The Metabolic Fate of Hydroxybenzoic Acids in Plants

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When fed to higher plants, hydroxybenzoic acids form *O*-glucosides unless they contain an *ortho*-hydroxyl group when glucose esters are formed. The properties and methods of identification of several of these derivatives are outlined.

Hydroxybenzoic acids are widely distributed throughout the Plant Kingdom [1–9]. As in the case of other phenolic compounds, including the cinnamic acids [10], the hydroxybenzoic acids occur in combined forms from which they are released by acid or base hydrolysis [7]. Remarkably, however, there are only a few known naturally occurring derivatives [10–15], most of which are *O*-glycosides. In some plants, the acids have also been shown to be present in an insoluble form, esterifying lignin hydroxyl groups [16].

As the first step in a survey of the occurrence of soluble hydroxybenzoic derivatives in plants, we have fed a number of different acids to leaf discs from several angiosperms and examined the extractable products formed.

Results and Discussion

Leaf discs from a variety of plants were studied, including *Raphanus sativus, Pisum sativum, Helianthus annuus, Tropaeolum majus, Lactuca sativa, Spinacea oleracea, Cucurbita pepo, Brassica oleracea, Gaultheria procumbens* and *Catalpa bignoides*. The products obtained from each acid, as judged chromatographically, appeared to be the same regardless of the species used. Most of our work, however, has been done using leaves from *Cucumis sativus*.

The hydroxy acids listed in Table 1 were fed to the discs under continuous light and the products extracted with methanol. The derivatives were isolated by a combination of paper and preparative thin layer chromatography using appropriate solvents (Table 1). The structures of the isolated compounds followed from their chromatographic and spectroscopic behaviours (Tables 1 and 2).

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Thus, each of the sugar derivatives (ester or *O*-glucoside) show, expectably, a lower R_f value in butanol-acetic acid-water (BAW) and a higher R_f in 20% acetic acid than their parent acids. Compounds having a free ionisable carboxyl group have a noticeably lower R_f in the neutral solvent butanol-ethanol-water (BEW) than in BAW, whereas the R_f values of esters is hardly changed: in the more basic solvent, isopropanol-*n*-butanol-*tert*-butanol-ammonia (IBBA), both carboxyl and some phenolic hydroxyl groups are ionised, resulting in a further reduction in R_f values, again the esters being less affected than the corresponding acids (Table 1). Compounds having a free carboxyl group can also be readily demonstrated both by their mobility on electrophoresis at pH 5.2, (Table 1) and by the variation in the λ_{max} of their UV spectra on changing the pH from 3.8 to 7.0. Here, all compounds having a free carboxyl group show a small but definite hypsochromic shift of 3 – 13 nm depending on the compound involved. The presence of a free *o*-hydroxy group was readily shown by the bathochromic shift obtained by the addition of aluminium chloride (Table 2). Spectra in the basic solution (pH 12.0) gives information about the disposition of free phenolic hydroxyl groups.

All the derivatives formed, with the exception of the main component from salicylic acid *(vide infra)*, gave the parent acid and glucose on hydrolysis. Determination of the aglycone : sugar ratio showed it to be 1 : 1 in all cases except for the derivative from α-resorcylic acid where it was 2 : 1.

Using such criteria, it can be seen that *m*-hydroxybenzoic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids gave monoglucose esters while α-resorcylic acid gives, presumably, an acylated glucose ester. On the other hand *o*-pyrocatechuic, β-resorcylic and gentisic acids gave the 3-, 4-, and 5-*O*-glucosides respectively, all of which have a free

Acid	Benzoic acid Substitution pattern	R_f values +								Derivatives	
		Free acid				Derivatives				Electro-phoretic mobility MSA*	Agly-cone—Sugar Ratio
		20% Ha	BAW	BEW	IBBA	20% Ha	BAW				
Acids forming esters											
m-hydroxybenzoic	3-hydroxy	0.80	0.66	0.48	0.57	0.88	0.48	0.48	0.57	0	1:0.93
p-hydroxybenoic	4-hydroxy	0.84	0.69	0.52	0.43	0.86	0.53	0.54	0.47	0	1:0.96
protocatechuic	3,4-dihydroxy	0.71	0.60	0.50	0.14	0.76	0.52	0.55	0.16	0	1:1.04
α-resorcylic	3,5-dihydroxy	0.72	0.63	0.37	0.30	0.86	0.47	0.51	0.33	0	1:0.50
vanillic	3-methoxy 4-hydroxy	0.84	0.72	0.47	0.26	0.88	0.51	0.51	0.26	0	1:1.04
syringic	3,5-dimethoxy 4-hydroxy	0.75	0.69	0.42	0.20	0.84	0.53	0.44	0.22	0	1:1.00
Acids forming glycosides											
salicylic	2-hydroxy	0.78	0.64	0.52	0.85	0.86	0.31	0.18	0.40	58	1:1.00
o-pyrocatechuic	2,3-dihydroxy	0.76	0.58	0.39	0.64	0.88	0.33	0.20	0.42	57	1:1.04
β-resorcylic	2,4-dihydroxy	0.80	0.69	0.42	0.48	0.84	0.41	0.16	0.23	56	1:1.04
gentisic	2,5-dihydroxy	0.78	0.64	0.41	0.66	0.86	0.32	0.20	0.40	58	1:1.01

Table 1. Some properties of hydroxybenzoic acid and their derivatives.

+ Solvents 20% HA and IBBA on celullose TLC and Solvents BAW and BEW on silica TLC. * Where M = distance moved by derivative/distance moved by salicylate X 100. The free acids gave MSA* values ranging from 71 (syringic acid) to 94 (*p*-hydroxybenzoic acid).

Compound	λ_{max} (n.m.) of							
	Derivative				Free acid			
	Acid	Neutral	Base	$AlCl_3$	Acid	Neutral	Base	$AlCl_3$
Acids forming esters								
m-hydroxybenzoic	239, 299	231, 291	231, 314	240, 299	233, 279	231,278	231, 292	233, 279
p-hydroxybenzoic	257	248	278	258	257	258	305	257
protocatechuic	261, 296	253, 292	234, 276, 305	264, 300	265, 300	265, 300	237, 293sh 325	265, 300
α-resorcyclic	251, 310	244, 300	229, 320	252, 310	255, 314	255,314	234, 274, 342	255, 314
vanillic	263, 293	253, 287	285, 300	263, 294	264, 295	267, 294	238, 321	264, 295
syringic	276	263	231, 303	277	273	273	243, 332	273
Acids forming glycosides								
salicylic	238, 303	234, 298	234, 298	239, 312	232, 317	232, 313	232, 313	328
o-pyrocatechuic	248, 319	242, 312	231, 328	251, 323	241, 310	235, 305	235, 305	317
β-resorcylic	257, 297	249, 294	234, 272, 299	258, 303	251, 291	244, 288	242, 288	254, 304
gentisic	240, 331	235, 323	349	241, 338	237, 322	232, 313	233, 312	232, 328

Table 2. λ_{max} of hydroxybenzoic acids and their sugar derivatives.

o-hydroxy group (Tables 1 and 2). Salicylic acid gave three compounds: the main compound, whose properties are given in the Tables, proved to be identical to gentisicacid-5-*O*-glucoside [17]; the others, formed in trace amounts, appeared to be the *O*-glucoside and glucose ester of salicylic acid itself.

The results obtained for the ester-forming acids are not surprising since it has been shown earlier that similarly substitued hydroxycinnamic acids when fed to plants yielded mainly the corresponding glucose esters [10, 18]. In two cases, however, *Lycopersicon esculentum* [10] and *Nicotiana tabacum* [18], phenolic *O*-glucosides were also formed. In these studies, the fate of *o*-coumaric acid was not investigated.

The finding that all the hydroxybenzoic acids having an *ortho* hydroxyl group form *O*-glucosides, indicates that the hydrogen bonding between the carboxyl group and the hydroxy group prevents ester formation. It is interesting to note that tannase,

gallotannin esterase, is inhibited by *o*-hydroxybenzoic acids[19] and such acids tend to inhibit esterification mechanisms in animals[20]. It should also be pointed out that, as mentioned earlier, many of the naturally occurring hydroxybenzoic acid derivatives so far identified are *O*-glucosides. It seems possible, therefore, that in many cases esterification may be a detoxification mechanism unrelated to normal metabolism. In this respect it is remarkable that when salicylic acid was fed to leaf discs of *Gaultheria procumbens*, which synthesises copious amounts of the *O*-primeveroside of methyl salicylate[12], the tissue rapidly died. On the other hand, this plant could readily detoxify protocatechuic acid to the corresponding ester.

Experimental

Materials: With the exception of *Gaultheria procumbens* and *Catalpa bignoides*, leaves of which were taken from specimens growing in the Royal Botanic Gardens, Kew, plants were grown from commercial seed in John Innes compost, or in vermiculite using Hoaglands solution, in a greenhouse at 15·20°. Fully developed first and second leaves were used in all experiments, usually taken when the plants were 3–4 weeks old.

Feeding and Extraction: Leaf discs (1 cm diam) were cut from leaves and floated in batches of 25 (ca. 0.5 gm) on solutions of the appropriate acid (0.01 M), which had been neutralised (sodium hydroxide), containing 1% sucrose. The discs were exposed to continuous light (1000 lux) at 25° for 36 hrs. The discs were gently washed with water and dropped into boiling 80% methanol (10 mls/gm). The extract was decanted and the discs extracted twice more. The combined extracts were concentrated to small volume (ca. 1 ml/gm).

Chromatography: (a) Isolation of derivatives. Derivatives were isolated by chromatography on Whatman's No. 3 paper in *n*-butanol-acetac acid water, 6 : 1 : 2 (BAW) followed by 2% acetic acid and *n*-butanol-ethanol-water 4 : 1 : 2·2 (BEW) and, where necessary, preparative TLC on cellulose (Whatman's CC41) in aqueous acetic acid (HA). The purity of each compound was judged by TLC on Schleicher and Schull microcrystalline cellulose or silica film-backed thin layer plates in the solvents given in Table 1. The compounds were detected under short wave UV (256 nm) or by spraying with diazotized *p*-nitroaniline.

(b) Qualitative chromatography: The R_f values of acids and derivatives were determined by TLC as described above in BAW, BEW, 20HA, isopropanol-*n*-butanol-*tert*-butanol-conc. ammonia-water (IBBA) 4 : 2 : 2 : 1 : 2 and benzene-acetic acid-water, 6 : 7 : 3. The compounds were detected by examination under short and long-wave UV light, with and without ammonia, and by spraying with diazotized *p*-nitroaniline and 1% ferric chloride.

Sugars were examined on microcrystalline cellulose TLC in ethyl acetate-pyridine-water, 6 : 3 : 2 and *n*-butanol-acetone-acetic-acid-water, 7 : 7 : 2 : 4[21]. Sugars were revealed using *p*-aminobenzoic acid or aniline phosphate.

Electrophoresis: Electrophoresis was carried out on silica gel TLC plates using 0.01 M sodium acetate pH 5.2 at constant voltage (75 v/cm) for 20 min on a cooled platten. The position of compounds was detected as above. Salicylic acid was used as a reference.

Spectroscopy: U.V. Spectra were determined, using a Unicam SP 1700 recording spectrophotometer, in 80% methanol buffered to pH 3.8, 7.0 and 12.0 using a combination of acetic acid and potassium hydroxide. Aluminium chloride spectra were determined in 5 mM solution of the salt in 80% MeOH.

Hydrolysis of Derivatives: (a) Qualitative. Purified compounds were hydrolysed at 100° for 20 min in 2 N HCl. The cooled solution was extracted with ether, and this extract used for examination of the benzoic acids. The aqueous layer was neutralised with weak base ion exchange resin ("Amberlyst" A-21) before examination of the sugars.

(b) Quantitative. Hydroxybenzoic acids were determined directly, after hydrolysis as above, in the acid solution by spectral means. Sugars were measured using the phenol-sulphuric acid reagent[22, 23] directly on an aliquot of the solution of the derivative.

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Über den Abbau von Flavonolen in pflanzlichen Zellsuspensionskulturen

Degradation of Flavonols in Plant Suspension Cultures

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Flavonols, 2,3-dihydroxyflavanones, catabolism, benzoic acids, plant cell cultures

The flavonols kaempferol, quercetin and isorhamnetin were labelled with ^{14}C by keeping seven day old *Cicer arietinum* L. plants in an atmosphere of $^{14}CO_2$ for five days. The purified (U-^{14}C) flavonols were applied to cell suspension cultures of *Cicer arietinum* L., *Phaseolus aureus* Roxb., *Glycine max* and *Petroselinum hortense*. Based on the rates of $^{14}CO_2$ formation and distribution of radioactivity after fractionation of the cells, the flavonols were shown to be catabolized to a very high extent.

All four cell suspension cultures possess the enzymatic activity transforming flavonols to the recently discovered 2,3-dihydroxyflavanones. Upon incubation of the flavonols datiscetin and kaempferol with enzyme preparations from *Cicer arietinum* L. cell suspension cultures, it was demonstrated that the enzymatically formed 2,3-dihydroxyflavanones are further transformed in an enzyme catalyzed reaction. Salicylic acid was found as a degradation fragment of ring B of the 2,3,5,7,2′-pentahydroxyflavanone derived from datiscetin. Neither phloroglucinol nor phloroglucinol carboxylic acid were observed as metabolites of ring A. These in vitro findings were further substantiated by in vivo data because the flavonols kaempferol, quercetin and datiscetin when applied to cell suspension cultures of *Cicer arietinum* L. and *Glycine max* gave rise to para-hydroxybenzoic acid, protocatechuic acid and salicylic acid, respectively. It was thus concluded that flavonols are catabolized via 2,3-dihydroxyflavanones with the B-ring liberated as the respective benzoic acid. The data are discussed in connection with earlier findings on the catabolism of chalcones, cinnamic and benzoic acids.

Im Rahmen unserer Untersuchungen über den Stoffwechsel von Flavonolen in höheren Pflanzen [1, 2] haben wir kürzlich ein Enzym aus der Kichererbse, *Cicer arietinum* L., beschrieben, das Flavonolaglyka in 2.3-Dihydroxyflavanone umwandelt (z. B. **1** in **4**, siehe Abb. 1) [3]. Nach unseren bisherigen Ergebnissen ist diese Enzymaktivität in höheren Pflanzen weit verbreitet [4]. Für *Cicer arietinum* L. konnte dabei gezeigt werden, daß das erste Auftreten dieses flavonolumwandelnden Enzyms zu den Entwicklungsprozessen des Keimlings gehört und gleichzeitig mit der ersten Ausbildung der Enzyme der Flavonolbiosynthese erfolgt. Wir sehen in diesem gleichzeitigen Auftreten der Enzyme für die Flavonolbiosynthese und den weiteren Flavonolumsatz einen weiteren Hinweis für unsere Vorstellung, nach der sich die stationären Konzentrationen von sekundären Pflanzeninhaltsstoffen aus dem Wechselspiel von fortlaufender Synthese und weiterem Umsatz ergeben [1, 2, 5].

Obwohl verschiedene Arbeitskreise Hinweise erbringen konnten, daß Flavonole in höheren Pflanzen einem Umsatz unterliegen [1, 2, 6–9], wurde bisher noch nicht zweifelsfrei bewiesen, daß höhere Pflanzen Flavonole vollständig über den Intermediärstoffwechsel wieder bis zu CO_2 abbauen können. Wir berichten nun in dieser Arbeit über die Oxidation der ^{14}C-markierten Flavonole Kämpferol (**1**), Quercetin (**2**) und Isorhamnetin (**3**) in Zellsuspensionskulturen von *Cicer arietinum* L., *Phaseolus aureus* Roxb., *Glycine max* und *Petroselinum hortense*.

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