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TOBACCO POLYPHENOLS. I. THE BIOSYNTHESIS OF *O*-GLUCOSIDES AND *O*-GLUCOSE ESTERS OF HYDROXYCINNAMIC ACIDS

V. C. RUNECKLES and K. WOOLRICH

Imperial Tobacco Co. of Canada Ltd., Montreal, Quebec, Canada

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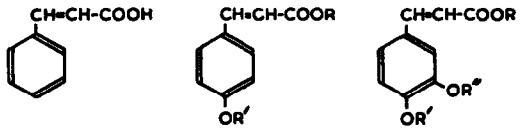
Abstract—Leaf disks of *Nicotiana tabacum* var. Delcrest metabolize *p*-coumaric and ferulic acids with the formation of the corresponding glucose esters and *O*-β-D-glucosides. In 40 hr more glucoside accumulates than glucose ester, when either acid is supplied. These findings are discussed in the light of recent reports that glucose esters are the predominant metabolic products of hydroxycinnamic acids in higher plants.

INTRODUCTION

THE recent studies by Harborne and Corner[1] have shown that, in addition to occurring naturally, the glucose esters of acids such as cinnamic (I), *p*-coumaric (II, R = R′ = H), caffeic (III, R = R′ = R″ = H) and ferulic (III, R = R′ = H, R″ = CH_3) are synthesized



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when the corresponding free acids are supplied to tissues of various higher plants. In addition, the 3-*O*-β-D-glucoside of caffeic acid has been found in wild potato berries[2] while both 3- and 4- *O*-β-D-glucosides are synthesized by tomato leaf tissue from free caffeic acid.[2] Prior to these observations, the only reported naturally occurring *O*-β-D-glucosides of hydroxycinnamic acids were linocinnamarin[3] (II, R = CH_3, R′ = glucosyl) and linocaffein[4] (III, R = CH_3, R′ = glucosyl, R″ = H), constituents of a complex polymer in flaxseed hulls and melilotoside (*O*-β-D-glucosyl-*o*-coumaric acid).[5]

The present communication describes the identification of the glucose esters and glucosides of *p*-coumaric and ferulic acids in tobacco leaf disks after supplying ^{14}C-labelled *p*-coumaric and ferulic acids as subtrates.

RESULTS

Radioautograms of two-dimensional chromatograms of extracts of tobacco leaf disks supplied with ^{14}C-labelled *p*-coumaric acid indicated the incorporation of considerable radioactivity into two components, A and B. Similar radioautograms of ^{14}C-labelled ferulic acid extracts revealed components C and D. By means of band chromatography, homogeneous samples of all four components were obtained, sufficient for the determination

[1] J. B. HARBORNE and J. J. CORNER, *Biochem J.* **81**, 242 (1961).
[2] J. J. CORNER and J. B. HARBORNE, *Chem. & Ind. (London)* 76 (1960).
[3] H. J. KLOSTERMAN, F. SMITH and G. O. CLAGETT, *J. Am. Chem. Soc.* **77**, 420 (1955).
[4] H. J. KLOSTERMAN and R. Z. MUGGLI, *J. Am. Chem. Soc.* **81**, 2188 (1959).
[5] W. KARRER, *Konstitution und Vorkommen der Organischen Pflanzenstoffe*, pp. 379–382, Birkhauser Verlag (1958).

of their chromatographic behaviour and fluorescence (Table 1) and ultraviolet spectra (Table 2). The spectral maxima in 50% methanol suggested that A and B were derivatives of *p*-coumaric acid, while C and D resembled ferulic acid.

TABLE 1. *Rf* VALUES AND FLUORESCENCE COLOURS OF COMPONENTS A, B, C, D AND REFERENCE COMPOUNDS

Component	Reference Compound†	*Rf* values × 100 at 25° in*								Fluorescence colours in§			
		BPW		KFW		BAW	2% HoAc	BzAW	BAm	LWUV‖		SWUV‖	
		‡		(3x)‡						—	+NH_3	—	+NH_3
A	PCG	54	67	45	32	61	75, 85	8	22	--	t	—	d.b.
B	GPC	31	39	29	19	60	68, 87	24	7	-	--	q.	q.
C	FEG	52	64	38	30	56	70, 81	6	19	b.	g.	b.	g.
D	GFE	21	17	27	17	56	61, 81	22	6	—	—	w.b.	w.b.
—	PCQ	35	42	65	56	73	64, 79	16	7	—	b.	—	d.b.
—	CAQ	26	34	41	33	61	55, 75	6	2	b.	g.	b.	g.

* See Experimental for solvent composition.
† PCG, 1-*p*-coumaroyl-glucose; GPC, glucosyl-*p*-coumaric acid; FEG, 1-feruloyl-glucose; GFE, glucosyl-ferulic acid; PCQ, *p*-coumaroyl-quinic acid; CAQ, caffeoyl-quinic acid (chlorogenic acid).
‡ Descending chromatography (otherwise ascending).
§ b., blue (d.b., dark blue; w.b., weak blue); g., green; t., turquoise; q., quench.
‖ LWUV, long-wave (366 mμ) or SWUV, short-wave (254 mμ) ultraviolet light.

By observing their spectra under conditions similar to those described by Jurd,[6] important differences were revealed as indicated in Table 2. Thus components A and C showed the typical bathochromic shift in alkaline solution, characteristic of a free phenolic hydroxyl group, with no hypsochromic shift in the presence of excess sodium acetate. Components B and D, on the other hand, showed this hypsochromic shift in the presence of both sodium acetate and sodium hydroxide, suggesting the presence of a free carboxyl group.

Acid hydrolysis of components A and B yielded *p*-coumaric acid while C and D gave ferulic acid. In each case the only other hydrolysis product detectable was D-glucose. Only A and C were cleaved by mild alkali, and subsequent studies revealed that these two components were only slowly hydrolyzed by β-glucosidase (emulsin) under conditions in

TABLE 2. SPECTRAL MAXIMA OF COMPONENTS B AND D AND REFERENCE COMPOUNDS IN 50% METHANOL AND IN THE PRESENCE OF SODIUM ACETATE AND SODIUM HYDROXIDE

Component	Reference Compound*	Maxima in mμ†		
		MeOH	NaOAc	NaOH
B	GPC	286	281	280·5
—	PC	290	286	333
—	PMC	288	284	284
D	GFE	284	281·5	281
—	FE	314, 290	309, 287	345, 304
—	DMC	310, 288	306, 278	306, 279

* GPC and GFE, see Table 1, footnote †; PC, *p*-coumaric acid; PMC, *p*-methoxycinnamic acid; FE, ferulic acid; DMC, 3,4-dimethoxycinnamic acid.
† See Experimental for composition of solutions.

[6] L. JURD, *Arch. Biochem. Biophys.* **66**, 284 (1957).

which B and D were rapidly hydrolysed to glucose and aglycone. In addition A and C were hydrolysed by an esterase preparation which showed no action towards B and D. As indicated in Table 3, hydrolysis with the appropriate agent yielded approximately equi-molecular amounts of glucose and aglycone.

TABLE 3. HYDROLYSIS PRODUCTS OF COMPONENTS A, B, C AND D; RATIOS OF AGLYCONE TO SUGAR

Component	Hydrolysis products	Method of hydrolysis*			
		Alkali	Acid	β-glucosidase	Esterase
A	*p*-coumaric acid, glucose	1·00 : 0·85	1·00 : 0·92	1·00 : 1·04	1·00 : 0·94
B	*p*-coumaric acid, glucose	—†	1·00 : 0·94	1·00 : 0·98	—†
C	ferulic acid, glucose	1·00 : 0·89	1·00 : 0·99	1·00 : 1·02	1·00 : 0·95
D	ferulic acid, glucose	—†	1·00 : 0·87	1·00 : 0·94	—†

* For details of methods of hydrolysis see Experimental.
† No reaction.

These results suggested that A and C were the 1-β-D glucose esters of *p*-coumaric and ferulic acids respectively. Comparisons with the chromatographic and spectral properties of synthetic 1-*p*-coumaroyl-β-D-glucose and 1-feruloyl-β-D-glucose[7] and with spectral data reported by Harborne and Corner[1] confirmed their identities.

In contrast, the spectral properties and behaviour towards alkali and emulsin of B and D suggested that they were β-D-glucosides. Accordingly, authentic samples of the two glucosides were prepared and their properties compared with isolated B and D (Tables 1 and 2).

Component B and *p*-coumaric acid glucoside could not be detected readily on paper chromatograms since neither fluoresced in ultraviolet light. Nor did either react with diazotized *p*-nitraniline.[8] However, component B could be located by radioautography. Chromatographic identity was accordingly demonstrated by preparing chromatograms of B and synthetic glucoside, separately and in mixture, spraying the developed chromatograms lightly with emulsin, and comparing R_f values measured by viewing the fluorescence of the released *p*-coumaric acid, and also by comparing the outlines of these fluorescent areas with those on radioautograms prepared from the same chromatograms.

Component D and ferulic acid glucoside fluoresced weakly in long-wave ultraviolet light, when exposed to ammonia fumes. However, in order to confirm their identity, similar procedures were employed to those described above for component B.

DISCUSSION

The extensive studies of Harborne and Corner[1] revealed the presence of glucose esters of hydroxycinnamic acids in a large number of higher plant species. Furthermore, such esters were found to be the predominant metabolic products formed when acids such as cinnamic or variously substituted hydroxy-and/or methoxy-cinnamic acids are supplied to the petioles of detached leaves of many of these species. In contrast, simple phenols give rise to glucosides in many species,[9,10] while Kosuge and Conn[11] showed that sweet-clover

[7] L. BIRKOFER, C. KAISER, W. NOUVERTNE and U. THOMAS, *Z. Naturforsch* **166**, 249 (1961).
[8] T. SWAIN, *Biochem. J.* **53**, 200 (1953).
[9] C. W. NYSTROM, N. E. TOLBERT and S. H. WENDER, *Plant Physiol.* **34**, 142 (1959).
[10] J. B. PRIDHAM and J. B. SALTMARSH, *Biochem. J.* **74**, 42P (1960).
[11] T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **234**, 2133 (1959).

leaves convert *o*-coumaric acid into its glucoside, melilotoside. However, Harborne and Corner report the finding of only two glucosides in their survey, those of caffeic acid.

The present evidence indicates that tobacco leaf tissue synthesizes both glucose esters and *O*-glucosides of *p*-coumaric and ferulic acids when these acids are supplied to disks of lamina. From measurements of the amounts of radioactivity incorporated into ester and glucoside (Table 4), it is apparent that appreciable synthesis of both types of glucose derivative occurs during the 40-hr feeding period, while in both cases more *O*-glucoside is synthesized than the ester.

TABLE 4. INCORPORATION OF ^{14}C INTO ESTERS AND GLUCOSIDES*

Substrate	Percentage of 80% *iso*-propanol extract†	
	Glucose ester	Glucoside
p-coumaric acid-2-^{14}C	11·0	19·6
ferulic acid-2-^{14}C	3·3	12·7

* Substrates supplied as 0·005 M solutions for 40 hr to 40 disks (1 cm dia.).
† Radioactivity in compounds expressed as a percentage of the total activity in the 80% *iso*-propanol extracts.

The present studies have been confined to a single species, and consequently it might be supposed that the predominant synthesis of glucosides of hydroxy-cinnamic acids is a peculiarity of tobacco. However, it seems more probable that such glucosides both occur in and can be synthesized by other species. That they have hitherto failed to be detected probably results from their lack of fluorescence and lack of reactivity towards the usual phenol reagents. For in both glucosides described in the present report, the glucose substituent effectively suppresses fluorescence and prevents coupling with phenol reagents. On the other hand, in both natural glucosides of caffeic acid[1,2] one hydroxyl group is unsubstituted and the two compounds are hence fluorescent.

EXPERIMENTAL

Substrates and reference compounds

Ferulic acid-2-^{14}C and *p*-coumaric acid-2-^{14}C were prepared essentially by the method of Vorsatz[12] from the appropriate aldehyde and malonic acid-2-^{14}C obtained by saponifying its diethyl ester (Atomic Energy of Canada Ltd., Ottawa, Ontario). *p*-Methoxycinnamic and 3 : 4-dimethoxycinnamic acids were similarly prepared from the appropriate aldehyde and unlabelled malonic acid.

The glucose esters of *p*-coumaric and ferulic acids were prepared according to Birkofer *et al.*[7] Reference chlorogenic acid (3-caffeoyl-quinic acid) was obtained from Mann Research Laboratories, New York, while the *p*-coumaroyl-quinic acid use was a preparation isolated from tobacco.[13]

Methyl-*O*-tetra-*O*-acetyl-β-D-glucosyl-*o*-coumarate (m.p. 145°, 163°) was prepared according to Klosterman *et al.*[3] Methyl-*O*-tetra-*O*-acetyl-β-D-glucosyl-ferulate (m.p. 140°) was prepared similarly from methyl ferulate and tetra-acetobromoglucose. Both were saponified with 5% barium hydroxide at room temperature for 2 hr, followed by treatment with Amberlite 1R-120(H) to remove barium ions.

[12] F. VORSATZ, *J. prakt. Chem.* **145**, 265 (1936).
[13] V. C. RUNECKLES, unpublished.

After filtration and concentration, *p*-*O*-*β*-D-glucosyl-coumaric acid (*p*-coumaric acid glucoside, II, R = H, R′ = glucosyl) (m.p. 192°) slowly crystallized. It has not been possible to crystallize the *O*-*β*-D-glucosyl-ferulic acid (ferulic acid glucoside, III, R = H, R′ = glucosyl, R″-CH_3) (cf. ref. [14]). Hydrolysis of both glucosides with acid and *β*-glucosidase yielded equimolecular amounts of hydroxycinnamic acid and glucose.

Plant material: feeding conditions

Disks of lamina, 1 cm diameter, were removed from mature leaves of *Nicotiana tabacum* var. Delcrest, grown on sand culture in a temperature-controlled greenhouse. Forty disks were washed with distilled water to remove debris, blotted gently, and floated on 0·005 M solutions of substrate prepared in a solution of 25 p.p.m. neomycin sulphate (to retard microbial growth) and adjusted to pH 6·0 with potassium hydroxide. The disks were kept at room temperature for 40 hr, illuminated by a single 300 watt tungsten lamp.

Preparation and fractionation of extracts

After 40 hr, the disks were washed with water and extracted with three changes of boiling 80% (v/v) *iso*-propanol. The combined extracts were reduced in volume at 35° *in vacuo*, and aliquots taken for two-dimensional chromatography on Whatman No. 1 paper in the solvent systems: (*a*) BPW (*n*-butanol, pyridine, water—14 : 3 : 3) and (*b*) KFW (methyl-*iso*-butyl ketone, 90% formic acid, water—14 : 3 : 2; triple development). All solvents were redistilled prior to use. Radioautographs were prepared using Kodak "No Screen" X-ray film. Chromatograms were examined in long- (366 mμ) and short- (254 mμ) wave ultraviolet light in the presence and absence of ammonia, and by spraying with diazotized *p*-nitraniline.[8]

Components A, B, C and D were isolated in greater amounts by means of band chromatography on Whatman No. 3 paper in BPW, followed by elution with cold 80% *iso*-propanol and subsequent band chromatography in KFW. In order to remove traces of contaminants, B and D were further chromatographed in water. By these procedures, chromatographically homogeneous fractions were obtained, as indicated by their behaviour in four additional solvent systems: 2% HOAc (2% aqueous acetic acid), BzAW (benzene, acetic acid, water—125 : 72 : 3), BAW (*n*-butanol, acetic acid, water—12 : 3 : 5) and BAm (*n*-butanol, 2N ammonia—1 : 1, upper layer). R_f values are presented in Table 1.

Hydrolysis procedures

Alkaline hydrolyses were carried out in aqueous 2 N sodium hydroxide at room temperature for 1 hr in a nitrogen atmosphere. Sodium ions were removed with Amberlite 1R-120 (H) ion exchange resin. Acid hydrolyses employed 0·1 N HCl on a steam bath for 30 min. *β*-Glucosidase (emulsin, Nutritional Biochemical Corp., Cleveland, Ohio) treatment was effected in sodium acetate buffer, pH 5·0, at room temperature for 2 hr, followed by treatment with Amberlite 1R-210 (H) resin to remove cations. An esterase solution was prepared from fungal hemicellulase (Nutritional Biochemical Corp.) and used according to the procedures of Levy and Zucker.[15]

In all cases, following treatment, the solutions were concentrated *in vacuo* at 35° and aliquots subjected to paper chromatography. *p*-coumaric and ferulic acids were identified by means of R_f values, ultraviolet spectra, fluorescence and reactions on paper to diazotized

[14] W. Fuchs, *Chem. Ber.* **88**, 1825 (1955).
[15] C. C. Levy and M. Zucker, *J. Biol. Chem.* **225**, 2418 (1960).

p-nitraniline, and were estimated spectrophotometrically at the appropriate wavelength (Table 2). Glucose was identified by cochromatography with authentic glucose-G ^{14}C of high specific activity, in BAW, BPW, KFW, using benzidine as locating reagent.[16] Following elution from quantitative chromatograms it was estimated by the method of Nelson.[17]

Spectroscopy

All spectral observations were made on 20μM solutions in 50% aqueous methanol, using a Unicam SP700 Spectrophotometer. Spectra in the presence of sodium acetate were obtained by adding solid anhydrous sodium acetate to the solutions and shaking until no further salt dissolved. Spectra of sodium salts were obtained by adding 0·1 ml 2N NaOH to 3·0 ml of solution.

In all cases where spectra were obtained on compounds isolated by paper chromatography, solutions obtained by eluting blank chromatograms were used as references.

Measurement of radioactivity

Radioactive components were eluted from chromatograms with cold 80% *iso*-propanol, concentrated *in vacuo* at 35° to known volume and aliquots counted on stainless steel planchets by means of a Model 750 Automatic Counting System (Baird-Atomic Inc., Cambridge, Mass.) employing a proportional flow counter with an ultra-thin (70 mg/cm^2) end window.

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[16] J. S. D. BACON and J. EDELMAN, *Biochem. J.* **48**, 114 (1951).
[17] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).