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A COMPARATIVE BIOCHEMICAL STUDY OF PHENOLASE SPECIFICITY IN *MALUS*, *PYRUS* AND OTHER PLANTS*

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Abstract—The transformation of monophenolic compounds to their *ortho*-dihydroxy analogues by the action of phenolase has been studied for enzyme sources from *Malus*, *Pyrus* and a few other plants. Dihydrochalcone glycosides of phloridzin type appear to be the most effective *in vitro* substrates for phenolase, even when these compounds do not occur in the plant concerned. Examination of a few plants selected at random indicates that the ability to hydroxylate phloridzin must be quite widespread in the plant kingdom. The flavanone naringenin is hydroxylated in much greater yield by phenolase from *Pyrus* rather than *Malus* leaves. Although dihydrochalcones are hydroxylated with equal facility by both blossom and leaf phenolases from *Pyrus*, in the case of naringenin it is only the leaf phenolase which exhibits any appreciable activity. Flavonoids of the flavone, flavonol and dihydroflavonol classes are not transformed to any appreciable extent as compared with the dihydrochalcones. Both the absolute and relative effectiveness of *p*-coumaric and 3-O-*p*-coumaroylquinic acids as phenolase substrates was found to be dependent upon the nature of tissue within the plant from which the enzyme was extracted.

INTRODUCTION

THE ENZYME phenolase (I.U.B. nomenclature is *o*-diphenol:O_2 oxidoreductase E.C. 1.10.3.1) is also known by many other names; some refer to an initial monophenol *o*-hydroxylating function and others (including the I.U.B. nomenclature) only to the ability to oxidize *o*-dihydroxy phenols to quinonoid forms. Phenolase preparations which lack or have attenuated hydroxylase ability are common but preparations in which the hydroxylase activity predominates are unknown. There is now much evidence that the two functions of phenolase, the hydroxylating and dihydroxyphenol oxidase activities, both form parts of a closely integrated system. Many excellent reviews are available[1-8] and the reader is referred to these for further information.

In plants phenolase has one very clear function, i.e. the oxidation of mono and dihydroxy phenols to brown or black complex polymeric material which accompanies cell-rupture

* Part of a thesis entitled "Studies on the Nature, Distribution and Biosynthesis of Phenolic Compounds in *Pyrus* and *Malus*", by J. S. Challice (July 1969).

[1] D. KERTESZ and R. ZITO, in *Oxygenases* (edited by O. HAYAISHI), p. 307, Academic Press, London (1962).
[2] J. B. PRIDHAM (editor), *Enzyme Chemistry of Phenolic Compounds*, Pergamon Press, Oxford (1963).
[3] E. E. CONN, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 399, Academic Press, London (1964).
[4] E. FRIEDEN, S. OSAKI and H. KOBAYASHI, *J. Gen. Physiol.* **49** (1), 213 (1965).
[5] H. S. MASON, in *Biochemistry of Copper* (edited by J. PEISACH *et al.*), p. 339, Academic Press, London (1966).
[6] D. W. BROOKS and C. R. DAWSON, in *Biochemistry of Copper* (edited by J. PEISACH *et al.*), p. 343, Academic Press, London (1966).
[7] B. G. MALSTRÖM and L. RYDEN, in *Biological Oxidations* (edited by T. P. SINGER), p. 415, Interscience, New York (1968).
[8] O. HAYAISHI, in *Biological Oxidations* (edited by T. P. SINGER), p. 581, Interscience, New York (1968).

caused either through attack by parasitic organisms or by mechanical damage.[9-13] In the intact plant cell it is evident that phenolase, phenolic substrate and oxygen are prevented from reacting together by spatial separation, enzyme inhibition, or both. It is still not completely clear whether or not phenolase can be assigned any biochemical function in the intact cell at particular stages of growth or whether it has any physiological function.

There have been many recent reports in the literature of the functioning of simple phenols, phenolic acids and flavonoids in growth regulation.[14-16] Certain monophenols have been found to enhance the activity of indole acetic acid (IAA) oxidase whilst the corresponding *o*-dihydroxy analogues inhibit the activity of this enzyme. The implication is that the balance between monophenolics and *o*-dihydroxyphenolics, which is dependent upon the operation of the hydroxylating activity of phenolase, controls the level of the growth hormone IAA which in turn influences the physical growth and differentiation of the plant. Galston[16] has shown that in the pea seedling a kaempferol glycoside is a product of etiolation metabolism whereas the corresponding *o*-dihydroxylated analogue (a quercetin glycoside) is produced in appreciable amounts only by photostimulated metabolism. In this connexion it is interesting to note that with cinnamic acid derivatives only the *cis*-isomers are active in growth regulation.[17,18] The *cis*-isomers are readily formed *in vitro* from the *trans*-forms by irradiation with light. The dihydrochalcones phloridzin and sieboldin, which occur in *Malus*,[19] have been shown by Stenlid[20] to be a potent stimulator and strong inhibitor respectively of IAA-oxidase activity. In this respect it is unfortunate that sieboldin (3-hydroxyphloretin 4'-glucoside) has only a limited occurrence within the genus *Malus* whilst the true hydroxylated phloridzin (3-hydroxyphloretin 2'-glucoside) has not been found in intact plant tissue. Little work has been reported on the relation between phenolase activity itself and growth response; a recent paper by Buban[21] deals with this aspect.

In the biosynthesis of phenolics in general it is still unclear at what stage (or stages) the second *o*-hydroxyl group is introduced into the aromatic ring. The initial *p*-hydroxyl is now known to be introduced quite early, at the phenylalanine or cinnamic acid stage, whilst in the flavonoids current evidence indicates that the second hydroxyl in the B-ring is introduced after the formation of the first C_{15} compound.[22-25]

[9] S. RICH (editor), *Perspectives of Biochemical Plant Pathology*, The Connecticut Agricultural Experiment Station Bulletin 663 (1963).
[10] R. N. GOODMAN, Z. KIRALY and M. ZAITLIN, *The Biochemistry and Physiology of Infectious Plant Disease*, van Nostrand (1967).
[11] J. RAA, *Natural Resistance of Apple Plants to* Venturia inaequalis. *A Biochemical Study of its Mechanism*. Ph.D. Thesis, Utrecht (1968).
[12] J. RAA and J. C. OVEREEM, *Phytochem.* **7**, 721 (1968).
[13] J. W. ANDERSON, *Phytochem.* **7**, 1973 (1968).
[14] H. W. SIEGELMAN, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 437, Academic Press, London (1964).
[15] G. ENGELSMA, *Philips Tech. Rev.* **28**, 101 (1967).
[16] A. W. GALSTON, in *Perspectives in Phytochemistry*, Chap. 10, Academic Press, London (1969).
[17] A. J. HAAGEN-SMIT and F. W. WENT, *Proc. Acad. Sci. Amst.* **38**, 852 (1935).
[18] E. N. UGOCHUKWU and R. L. WAIN, *Ann. appl. Biol.* **61**, 121 (1968).
[19] A. H. WILLIAMS, in *Comparative Phytochemistry*, Chap. 17, Academic Press, London (1966).
[20] G. STENLID, *Physiol. Plant.* **21**, 882 (1968).
[21] T. BUBAN, *Bot. Kozlem* **55**, 101 (1968).
[22] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, Chap. 8, Academic Press, London (1967).
[23] H. GRISEBACH, in *Recent Advances in Phytochemistry*, Vol. 1, Appleton-Century-Crofts, New York (1968).
[24] E. WONG, *Phytochem.* **7**, 1751 (1968).
[25] E. WONG and H. GRISEBACH, *Phytochem.* **8**, 1419 (1969).

The object of the work now reported is to examine qualitatively the substrate specificities of phenolase preparations from different parts of the plant in the hope of throwing some light on the problems mentioned. Comparative studies of this nature would seem a necessary preliminary to more specialized quantitative studies which must inevitably be considerably restricted in their scope. The investigations now reported should also be of assistance in defining reliable criteria for the hydroxylase activity of phenolases in general.

RESULTS AND DISCUSSION

General

Incubations of various monophenolic substrates with cell-free extracts of acetone powders prepared from the appropriate plant tissues were carried out generally at pH 4·5 in the presence of ascorbic acid to stop the phenolase-catalysed reaction at the *o*-dihydroxyphenol stage. The unchanged substrate and transformation product (if any) were recovered by solvent extraction and examined by paper or thin-layer chromatography. Tables 1 and 2 show the

TABLE 1. TRANSFORMATION OF MONOPHENOLIC COMPOUNDS* TO *ortho*-DIHYDROXY ANALOGUES BY PHENOLASE† FROM *Malus pumila* TISSUES

	Mature leaves			Young leaves		Immature whole fruit	Mature fruit flesh‖	Blossom petals
Monophenolic compound	1	2	3	4	5	2	2	6
Phloretin	v. good	good	good	mod.	good	poor	good	good
Phloretin 2′-glucoside (Phloridzin)	good	v. good	v. good	good	v. good	poor	good	v. good
Phloretin 2′-rhamnoside							v. good‡	
Phloretin 4′-glucoside (Trilobatin)§							v. good‡	
Dihydrokaempferol (Aromadendrin)	nil						nil‡	
Dihydrokaempferol 3-rhamnoside (Engelitin)	nil						v. poor‡	
Naringenin		v. poor	v. poor					v. poor
Naringenin 7-rhamnoglucoside (Naringin)	nil	nil					nil‡	nil
Apigenin 7-glucoside			nil				nil‡	
Kaempferol		nil	v. poor					nil
p-Hydroxypropiophenone		nil					nil‡	nil
3-*O*-*p*-Coumaroylquinic acid	v. poor	good		mod.	nil		v. poor	poor
p-Coumaric acid	v. poor	v. good	good	mod.	mod.		v. poor	good
Tyrosine		nil				nil	nil	nil

Key: 1 = MIII rootstock; 2 = var. Bramley; 3 = var. Stoke Red; 4 = var. Bramley (sampled in May); 5 = var. Bramley (sampled in August); 6 = var. Cheddar Cross.

* Designation of substrate effectiveness: nil means approx. transformation of 0%; v. poor trace 5%; poor 5–10%; mod. 10–25%; good 25–50%; v. good 50–100%; blank space = substrate not tested.

† At pH 4·5 unless stated otherwise, see Experimental section for further details.

‡ pH 6·0.

§ In *M. sargentii*, *M. trilobata*, *M. sieboldii arborescens* and an unknown *Malus* hybrid, phloridzin is replaced by the isomeric trilobatin; both compounds, however, serve equally well as phenolase substrates when incubated with acetone powder extracts of each of these four *Malus* specimens.

‖ With this enzyme source the following additional substrates were transformed thus: *p*-hydroxybenzyl alcohol (v. poor); *p*-hydroxybenzoic acid (nil); arbutin (v. poor); cinnamic acid (nil); salicin (nil)—all at pH 6·0.

TABLE 2. TRANSFORMATION OF MONOPHENOLIC COMPOUNDS* TO *ortho*-DIHYDROXY ANALOGUES BY PHENOLASE† FROM *Pyrus communis* TISSUES

Monophenolic compound	Mature leaves 1	Mature leaves 2	Young leaves 1	Immature whole fruit 2	Mature fruit flesh‖ 2	Blossom petals 3
Phloretin	good		mod.	good	good	good
Phloretin 2′-glucoside (Phloridzin)	v. good	v. good	v. good	mod.	good	v. good
Phloretin 2′-rhamnoside					good‡	
Phloretin 4′-glucoside (Trilobatin)	v. good		v. good		v. good‡	v. good
Dihydrokaempferol (Aromadendrin)					nil‡	
Dihydrokaempferol 3-rhamnoside (Engelitin)					v. poor‡	
Naringenin	mod.	mod.				v. poor
Naringenin 7-rhamnoglucoside (Naringin)		nil			nil‡	nil
Apigenin§	nil		nil			
Apigenin 7-glucoside§	v. poor		nil		nil‡	
Kaempferol	poor	nil				nil
p-Hydroxypropiophenone		nil			nil‡	nil
3-*O*-*p*-Coumaroylquinic acid	v. good		good		v. poor	mod.
p-Coumaric acid	v. good		good		poor	good
Tyrosine		nil		nil	nil‡	nil

Key: 1 = var. Jaspoideae; 2 = var. Conference; 3 = var. Holme Letty.

* Designation of substrate effectiveness: see Table 1.

† At pH 4·5 unless stated otherwise, see Experimental section for further details.

‡ pH 6·0.

§ These flavones were not hydroxylated by phenolase from the leaves of *P. betulaefolia* (Kew specimen, flavone-containing) and *P. betulaefolia* (Oregon specimen, flavones absent) although phloridzin functioned as a v. good and mod. substrate respectively in parallel incubations.

‖ With this enzyme source the following additional substrates were transformed thus: *p*-hydroxybenzyl alcohol (v. poor), *p*-hydroxybenzoic acid (nil), arbutin (v. poor), cinnamic acid (nil)—all at pH 6·0.

TABLE 3. TRANSFORMATION OF DIHYDROCHALCONES* TO *ortho*-DIHYDROXY ANALOGUES BY PHENOLASE† FROM LEAVES OF PLANTS WHICH DO NOT THEMSELVES CONTAIN DIHYDROCHALCONES

Subclass	Order	Family	Genus and species	Phloridzin	Trilobatin	Phloretin
Monocotyledons	Arales	Araceae	*Arum maculatum*	poor	poor	
Dicotyledons (Archichlamydeae subdivision)	Ranales	Ranunculaceae	*Ranunculus ficaria*	v. good	v. good	
	Umbellales	Umbelliferae	*Smyrnium olusatrum*	mod.	mod.	
	Rosales	Rosaceae	*Prunus laurocerasus*	mod.	good	good
			Cydonia oblonga	v. poor	good	mod.
	Centrospermae	Chenopodiaceae	*Beta vulgaris*[26]	transformed		
			Spinacia oleracea[27]	transformed		

Key: as in Tables 1 and 2, see Experimental section for further details.

In additional incubations with *P. laurocerasus* phenolase, dihydrokaempferol, dihydrokaempferol 3-rhamnoside and naringenin 7-rhamnoglucoside did not function as substrates; 3-*O*-*p*-coumaroylquinic acid and *p*-coumaric acid were transformed only in trace amounts.

[26] J. NEUMANN and M. AVRON, *Plant Cell Physiol., Tokyo* **8**, 241 (1967).

[27] S. IZAWA, G. D. WINGET and N. E. GOOD, *Biochem. Biophys. Res. Commun.* **22**, 223 (1966).

results obtained from incubations with extracts from various tissues of apple and pear; Table 3 gives the results of tests on widely differing plants for the ability to hydroxylate some dihydrochalcones.

Substrate Specificities

The most striking fact to emerge from these results is the general effectiveness of phloretin and phloretin glycosides as phenolase substrates; such C_{15} compounds are classed here as monophenolic substrates because the additional hydroxyl is always introduced *ortho-* to the existing *para*-hydroxyl in the B-ring only. It will be seen that although the dihydrochalcones function well as phenolase substrates in both *Malus* and *Pyrus* (although these compounds occur only in the former) the flavanone naringenin is transformed much better by phenolase from *Pyrus* rather than *Malus*. Although Henke[28] claimed to have found naringenin glycosides in the leaf of many *Malus* species, we have been unable to substantiate his claim except in one isolated instance involving an unknown *Malus* hybrid.[29] Eriodictyol 7-glucoside has been found in small amounts in the bark of the common apple tree[19] but never in the leaves of any *Malus* species. A survey of the leaf phenolics of most species of *Pyrus* has not revealed the presence of any flavanones.[30,31] The tables show that a number of compounds which might have been expected to function as substrates were not in fact transformed at all or only in very small amounts. It is of interest to note that compounds belonging to the dihydroflavonol, flavonol and flavone classes are apparently of this type. It was surprising to find that tyrosine would not function as a substrate; some animal phenolases have been shown to exhibit high degrees of specificity for tyrosine[3]—in fact the term "tyrosinase" is often used as a synonym for phenolase irrespective of whether it comes from plant or animal sources. Walker[32] has also observed that tyrosine does not function as a substrate for apple fruit phenolase whilst, in contrast, Robb *et al.*[33] showed that the tyrosine → DOPA transformation was effected by phenolase from the broad bean (*Vicia faba*). Macrae and Duggleby[34] state that tyrosine is transformed only very slowly by potato tuber phenolase. *p*-Coumaric acid and 3-O-*p*-coumaroylquinic acid could both serve as quite effective substrates with some *Malus* and *Pyrus* tissues. The results obtained with arbutin were somewhat indefinite but Sato[35] has demonstrated that *o*-hydroxylation does take place with phenolase from a number of arbutin-containing plants. In view of the fact that cinnamic acid hydroxylase is known to be very readily denatured,[36,37] it was not surprising to find that the ability to transform cinnamic acid to *p*-coumaric acid, when tested for, was entirely lacking. It is interesting to note that extreme insolubility of a compound in aqueous media does not necessarily preclude its functioning as an effective phenolase substrate, as is shown by the transformation of phloretin and naringenin by *Malus* and *Pyrus* phenolases and by the transformation of tyrosine by mushroom tyrosinase. The 7-rhamnoglucoside of naringenin, in contrast to the aglycone, was not transformed at all. Glycosidation of a compound is clearly not essential for the functioning of that compound as a substrate, although it does

[28] O. Henke, *Flora (Jena)* **153**, 358 (1963).
[29] A. H. Williams, *Ann. Rep. Long Ashton Res. Sta.*, p. 39 (1967).
[30] J. S. Challice and A. H. Williams, *Phytochem.* **7**, 119 (1968).
[31] J. S. Challice and A. H. Williams, *Phytochem.* **7**, 1781 (1968).
[32] J. R. L. Walker, *Australian J. Biol. Sci.* **17**, 360 (1964).
[33] D. A. Robb, T. Swain and L. W. Mapson, *Phytochem.* **5**, 665 (1966).
[34] A. R. Macrae and R. G. Duggleby, *Phytochem.* **7**, 855 (1968).
[35] M. Satô, *Phytochem.* **2**, 385 (1963).
[36] P. M. Nair and L. C. Vining, *Phytochem.* **4**, 161 (1965).
[37] D. W. Russell and E. E. Conn, *Arch. Biochem. Biophys.* **122**, 256 (1967).

seem to enhance the effectiveness as substrates in the case of the dihydrochalcones. In some of the incubations of phloridzin and (in particular) trilobatin with *Malus* and *Pyrus* phenolases the transformations to the *o*-dihydroxy analogues were either complete or else very nearly so; this seems hard to reconcile with the general principle that the hydroxylase activity is competitively inhibited by *o*-dihydroxyphenols.[5] The finding also apparently conflicts with Kertesz and Zito[1] who state that a considerable amount of the monohydroxyphenol remains unoxidized in the ascorbic acid–phenolase system.

Tables 2 and 3 show that the ability of phenolases to hydroxylate dihydrochalcones is not restricted to those sources where these compounds occur naturally. It would appear that the ability to hydroxylate these compounds is quite widely distributed within the angiosperms. It is interesting to note that *Cydonia oblonga* (the quince) is the only species listed in Table 3 with phenolase that does not efficiently transform phloridzin; strangely enough trilobatin is transformed in high yield and phloretin is noticeably a better substrate than phloridzin.

In a few *Malus* species[19] phloridzin (phloretin 2′-glucoside) is replaced by the isomeric trilobatin (phloretin 4′-glucoside) and by sieboldin, which raised the question of whether trilobatin would function as a better phenolase substrate than phloridzin in these cases; but as shown in Table 1 both isomers were transformed equally well in all cases. A previous survey of *Pyrus* phenolics[30,31] has shown that flavones do not occur in the common pear (*P. communis*); hence additional incubations were carried out with apigenin and apigenin 7-glucoside and an acetone powder of the leaves of the E. Asian flavone-containing *P. betulaefolia*. The results (Table 2) show that these flavones are not transformed although in a parallel incubation phloridzin functions as a very good substrate.

Preliminary experiments have shown that although phloridzin is transformed generally in greater yield than phloretin by pear blossom phenolase, there is a very pronounced lag (*ca.* 10 min or more) at pH 6·0 in the case of phloridzin only; at pH 4·5 this lag is considerably diminished.

Differences in Substrate Specificities between Different Tissues

The substrates studied in detail were phloretin, phloridzin, *p*-coumaric acid and 3-O-*p*-coumaroylquinic acid. As regards the dihydrochalcone transformation in *Malus* the phenolase activity is very low in the immature fruitlets whilst the activity in the mature fruit flesh is quite high; in *Pyrus* there is high phenolase activity in both immature and mature fruit. With phloretin as substrate, incubations were run at both pH 4·5 and pH 6·0 for the different tissue types as listed in Tables 1 and 2 and the results indicated that, whilst transformation was equally effective at both pH levels for the leaves and blossom petals, in the immature fruitlets and mature fruit flesh transformation occurred only at pH 4·5. With phloridzin as substrate the transformations occurred with nearly equal facility at both pH levels for all tissue types. The most obvious interpretation of this phenomenon is that the actual shape of the pH *versus* activity function must vary with both substrate and source of phenolase; in the phloretin–fruit phenolase system there must be a sharp maximum around pH 4·5 in the pH-activity function whilst in the other cases mentioned the maximum is noticeably broader, thereby spanning both pH 4·5 and 6·0. Such variation in the shape of the pH-activity function has been observed for different substrates with potato tuber phenolase.[38] Although dihydrochalcones are transformed equally by both blossom and leaf phenolase from *Pyrus*, with naringenin only leaf phenolase shows any appreciable activity.

[38] F. A. M. ALBERGHINA, *Phytochem.* 3, 65 (1964).

Another form of tissue specificity is evident in the hydroxylation of 3-O-*p*-coumaroylquinic acid and *p*-coumaric acid respectively; with blossom petals of both *Malus* and *Pyrus* *p*-coumaric acid seems a more effective substrate than its quinic acid ester. In further experiments these two compounds do not show any relative differences as phenolase substrates for mature leaves, young leaves sampled early in the season and in the mature fruit flesh. A surprising observation was that with young Bramley leaves sampled late in the season 3-O-*p*-coumaroylquinic acid would not function at all as a substrate whilst *p*-coumaric acid was effective. Whilst the mature fruit flesh can effect the hydroxylation of dihydrochalcones in high yields, this tissue is very low in activity with respect to the hydroxylation of the *p*-hydroxycinnamic acids. The first *Malus* mature leaf specimen (listed in Table 1) has apparently selectively lost the ability to hydroxylate *p*-hydroxycinnamic acids; in this particular case the leaves were stored for some months in the deep freeze prior to preparation of the acetone powder. Other leaf specimens, when the acetone powders were prepared from fresh leaves, retained the ability to hydroxylate *p*-hydroxycinnamic acids.

General Discussion

The investigations which have been described, although only of an exploratory nature, indicate that the substrate specificity of phenolase is not only a function of the particular plant in which it occurs but also of the particular organ and stage of growth of the plant. This may be regarded as evidence that the plant phenolases serve some physiological function. Observed differences in phenolase specificities could be due either to conformational differences reflected perhaps in differing isoenzyme specificities, or the presence of naturally occurring inhibitors with either selective spatial distribution or selective inhibitory properties. In the present study it is the ability of individual phenolics to function as substrates for a particular phenolase that has been investigated; this is not necessarily a measurement of the amount of active enzyme present.

It was rather surprising to find that dihydrochalcones function as such efficient and general substrates with the range of phenolases studied; dihydrochalcones are a relatively rare class of compound in the plant kingdom and seem to be metabolic end-products; they are not an intermediate stage in the biosynthesis of other flavonoid compounds. The results of the present study suggest that the second hydroxyl in the B-ring of flavonoids may be introduced in a C_{15} precursor with a saturated middle-ring (e.g. chalcone epoxide, flavanone or dihydroflavonol?) rather than at the flavone or flavonol stages where the middle-rings are unsaturated. Although apigenin and kaempferol could, under certain circumstances, function as phenolase substrates in the present investigations the transformations were very poor in comparison with other compounds. Recently, some workers[39] have succeeded in hydroxylating naringenin, dihydrokaempferol and kaempferol with a phenolase preparation from spinach beet leaves; here the enzyme was in a purified rather than the impure "native" state used in the present investigations. Without kinetic data from experiments with highly purified enzyme preparations little more can be said upon this subject.

The results obtained with *p*-coumaric acid and 3-O-*p*-coumaroylquinic acid may have some relevance to chlorogenic acid biosynthesis. Until comparatively recently 3-O-*p*-coumaroylquinic acid was thought to be the sole precursor of chlorogenic acid[40,41] but later

[39] P. F. T. VAUGHAN, V. S. BUTT, H. GRISEBACH and L. SCHILL, *Phytochem.* **8**, 1373 (1969).
[40] K. R. HANSON and M. ZUCKER, *J. Biol. Chem.* **238**, 1105 (1963).
[41] K. R. HANSON, *Phytochem.* **5**, 491 (1966).

it was shown that caffeic acid could also function as a precursor.[42] Steck[43] has shown that in tobacco leaves the major route has 3-O-*p*-coumaroylquinic acid as the immediate precursor of chlorogenic acid whilst the secondary route involves caffeic acid in this role; thus, in the major route, the quinic acid ester is the phenolase substrate, whilst in the secondary route *p*-coumaric acid itself is the substrate for this enzyme. Since it has been found that the relative activity of phenolase towards these two substrates varies with the actual part of the plant, the relative importance of the two pathways of chlorogenic acid biosynthesis may also vary with the part of the plant. Whether or not this has any significance for growth-control mechanisms in the plant is an intriguing question.

EXPERIMENTAL

Preparation of Acetone Powders

Fresh plant material was homogenized with acetone at −12° in a Waring Blender, filtered, and the solid matter then twice re-homogenized with acetone, filtered, and air-dried overnight. The dried acetone powders (*ca.* 20 g from 60 g f.w.) were stored at room temp. These preparations still contained indigenous phenolic glycosides and other acetone-insoluble matter. Phenolic-free "protein powders" were prepared (when necessary) as follows: 4 g acetone powder (from leaves) +2 g ascorbic acid was shaken with 100 ml ice-cold water for 10 min and the slurry squeezed through fine mesh cotton directly into 1·2 l. acetone at −12°. The residue was re-extracted with 20 ml 2% ascorbic acid and the liquor added to the same acetone. The precipitated protein was filtered, well washed with cold acetone and air-dried overnight on absorbent paper (yield = *ca.* 0·5–0·8 g white powder); these "protein powders" could only be prepared from leaf acetone powders. Both the crude acetone powders and purified "protein powders" retained high phenolase activities even after many months' storage.

Extraction of Phenolase

Experiments showed that the maximum activity was extracted from the crude acetone powders by a pH 7 phosphate–citrate buffer; plain H_2O extraction resulted in only very low activity, comparable with that obtained with buffers of pH 4–5. Addition of 2% Triton X-100 to the extracting medium did not give significantly greater solubilization of phenolase activity. Phenolase solutions were prepared immediately before required, as follows: 0·1 g crude acetone powder + 0·4 g 1,5-gluconolactone (β-glucosidase inhibitor) was shaken with 10 ml pH 7 phosphate–citrate buffer, stood for 10 min, then squeezed through fine cotton and filtered through filter paper. The phenolase could not be solubilized to the same extent from the purified protein powders; hence an equivalent amount of the powder was suspended in buffer containing 4% 1,5-gluconolactone—such preparations showed phenolase activities roughly comparable to those of the crude solubilized extracts.

Assay of Phenolase Activity

When required, for the comparison of phenolase extraction methods etc. as above, assays were made by the colorimetric measurement of the rate of DOPA-chrome formation from DL-DOPA.[44]

Incubation of Monophenolic Substrates with Phenolase

2 mg substrate was added to 15 ml pH 7 phosphate–citrate buffer, and ascorbic acid was then added at 1% for a final incubation pH of 6·0 and at 3% for a final pH of 4·5. 5 ml phenolase prep. was added with vigorous shaking and after checking pH values the tubes were left for 17 hr to allow the reactions to proceed to completion. As a check on the activity of the enzyme sources, parallel incubations were run with phloridzin with every experiment. Blank incubations with enzyme and/or substrate only were run each time.

In the case of tyrosine, its limited solubility necessitated a modified procedure without ascorbic acid; transformation was measured by the characteristic colour of DOPA-chrome. When L-tyrosine was incubated with mushroom tyrosinase (Koch–Light) a strong DOPA-chrome colour was formed; mushroom tyrosinase also hydroxylates phloridzin in good yield.

Recovery of Unchanged Substrate and Transformation Product

After 17 hr, the incubation medium was acidified (2 N H_2SO_4) and extracted with 2 × 20 ml EtAC. After H_2O-washing of the combined EtAC layers to remove acid, the solvent layer was taken to dryness *in vacuo* at 50° and the residue disolved in 1 ml EtOH.

[42] O. L. GAMBORG, *Can. J. Biochem.* **45**, 1451 (1967).
[43] W. STECK, *Phytochem.* **7**, 1711 (1968).
[44] S. M. CONSTANTINIDES and C. L. BEDFORD, *J. Food. Sci.* **32**, 446 (1967).

Chromatographic Examination of Unchanged Substrate plus Transformation Product

The final extracts (for paper chromatography $5 \times 10\,\mu l$; for TLC $5 \times 2\,\mu l$) were examined chromatographically. Standard phenolics were spotted in graded concentrations alongside to facilitate visual estimation of extent of conversion of substrate expressed as shown in Table 1. For paper chromatography the solvents used were: sec-BuOH:HAc:H_2O, 70:2:28, by vol. (SBA) and 2%, v/v HAc,[30,31] in each of these the transformation product ran a little slower than the unchanged substrate; spots were visualized by u.v. light and by diazo-coupling as previously described.[30,31] For TLC, silica gel G/NaOAc plates were employed with $CHCl_3$: EtAc:HCOOH, 50:40:10, by vol. as solvent;[39] spots were visualized as previously described. Here again, transformation product always ran slower than the original substrate. Generally speaking, paper chromatography gave the best separations in the case of phenolic glycosides whilst TLC gave superior separations with the phenolic aglycones. Flavonoid aglycone pairs such as quercetin/kaempferol, 3-OH phloretin/phloretin and eriodictyol/naringenin could be separated in reverse order to that in paper chromatography and TLC by paper electrophoresis using pH 8·8 borate buffer for 4–18 hr on Whatman 3MM paper (0·5 mA/cm).[45] A Shandon "Vokam" power supply on constant current was employed.

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