUS* SPECIES*

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Abstract—Phenylalanine-G-^{14}C and tyrosine-G-^{14}C were administered to shoots taken from the normal and variant forms of *Eucalyptus sideroxylon* and variant forms of *E. dalrympleana* and *E. gulfoylei*. The conversion of these amino acids to polyphenols was much less with the normal form than with the variant form. The degree of incorporation of radioactivity into polyphenols was in the order of chlorogenic and *p*-coumarylquinic acids, catechin, dihydrokaempferol rhamnoside, flavonol glycosides and stilbenes. The biosynthesis of different classes of monomeric polyphenols appears to proceed by parallel pathways and the activity of a compound is dependent not only on its relative position in a biosynthetic pathway but on its relative rate of conversion to polymers in the dynamic plant system. Evidence indicates that quercetin is formed by hydroxylation of kaempferol, that rhamnosides are formed more readily than glucosides and that in the formation of disaccharides, the monosaccharides are added one at a time. It is postulated that cyclization of acetate units in flavonoids and stilbenes is a rate-determining step which results in an accumulation of *p*-coumarylquinic and chlorogenic acids when an unusually large amount of material passes through the prephenic acid pathway. Tyrosine was largely converted to sugars and apparently acetate units were degradation products; no evidence for tyrase was found in *E. sideroxylon* leaves. The conversion of phenylalanine to sugar and acetate also took place but to a much smaller extent.

INTRODUCTION

THE EARLY stages of the biosynthesis of aromatic compounds and their precursors have been mainly determined by studying mutants of micro-organisms and determining the nature of the compounds accumulated at the blockages in the biosynthetic pathways. At later stages of biosynthesis, blockages in the normal pathway may enable alternative routes to operate and produce end-products other than a normal intermediate.

A few cases are known of natural mutants in plants which have a marked different chemical composition. A study of biosynthesis in such normal and mutant forms should provide a new approach to the problem of the interrelationship of pathways to different polyphenols. Moreover, it is desirable to know the factors responsible for the changes in the normal biosynthetic pattern and the way in which they act. The possibility of being able to modify the composition of the polyphenolic complex in a plant tissue by decreasing the amount of certain components may have obvious commercial advantages.

The leaves of *Eucalyptus sideroxylon*[1] contain *p*-coumarylquinic and chlorogenic acids, catechin, engelitin (dihydrokaempferol 3-rhamnoside), nicotiflorin (kaempferol 3-rutinoside), quercitrin (quercetin 3-rhamnoside), isoquercitrin (quercetin 3-glucoside) and rutin (quercetin 3-rutinoside). In addition, leaves from the variant form[1] of this species contain in addition piceid (3,4′,5-trihydroxystilbene 3-glucoside), rhapontin (3,3′,5-trihydroxy-4′-methoxystilbene 3-glucoside) and astringin (possibly a pentahydroxystilbene 3-glucoside).

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[1] W. E. HILLIS and K. ISOI, *Phytochem.* 4, 541 (1965).

The purpose of this paper is to report the amount of radioactivity incorporated into the above compounds after feeding shoots with ^{14}C-labelled phenylalanine and tyrosine, and to postulate their biosynthetic pathways (and their interrelationship) in the normal and variant forms of this *Eucalyptus* species.

RESULTS AND DISCUSSION

Expression of the Incorporation of Activity

At the outset of this work it was considered possible that the relative positions in the biosynthetic pathways of a number of closely related compounds could be deduced from the degree of incorporation of activity from a relatively simple precursor. Such an approach would obviate the criticisms that more complex precursors cannot permeate to the sites of synthesis in the cell or that some compounds may be modified in the cell to products which then enter the normal pathway. However, it became apparent during the course of the work that the methods used did not permit the calculation of a satisfactory expression of the degree of incorporation into a dynamic system. It is commonly supposed that flavonoids are end-products of biosynthesis, but this is probably incorrect, particularly with some classes such as leuco-anthocyanins and catechins which readily polymerize to form condensed tannins. In most cases monomeric polyphenols form only a minor portion of the extractable material from plant tissues. While flavans probably give rise to the major portion of polymeric material, other polyphenols may also co-polymerize. Data[2] showing a loss of activity in rutin isolated after a metabolic period of 48 hr, suggest to the authors that polymerization and other changes of this compound may have taken place.

Watkin and Neish[2] have stated that the dilution of a labelled precursor in the biosynthesis of a particular polyphenol is due to (*a*) dilution in the endogenous pool of the precursor, (*b*)

TABLE 1. THE CONVERSION OF LABELLED SUBSTRATES BY *Eucalyptus* SPECIES

Species used	*E. sideroxylon*					*E. dalrympleana*	*E. guilfoylei*
Sample	a*	b	c	d	e		
Wt. taken (g)	7·5	6·2	5·1	12·2	9·0	1·0	1·0
Solids content %	49·9	44·7	50·0	46·2	—	—	—
Compound administered	L-Phenylalanine-G-^{14}C				L-Tyrosine-G-^{14}C		
Activity administered (mc)	0·025	0·025	0·05	0·05	0·05	0·005	0·005
Date of feeding (1964)	28 Jan.	28 Jan.	25 Feb.	2 Jan.	4 Feb.	7 Feb.	11 Feb.
	% Distribution of radioactivity in oil-free leaf extract						
p-Coumarylquinic acid	36	16	7·1	2·2	4·2	5·0	12·0
Chlorogenic acid	—	12	30	1·9	6·0	—	—
Catechin	—	2·3	0·4	0	0·2	—	—
Engelitin	0	11	—	3·2	—	—	0
Flavonol glycosides	7·0	5·0	4·3	1·9	1·0	7·4	1·4
Piceid	0	2·3	1·9	2·3	1·0		
Rhapontin	0	2·6	1·0	5·4	4·7	8·8	17·0
Astringin	0	5·2	0·7	2·4	1·8		
Sugars	0	0	0	18·4	2·2	7·9	8·9

* Normal type, all other samples were variants.

[2] J. E. WATKIN and A. C. NEISH, *Can. J. Biochem. Physiol.* 38, 559 (1960).

TABLE 2. THE DISTRIBUTION OF ACTIVITY IN DIFFERENT FRACTIONS OF *E. sideroxylon* LEAVES

Compound administered (6 May 1964)	L-Phenylalanine-G-^{14}C		L-Tyrosine-G-^{14}C	
Weight of compound (mg)	0·45	0·45	0·33	0·33
Activity of compound (mc)	0·025	0·025	0·025	0·025
Type of species	Normal	Variant	Normal	Variant
Weight of test sample (g)	6·00	5·75	5·80	6·15
Solids content (%)	51·9	48·2	51·9	48·2
I Fraction†				
Weight (mg)	300	485	261	573
Activity (counts/min/mg)	6·0	107	13·8	71·8
Total activity (counts/min)	1,810	51,800	3,620	41,200
Activity (%)*	23·0	23·0	27·0	32·7
II Fraction†				
Weight (mg)	259	84	259	80
Activity (counts/min/mg)	4·6	326	1·9	55·7
Total activity (counts/min)	1,190	27,400	492	4,460
Activity (%)*	15·6	12·1	3·6	3·6
III Fraction†				
Weight (mg)	524	593	503	581
Activity (counts/min/mg)	8·8	246	18·3	138
Total activity (counts/min)	4,610	146,000	9,200	80,200
Activity (%)*	60·6	64·9	69·1	63·7
Wax Fraction				
Weight (mg)	347	334	323	343
Activity (counts/min/mg)	3·1	48	7·0	81
Petrol-soluble Fraction				
Weight (mg)	65	30	49	32
Activity (counts/min/mg)	4·8	43	15	725

† The methanol extracts of the leaves were concentrated, freed from wax, extracted with petrol, and the remaining material separated on No. 3 Whatman paper with 6% acetic acid. I Fraction, R_f 0·0–0·2; II Fraction, R_f 0·2–0·5; III Fraction, R_f 0·5–1·0.

* Expressed as a percentage of the total activity of the three fractions.

dilution by the compound formed from unlabelled precursors during the experiment, and (*c*) dilution by the preformed compound. It is evident from the previous statements, that in a dynamic plant system, not only the size of the pool[3] of a particular compound but the rate of turnover of this pool must also be considered. Both of these aspects, and particularly the latter, can differ from compound to compound and can be influenced by environmental conditions.

The results obtained in this study are expressed in Tables 1 and 2 as a percentage of the total activity in the leaf extract and in Table 3 as specific and total activities. It should be noted, however, that purification of the complex mixture resulted in losses so that the values for the total activities are low, and also that the amounts and specific activities of the individual compounds were too low to permit purification to constant activity (Table 3). Chromatographic and spectral examination indicated that almost all the compounds were pure, and since the different leaf samples (Table 2) were treated in an identical manner the results

[3] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* 37, 537 (1959).

Table 3. Activity of components isolated from *E. sideroxylon* leaves

Substrate	L-Phenylalanine-G-^{14}C				L-Tyrosine-G-^{14}C			
Type of Species	Normal		Variant		Normal		Variant	
Activity*	Specific	Total	Specific	Total	Specific	Total	Specific	Total
Compound								
p-Coumarylquinic acid	3·2	48·8	350**	8,750	7·3	138	41·8**	335
Chlorogenic acid	5·0	46·5	732	14,500	2·0	16·7	16·9	269
Catechin	1·4	13	214	4,270	2·28	106	16·4	316
Engelitin***	—	—	127	1,290	—	—	4·4	34
Nicotiflorin	0·67	4	52	352	0·89	9	(18·1)††	(60)
Quercitrin	2·0	7†	74	458	1·5	19	12·8	160
Isoquercitrin	1·2	26	49	308	0·85	8	8·2	42
Rutin	0·18	30	31	85	0·49	11	(10·5)††	(48)
Piceid	—	—	17·2	2,420	—	—	4·1	650
Rhapontin	—	—	11·9	3,180	—	—	3·6	760
Astringin	—	—	19·0	442	—	—	6·0	196
Amino acids†††	0·72	54	21·1	1,106	3·2	237	20·9	471
Glucose	0·31‡	101	1·35	380	3·7‡	2,080	20·0	1,598
Fructose			0·95	174			24·5	2,150
Sucrose	—	—	—	—	—	—	215	22,608
"Shikimic acid"	0·65	38	185	10,500	9·5	405	36·4	—
Chlorophyll§	3·1	—	79·5	—	9·6	—	177	—

* As counts/min/μmole or total counts/min.

** A II sub-fraction possessed the same chromatographic and similar spectral properties but with specific activities of 154 and 7·5 counts/min/μmole and total activities of 2230 and 309 counts/min for phenylalanine and tyrosine feedings respectively.

*** A III sub-fraction possessed the same chromatographic and similar spectral properties but with specific activities of 57·2 and 3·6 counts/min/μmole and total activities of 488 and 41 counts/min for phenylalanine and tyrosine feedings respectively.

† Loss during recovery.

†† Final purification inadequate.

††† Activity of mixture determined and calculated as aspartic acid.

‡ Activity of mixture.

§ Crude material collected from the fractionation of I Fraction (Table 2) with BAW. Activity expressed as counts/min/mg.

(Table 3) are mutually comparable. Taking both the specific and total activities in conjunction, the differences between the components indicate certain trends in biosynthesis. A significant amount of the activity was present in the polymeric material containing ellagitannins and leucoanthocyanins, but these fractions were not examined further.

Incorporation of Activity by Different Physiological Forms

The results from different feeding trials using samples of different species followed by separation into broad fractions, suggested that the normal form of *E. sideroxylon* (Table 1) incorporated a lower amount of activity into polyphenols than did the variant form. A more detailed examination was made when leaves from both forms were fed simultaneously with labelled substrates (Table 2).

The leaves of the normal forms had been collected in low (22 in./year) rainfall regions and were more leathery than those from the garden-grown variant form. Consequently, it was not unexpected that the rate of uptake of the precursor solution was slower with leaves of this

normal form. However, the difference in the degree of incorporation into polyphenols by the two forms is much greater than would be expected from the above cause.

We were unable to find labelled phenylalanine or tyrosine in the extracts of the leaves after a 24 hr metabolic period. The age of the two types of leaves, their extractives content and the relative degree of incorporation of radioactivity into the three fractions of the extractives (after feeding with phenylalanine, Table 2) are about the same. However, the sum of the total activities incorporated in the different fractions (Table 2) differs for the two types of leaves and one possible explanation is that the amount of photosynthetic products appropriated by different phases of metabolism differs in the fully enlarged leaves of the two types. Apparently more of these products are used in normal leaves for purposes other than the formation of polyphenols than are used in variant leaves. Zaprometov[4] has shown the rate of incorporation of activity into catechin to decrease sharply with the age of the tea leaf. Different metabolic states in the two forms of *E. sideroxylon* leaves may also be responsible for the different degrees of incorporation.

Incorporation of Activity from Phenylalanine and Tyrosine

In the variant leaves the degree of conversion of labelled phenylalanine into labelled methanol soluble extractives was much higher than the corresponding conversion of tyrosine (Table 2). Normal leaves appear to be able to convert more tyrosine than phenylalanine into extractives but this may be due to the slower absorption of the phenylalanine solution which was not completely taken up (see Experimental).

Compared with phenylalanine the degree of conversion of tyrosine to the flavonoid fraction (Fraction II, Table 2) is relatively much lower than to other fractions. Tyrosine[5–8] and, to a lesser extent, phenylalanine[8] have been found to be glucogenic amino acids when administered to leaf discs from several plants although sugar formation is much less when the substance is fed to whole leaves than to discs (V. C. Runeckles, personal communication). The formation of labelled sugars from these amino acids has been confirmed in this present work (see Table 3).

The relative activities of the various components (Tables 2 and 3) was supported by the radioautographs of chromatograms of the extractives.

Relative Activity of Leaf Extractives

The degree of incorporation of activity into different polyphenols was in the order of chlorogenic and *p*-coumarylquinic acids, catechin, engelitin, flavonol glycosides and the stilbenes. The high activity of the acids is expected as they are synthesized early in the pathway (Fig. 1). The evidence of Runeckles[9] and others indicates that *p*-coumarylquinic acid can be an intermediate in the formation of polyphenols. Presumably hydrolysis occurs and *p*-coumaric acid itself re-enters the main pathway (Fig. 1) where it is a good precursor of polyphenols such as quercetin.[10] Quinic acid can either re-enter the shikimic acid pathway or be utilized for respiration.[11,12]

[4] M. N. Zaprometov, *Soviet Plant Physiol.* **10**, 73 (1963).
[5] A. Hutchinson, C. D. Taper and G. H. N. Towers, *Can. J. Biochem. Physiol.* **37**, 902 (1959).
[6] I. Imaseki, R. Oneyama and M. Tajima, *Yakugaku Zasshi* **80**, 1802 (1960).
[7] R. K. Ibrahim, S. G. Lawson and G. H. N. Towers, *Can. J. Biochem. Physiol.* **39**, 873 (1961).
[8] V. C. Runeckles, *Can. J. Botany* **41**, 823 (1963).
[9] V. C. Runeckles, *Can. J. Biochem. Physiol.* **41**, 2249 (1963).
[10] E. W. Underhill, J. E. Watkin and A. C. Neish, *Can. J. Biochem. Physiol.* **13**, 219 (1957).
[11] S. Yoshida, *Botan. Mag. Tokyo* **77**, 10 (1964).
[12] L. H. Weinstein, C. A. Porter and H. J. Laurencot, *Contrib. Boyce Thompson Inst.* **21**, 201 (1961).

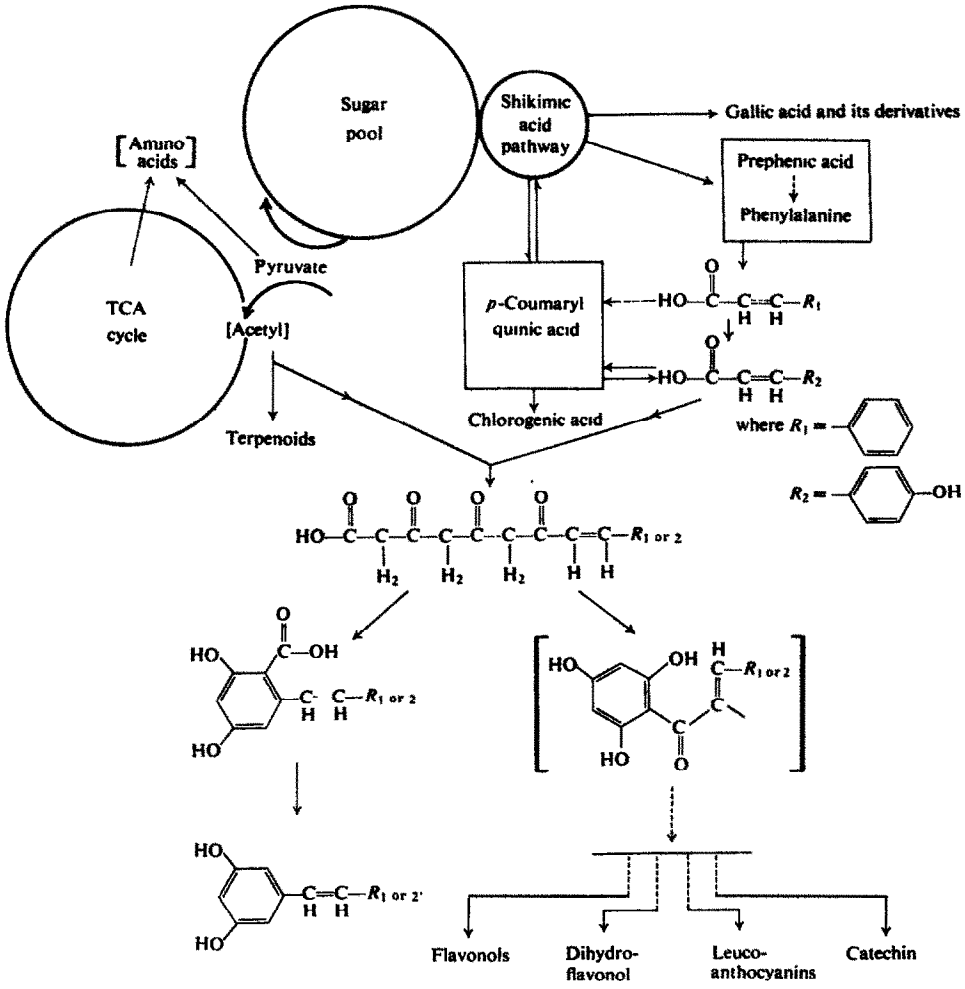

FIG. 1. A POSSIBLE RELATIONSHIP BETWEEN DIFFERENT BIOSYNTHETIC PATHWAYS.

The biosynthesis of chlorogenic acid proceeds via *p*-coumarylquinic acid[9,13,14] but in contrast to *p*-coumaric acid, caffeic acid is a poor precursor for chlorogenic acid[9] and quercetin.[10] It is possible that caffeic acid cannot permeate to the site of the appropriate enzyme systems and the available evidence shows that it can enter the biosynthetic pathway only after metabolic fragmentation. Consequently the route from *p*-coumarylquinic acid to chlorogenic acid is an irreversible one and the latter compound can only be used in oxidation and polymerization reactions. On the other hand, since *p*-coumarylquinic acid is an intermediate in the early stages of the pathway for flavonoids and stilbenes (Fig. 1) and can be readily turned-over in a dynamic system it is to be expected its activity would be appreciably different from that of chlorogenic acid (Table 3).

The activity of the stilbenes is much lower than that of chlorogenic and *p*-coumarylquinic

[13] K. R. HANSON and M. ZUCKER, *J. Biol. Chem.* **238**, 1105 (1963).
[14] P. N. AVADHANI and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **39**, 1605 (1961).

acids (Table 1) and this observation is comparable to the findings of Ibrahim and Towers[15] that the "major portion" of the activity from administered phenylpropanoid compounds was diverted away from the synthesis of hydragenol (a stilbene-like compound) to the formation of two coumarins. The latter would be formed directly from *p*-coumaric acid.[16]

Hydroxy stilbenes are derived from a phenylpropanoid precursor, which originated from the shikimic-prephenic acid pathway, and three acetate units.[17–19] Presumably this resultant intermediate in stilbene formation is the same as that in flavonoid formation.[20] The number of stages in the formation of stilbenes from the β-polyketo chain precursor is less than in the formation of catechin and dihydroflavonols (e.g. engelitin), yet the specific activity of the stilbenes is lower than any other group of polyphenols. Decarboxylation of the stilbene precursor could be a rate-determining step but no evidence for this type of compound has yet been found in eucalypt leaves. Siegelman and Hendricks[21] have postulated the rate-determining steps in the synthesis of the A or phloroglucinol ring in anthocyanins. Synthesis of the A ring in catechin proceeds more slowly than that of the B ring[22] and presumably this rate is the same for other flavonoids. It is possible that this rate could be appreciably different in stilbene formation owing to the different manner of cyclization of the A ring and the lower activity of the stilbenes indicates that it could be slower.

It is considered that after certain periods of metabolism the specific activity of different classes of polyphenols is dependent not only on the amount present but also on its rate of turnover to polymeric and other materials. For this viewpoint to be correct the biosynthesis of different classes of polyphenols must proceed by parallel pathways.

Catechin isolated from the different samples possessed a relatively high activity. There were large losses during purification of the small amounts of this compound and the figures given (Table 3) are the highest activities recorded. The samples were not completely pure but further attempts at purification were unsuccessful and resulted in lower activities. It is probable that the actual activities of pure catechin are higher than those given. A relatively high activity in catechin has been found previously[23,24] and Zaprometov[22] concluded from the activities of the compounds that catechin is formed at a much greater rate in the tea plant than quercetin in buckwheat. Catechin is a component of a wide range of plants and tissues and a considerable amount is formed in tea leaves from labelled carbon dioxide in 15 min.[25] From our studies we postulate that catechin has a physiological function and is being continuously formed and rapidly converted to other products. Labelled catechin fed to leaves was converted to polymerized material but not to other flavonoids.[24] There is much evidence to indicate that catechin or leucoanthocyanin or both are the precursors of condensed tannins which are found in the tissues of most plants.[26] The high activity of catechin relative to other flavonoids and stilbenes could be due to its more rapid turn-over so that there is little dilution by preformed material. This seems a more likely explanation than that of more rapid forma-

[15] R. K. Ibrahim and G. H. N. Towers, *Can. J. Biochem. Physiol.* **40**, 449 (1962).
[16] S. A. Brown, *Phytochem.* **2**, 137 (1963).
[17] W. E. Hillis and M. Hasegawa, *Chem. & Ind. (London)* 1330 (1962).
[18] G. Billek and W. Ziegler, *Monatsch. Chem.* **93**, 1430 (1962).
[19] E. von Rudloff and E. Jorgensen, *Phytochem.* **2**, 297 (1963).
[20] A. C. Neish, *Ann. Rev. Plant Physiol.* **11**, 55 (1960).
[21] H. W. Siegelman and S. B. Hendricks, *Plant Physiol.* **33**, 409 (1959).
[22] M. N. Zaprometov, *Biokhimiya* **27**, 366 (1962).
[23] W. E. Hillis and M. Hasegawa. Unpublished data.
[24] M. Hasegawa and M. Yasue, *J. Japan For. Soc.* **44**, 244 (1962).
[25] M. N. Zaprometov, *Section Rep. 5th Biochem. Congress*, Moscow, 1961.
[26] D. E. Hathway, In *Wood Extractives* (Edited by W. E. Hillis), p. 211. Academic Press, New York (1962).

tion because coniferyl alcohol, which could be a more direct precursor, was poorly incorporated in related studies.[27]

Small amounts of an iso-flavanone-like compound[1] and other compounds were isolated but incompletely purified. Their activities were much lower than comparable purified components.

Hydroxylation of Polyphenols

The lack of correlation between the pattern of hydroxyl groups in the B rings of the stilbenes and of the flavonoids[28] shows that, with the possible exception of the 4'-hydroxyl, the B ring is hydroxylated after combination with the acetate groups and after divergence of the pathways to different polyphenols. This is in agreement with the conclusions that the pathways of aurone synthesis and of flavonoids diverge at a relatively early stage.[29,30] It is considered that oxidation of the B ring takes place after ring closure as otherwise dihydroquercetin derivatives would be expected in addition to the dihydrokaempferol derivative, engelitin.

Although catechin, cyanidin and quercetin are the most common members of their classes, genetic evidence indicates the ease of formation is in the order of 1 then 2 and then 3 hydroxyl groups in the B ring.[31] In agreement with this, the kaempferol compound (nicotiflorin) has a higher activity than the corresponding quercetin derivative (rutin: Table 3). The lower activity found in rutin as compared with quercitrin (Table 3) is in agreement with the findings of Harborne[32] that monosaccharides are added one at a time under genetic control. Furthermore, the activity of the 3-rhamnoside (quercitrin) is higher than that of the 3-glucoside (isoquercitrin, Table 3) and this observation together with the presence of the 3-rhamnoside of dihydrokaempferol indicates that, in this species, glycosylation with rhamnose proceeds more rapidly than with glucose.

The activities of the different stilbenes do not follow the trends shown by the flavonoids. Astringin (possibly a pentahydroxy stilbene) has a higher activity than the other two stilbene glucosides (Table 3). This may be due to a rapid turnover of the compound in the leaves. The instability of astringin resulted in low recovery yields and low total activity. The activity of rhapontin suggests a relatively rapid methylation of the 4'-hydroxyl.

Accumulation of Cinnamic Acid Derivatives and Shikimic Acid

Chlorogenic and *p*-coumarylquinic acids are usually present in greater quantities in the variant form than in the normal forms of the same species or the normal form of closely related species.[1,28] In view of the position of *p*-coumarylquinic acid in the biosynthetic pathway (Fig. 1), it could be a storage form of the B ring of flavonoids and stilbenes when there is an increased metabolic activity or a decreased formation of gallic acid and its derivatives. In this context, it is noteworthy that chlorogenic acid frequently accumulates when certain tissues are affected by virus disease.[33]

Formation of the A ring of catechin appears to be a rate-determining step.[22] Consequently,

[27] H. GRISEBACH, *Z. Naturforschung* **13b**, 335 (1958).
[28] W. E. HILLIS and K. ISOI, In preparation.
[29] E. C. JORGENSEN and T. A. GEISSMAN, *Arch. Biochem. Biophys.* **54**, 72 (1955).
[30] J. B. HARBORNE, *Phytochem.* **2**, 327 (1963).
[31] J. B. HARBORNE In *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 593. Pergamon Press, Oxford (1962).
[32] J. B. HARBORNE, *Biochem. J.* **84**, 100 (1962).
[33] I. URITANI, In *Biochemistry of Plant Phenolic Substances*, Symposium (Edited by G. JOHNSON and T. A. GEISSMAN), p. 98. Colorado State University (1961).

if the balance of metabolism is altered in favour of a larger amount of material passing through the prephenic acid pathway (Fig. 1), this rate-determining step could cause the accumulation of *p*-coumarylquinic and chlorogenic acids. The possibility that the presence of these acids is associated with increased formation of flavonoids and stilbenes is being investigated.

Labelled shikimic acid quickly accumulated when seedlings were fed with labelled glucose.[28] On the other hand, labelled phenylalanine and tyrosine were quickly converted to other components. Consequently, there also appears to be a rate-determining step between shikimic acid and these aromatic amino acids.

Enzyme Activity

The detection of tyrase (L-tyrosine ammonia-lyase) in the leaves of an *E. sideroxylon* variant has been previously reported.[34] We have been unable to detect it in leaves collected from the identical tree at different times throughout one year. Phenylalanine deaminase (L-phenylalanine ammonia-lyase) activity was also absent, although weak tyrase activity was detected in leaves of *E. dalrympleana* and *E. salmonophloia* which contained stilbenes. Leaves of eucalypts contain appreciable quantities of polyphenols, and it has already been demonstrated that some classes of polyphenols can deactivate certain enzymes, e.g. leucoanthocyanins,[35,36] stilbenes[37] and ellagitannins.[38] During preparation of the acetone powder of the leaf, when the membrane containing the polyphenols[39] would be ruptured, the polyphenols would be able to come into contact with the enzymes.[40] It seems probable that even with the conditions used, enzymes could be deactivated owing to the large amount and type of polyphenols present. Consequently, detection of enzyme activity in such materials is more dependent on experimental conditions than is usual and this aspect is under investigation.

Owing to the difficulties in detecting enzyme activity, and following the suggestion of Brown[41] that the relative proportions of the two deaminase enzymes of tyrosine and phenylalanine might be different in different plants, an attempt to assess the activity of these enzymes was made from an examination of the activity of different compounds formed (Table 3). It can be seen that there is no significant difference in the ratio of the activities of the components after feeding with the different substrates and it is apparent there is no association of stilbene formation with possible tyrase activity. The activity of the components from the leaves fed with tyrosine are much lower than the corresponding activities in the phenylalanine experiment. Ibrahim and Towers[42] also found tyrosine, as compared with phenylalanine, to be very poorly incorporated in the stilbene-like compound hydragenol formed in cuttings of *Hydrangea macrophylla.*

The activity of the carbohydrate fraction formed from tyrosine after 24 hr is high (Table 3) and activity has also been detected in the amino acids isolated. The latter were identified as glutamic and aspartic acids, α-alanine and glycine. Similar results were obtained by Ibrahim *et al.*[7] on feeding several plants with tyrosine, and they suggested tyrosine is degraded to

[34] D. E. Bland, *Biochem. J.* **88**, 523 (1963).
[35] W. G. C. Forsyth, V. C. Quesnel and J. B. Roberts, *J. Sci. Food Agri.* **9**, 181 (1958).
[36] R. J. W. Bryde, A. H. Fielding and A. H. Williams, In *Phenolics in Plants* (Edited by J. B. Pridham), p. 95. Pergamon Press, London (1960).
[37] H. Lyr, *Flora (Jena)* **152**, 570 (1962).
[38] D. E. Hathway and J. W. T. Seakins, *Biochem. J.* **70**, 158 (1958).
[39] A. B. Wardrop and J. Cronshaw, *Nature* **193**, 90 (1962).
[40] E. A. H. Roberts, *J. Sci. Food Agri.* **9**, 381 (1958).
[41] S. A. Brown, *Can. J. Botany* **39**, 253 (1961).
[42] R. K. Ibrahim and G. H. N. Towers, *Can. J. Biochem. Physiol.* **40**, 627 (1960).

acetate which in turn is converted to glutamic and aspartic acids or to sugars. Another interpretation is that the four carbon dicarboxylic acids formed on degradation of tyrosine could yield phosphoenolpyruvate (and hence carbohydrate) more readily than would acetate. The dicarboxylic acids would also form acetate. A distinctive feature of the compounds formed after feeding tyrosine to the variant sample is that the activity in the A ring is much greater than that in the B ring of the stilbene molecule (Table 4). Consequently, there is little evidence for the presence of a deaminase for this amino acid which apparently is metabolically degraded to simpler units. As the A ring can be formed directly from acetate units, it is to be expected that the activity of this ring is much higher than that of the B ring which would arise by way of phosphoenolpyruvate–sugars–shikimic acid–prephenic acid (Table 4).

The activity of the carbohydrate fraction formed by phenylalanine is much lower than that of tyrosine (Table 3), but nevertheless is appreciable.[8] Partial conversion of phenylalanine to acetate units could account for the small proportion of activity found in the A ring of stilbenes (Table 4) and after feeding with differently labelled phenylalanines.[17] The formation of sugar would account for the activity found in the quinic acid moieties of chlorogenic and *p*-coumaryl-quinic acids (Table 4). The amount of radioactivity in the 3,5-dihydroxybenzoic acid from rhapontin was higher than that from piceid (Table 4). One possible explanation is that the acetate units do not combine with the phenylpropanoid precursor until it is completely substituted, and that in the 24 hr metabolic period there is relatively less of the active 3-hydroxy 4-methoxybenzoic acid moiety available for rhapontin synthesis than of the 4-hydroxybenzoic acid moiety for piceid. This deduction is contrary to the conclusions made earlier concerning flavonoid biosynthesis and no supporting evidence could be found for the deduction. Another possible explanation is the different turn-over rates of piceid and rhapontin result in different rates of biosynthesis. As the relative intensities of radioactivity in the two pools supplying the A and B rings would differ during 24 hr, the different rates of biosynthesis would become apparent in the distribution of activity in the stilbenes.

Further evidence of the degradation of tyrosine and phenylalanine to acetate is shown by the activity of the crude terpenoid oils (petrol-soluble fraction, Table 2) which are formed from acetic and mevalonic acids.[43] The activity of the wax fraction is probably partly due to occluded terpenoids as a partial purification of one sample reduced the activity. When tyrosine is fed, the activity of the terpenoid oils is appreciably higher than that of the other fractions (Table 2) and this would be expected if the first step of its utilization is degradation to acetate.

In view of the large amount of activity in the sugars and the presence of activity in the polyphenols after feeding tyrosine to variant leaves, it is not unexpected that active shikimic acid was isolated. The low activity of the sugars relative to the polyphenols formed after phenylalanine feeding is in agreement with the view that little degradation of this amino acid to acetate takes place. However, the activity of shikimic acid is remarkably high (Table 3). In contrast to the polyphenols, the purity of the shikimic acid cannot be assessed from the u.v. spectrum and radioautograms show the presence of a highly active impurity with very similar chromatographic properties.

This work illustrates the difficulties in drawing conclusions from the activity of a particular end-product after feeding and metabolizing a possible precursor for more than a certain period. The activity may be due to incorporation of degradation products of the supposed precursor in the biosynthetic pathway and not due to direct utilization of this compound.

[43] W. Sandermann, *Holzforschung* **16**, 65 (1962).

TABLE 4. THE DISTRIBUTION OF ACTIVITY WITHIN COMPOUNDS AFTER FEEDING VARIANT LEAVES WITH DIFFERENT SUBSTRATES

Compound	*p*-Coumarylquinic acid		Chlorogenic acid		Piceid		Rhapontin	
Acid formed on degradation	Quinic	*p*-Coumaric	Quinic	Caffeic	3,5-Dihydroxy benzoic	4-Hydroxy benzoic	3,5-Dihydroxy benzoic	3-Hydroxy 4-methoxy benzoic
Substrate	L-Phenylalanine-G-^{14}C							
Activity %*	3	97	19	81	10	90	24	76
Substrate	L-Tyrosine-G-^{14}C							
Activity %*	79	21	—	—	84	16	100	0

* Calculated from counts/min/μmole of purified degradation acids.

EXPERIMENTAL

The samples were taken from adult trees of *Eucalyptus sideroxylon* A. Cunn. ex Woolls, *E. dalrympleana* Maiden and *E. gulfoylei* Maiden. A garden-grown specimen of a variant of *E. sideroxylon* provided samples b, d and e, Table 1, and variant samples Table 2. All other samples were collected from trees growing in their natural habitat.

Paper Chromatography

All samples were examined by two-dimensional chromatography on Whatman No. 1 paper using the solvent systems (*a*) BAW, *n*-butanol–acetic acid–water (6:1:2) followed by (*b*) 6% acetic acid in a constant temperature (20°) room. Radioautographs were prepared using Kodak "No Screen" X-ray film and exposure times of 130 days. Chromatograms were examined in long- (366 mμ) and short- (254 mμ) wave u.v. light in the presence and absence of ammonia, by spraying with diazotized *p*-nitroaniline[44] and when appropriate with vanillin-hydrochloric acid[45] and periodate-aniline.[46]

The components were separated by means of band chromatography on Whatman No. 3 paper. All paper was washed with the relevant solvent before use. In addition to the above solvents, BEW, *n*-butanol–ethanol–water (4:1:2·2) was also used for certain fractions.

The amino acids and sugars were identified by direct comparison with authentic samples using as chromatographic solvents BAW, phenol saturated with water and in addition phenol–ethanol–ammonia (15:4:1) for amino acids and butyl acetate–pyridine–ethanol–water (8:2:2:1) for sugars. Ninhydrin was used as a chromogenic spray for the amino acids and aniline-diphenylamine[47] and aniline phosphate[48] for the sugars.

Administration of Radioactive Phenylalanine and Tyrosine

The shoots were taken from adult trees and possessed defect-free leaves that had recently reached full size. The aqueous solutions (1·5 ml or less) of the compounds were fed through freshly cut ends and water added after the solution was absorbed. The shoots were placed in a glasshouse for 24 hr and during the night period were illuminated with cool white fluorescent light with an intensity of approximately 1000 ft-c. The day temperature range was 25°–30° for different experiments and the night temperature 15°–20°. With samples b–e (Table 1), the solutions were taken up at an approximate rate of 0·10 ml/hr/g shoot weight, and with the normal-type sample (No. a, Table 1) absorption took place at about one-half this rate. With samples collected in autumn (Table 2), the variant-type samples absorbed the solutions at about 0·10 ml/hr/g but with the normal-type samples the rate was 0·40 ml/hr/g. A trace (less than 2%) of the phenylalanine solution fed to normal leaves in the latter experiment was not taken up.

Fractionation of Leaf Extracts

At the end of the metabolic period the leaves were removed from the shoots, cut up and plunged into boiling ethanol. The extract was removed and the residue extracted with methanol in a soxhlet-type extractor for 8 hr. The extracts were concentrated in a rotary evaporator, the residue heated with a small portion of water, filtered hot and the aqueous

[44] T. Swain, *Biochem. J.* **53**, 200 (1953).
[45] E. C. Bate-Smith and T. Swain, *Chem. & Ind. (London)* 377 (1953).
[46] S. Yoshida and M. Hasegawa, *Arch. Biochem. Biophys.* **70**, 377 (1957).
[47] G. Harris and I. C. MacWilliam, *Chem. & Ind. (London)* 249 (1954).
[48] A. S. F. Ash and T. M. Reynolds, *Australian J. Biol. Sci.* **7**, 435 (1954).

extraction continued until all soluble material was removed from the wax fraction. The aqueous extracts were extracted repeatedly with petrol (b.p. 60°–80°) and then applied to washed sheets of Whatman No. 3 paper (14 × 22·5 in.) as streaks and chromatographed with 6% acetic acid. The chromatograms were cut into three bands with R_f 0·0–0·2, 0·2–0·5, 0·5–1·0, and designated I, II and III Fractions respectively (Table 2). The bands were cut up, extracted with methanol for 8 hr in a soxhlet apparatus, the extracts concentrated and each extract applied to washed Whatman No. 3 papers as streaks and chromatographed for a second time with BAW. The bands obtained at this stage yielded the crude compounds listed in Table 1.

The various bands of mixed flavonoids from II Fraction (Table 2) were extracted and chromatographed for a third time with 6% acetic acid. The individual flavonoids were separated as bands and chromatographic examination, spectral properties and colour reactions of the extracts indicated that, with the exception of catechin, the flavonoids were pure. Examination of the catechin fractions indicated that during the last stages of purification, the ratio of polymeric material increased. The stilbenes from I Fraction were purified for a third time by chromatography using BEW.

Chromatographic separation of III Fraction with BAW yields sub-fraction IIIa, containing engelitin and *p*-coumarylquinic acid; IIIb, chlorogenic and *p*-coumarylquinic acids; IIIc, chlorogenic acid; IIId, compound *x* and a chlorogenic acid-like compound; IIIe, shikimic acid and compound *y*; IIIf, sugars and amino acids. Fractions IIIa–IIId were further purified chromatographically with 6% acetic acid and again with BEW. In addition to the major components, Fractions IIIa and IIIb contained small amounts of a compound similar in behaviour to *p*-coumarylquinic acid but with higher R_f values in BAW and which appear to be *p*-coumarylshikimic acid. The band with the shikimic acid colour reaction in Fraction IIIe was further purified chromatographically with 6% acetic acid and again with BAW. Fraction IIIf was dissolved in water and passed through a small Amberlite IR-120(H+) column to separate the sugars and amino acids, the latter being removed from the column with 4% ammonia. The sugars obtained from variant-type leaves were further separated on washed No. 3 Whatman paper with BAW.

All samples and bands were extracted with redistilled methanol and the extracts evaporated under vacuum at 40° in a rotary evaporator. The activity of all extracts was determined by placing an aliquot on stainless-steel planchets and measuring the radioactivity with an end-window tube (Phillips 18505) and electronic counter (Philips PW4035). The spectra of ethanol solutions of the extracts were compared with those of pure compounds[1] and this, together with chromatographic examination, indicated that the compounds in Table 3 were pure. Chromatographic examination showed that the stilbenes were a mixture of *cis* and *trans*-isomers.

Degradative Procedures

The quinic acid esters were hydrolysed by an esterase preparation as described by Levy and Zucker[49] and the products separated chromatographically by BAW. Quinic acid was detected by its reaction with aniline following periodate oxidation[46] and the aromatic moieties by their fluorescence under u.v. light in the absence and presence of ammonia.

Piceid or rhapontin (about 50 mg) was acetylated with acetic anhydride (5 ml) and pyridine (0·25 ml) at room temperature for 1–2 days. The mixture was evaporated under vacuum at 70°, the residue dissolved in acetone, shaken for 2 hr at room temperature with excess of an aqueous 2% solution of potassium permanganate, then an equal volume of water added and

[49] C. C. Levy and M. Zucker, *J. Biol. Chem.* 235, 2418 (1960).

the liquor filtered. Unoxidized material was removed by extraction with ether, and the aqueous solution acidified and extracted with ether. The evaporated extract was dissolved in a 2% potassium hydroxide–methanol solution, acidified, extracted with ether, the extract washed with water and, after evaporation, purified on chromatoplates of Silica Gel G.F. 254 (E. Merck, A. G. Darmstadt) using chloroform–ethyl acetate–formic acid (5:4:1) to yield 4-hydroxybenzoic acid. The aqueous solution left by the final ether extraction was evaporated to dryness under vacuum, 2N HCl (10 ml) added and heated on a steam bath for 0·5 hr. Ether extraction removed 3,5-dihydroxybenzoic acid, which was purified as above, and glucose was obtained from the aqueous residue.

Enzyme Activity

Tests for tyrase (L-tyrosine ammonia-lyase) and phenylalanine deaminase (L-phenylalanine ammonia-lyase) were done according to the methods of Neish [50] and Koukol and Conn [51] respectively. Parallel tests were run on oat shoots.

[50] A. C. Neish, *Phytochem.* **1**, 1 (1961).
[51] J. Koukol and E. E. Conn, *J. Biol. Chem.* **236**, 2692 (1961).