Studies on Flavonoid Metabolism

Biosynthesis of (+)-[¹⁴C]catechin by the Plant *Uncaria Gambir* Roxb.

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1. The formation of (+)-[¹⁴C]catechin has been demonstrated in *Uncaria gambir* after the administration of ¹⁴CO₂ and [¹⁴C]acetate. 2. By alkaline degradation to phloroglucinol and protocatechuic acid it has been shown that administration of ¹⁴CO₂ resulted in equal labelling of the A and B rings of catechin, whereas [¹⁴C]-acetate gave rise to labelling largely in the A ring. 3. Incorporation of ¹⁴C from both ¹⁴CO₂ and [¹⁴C]acetate into (+)-catechin was greater in young than in older leaves.

The pathway of flavonoid biosynthesis has been studied in several species of higher plant, including buckwheat (Underhill, Watkin & Neish, 1957; Watkin, Underhill & Neish, 1957; Geissman & Swain, 1957), red cabbage (Grisebach, 1957, 1958); *Eucalyptus* species (Hillis & Isoi, 1965), tea (Zaprometov, 1958, 1962), wild cherry (Compte, Ville, Zwingelstein, Favre-Bonvin & Mentzer, 1958) and red clover (Grisebach, 1959). The evidence obtained agreed with the hypothesis of Birch & Donovan (1953) that the A ring of the flavonoid molecule is formed by head-to-tail condensation of three acetyl (or malonyl) units, whereas ring B and C-2, C-3 and C-4 originate from an intact phenylpropane unit.

In furtherance of our metabolic studies on the degradation of (+)-catechin in the mammal (Griffiths, 1964), [¹⁴C]catechin as well as ring A-labelled [¹⁴C]catechin was required for oral administration to the guinea pig and rat. Although biosynthesis of (+)-catechin from labelled precursors has been demonstrated in the leaves of the tea plant (Zaprometov, 1958, 1962) and the cherry tree (Compte *et al.* 1958), the amount of (+)-catechin present is small (0.35% of the dry weight of the tea leaf; Vuataz, Brandenburger & Egli, 1959). *Uncaria gambir* Roxb. was selected as the experimental material since it contains a high content of (+)-catechin (Perkin & Yoshitake, 1902; Hathway & Seakins, 1957) and only traces of other polyphenols. *U. gambir* is indigenous in Malaya and Indonesia and was formerly cultivated extensively in these areas as a commercial source of tanning materials (Grist, 1926, 1950).

MATERIALS AND METHODS

Materials. (+)-Catechin was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Specimens of penta-acetyl-(+)-catechin (m.p. 131–132°) and 3',4',5,7-tetramethyl-(+)-catechin (m.p. 143–144°) were kindly given by Dr K. Weinges. Sodium [¹⁴C]acetate and Na₂¹⁴CO₃ were purchased from The Radiochemical Centre, Amersham, Bucks.

Plants. Young plants of *U. gambir*, obtained from the Department of Agriculture, State of Trengganu, Malaysia, were maintained in Singapore on loam soil.

Chromatography. Solvent systems were: *A*, butan-1-ol-acetic acid-water (4:1:5, by vol.); *B*, water; *C*, 1 ml. of ethylene glycol added to 50 ml. of the organic phase of a butan-1-ol-water mixture (4:1, v/v) (Freudenburg & Weinges, 1958); *D*, benzene-acetic acid-chloroform, (1:6:3, by vol.); *E*, 20% (w/v) KCl; *F*, propan-2-ol-acetic acid-water (1:2:5, by vol.); *G*, aq. 2% (v/v) acetic acid.

The reagent of Barton, Evans & Gardner (1952) and diazotized *p*-nitroaniline (Smith, 1960) were used for the detection of phenolic compounds. The reagents of Roux & Maihs (1960) were used for (+)-catechin: FeNH₄(SO₄)₂; vanillin-toluene-*p*-sulphonic acid; bis-diazotized benzidine; ammoniacal AgNO₃. *R_f* values are given in Table 1.

Administration of ¹⁴C-labelled compounds. Leafy shoots were cut under water with a razor blade and the cut ends were immersed in 1 ml. of water containing sodium [¹⁴C]acetate (0.5 mc) in a small bottle. One or two cuttings (about 10 g.) were used in each experiment. The solution (1 ml.) was usually absorbed within 1–2 hr. Three successive 1 ml. portions of water were added to the bottle as it approached dryness. The cuttings were then transferred to a beaker containing about 100 ml. of tap water and kept in the sun for 24 hr.

Experiments with ¹⁴CO₂ were carried out in an air-tight glass chamber. In this, cuttings of leafy shoots (about

10g.) were kept in a beaker containing a few millilitres of tap water saturated with CO_2 . The chamber was evacuated to a negative pressure and $^{14}\text{CO}_2$ was generated in it by slowly running lactic acid (50%, v/v) into aq. $\text{Na}_2^{14}\text{CO}_3$ (1mc). The shoots were exposed to tropical sunlight for 12hr. Air was then admitted to the chamber and drawn through a series of three traps containing 20ml. of 4N-NaOH to trap excess of $^{14}\text{CO}_2$.

Isolation of (+)-catechin. Freshly harvested leaves (49.6g.) were cut into small pieces and extracted with 250ml. of ethanol in a homogenizer for 10min. The suspension was filtered and the residue re-extracted with ethanol until the final extract was colourless (usually two to three extractions). The pooled ethanolic extracts were then concentrated *in vacuo* at 40° to a small volume in a stream of O_2 -free N_2 .

The concentrated ethanolic extract was applied as long bands on 12 sheets of Whatman no. 17M paper (40cm. $\times$ 45cm.). Authentic (+)-catechin was applied laterally to each band. The papers were developed (40cm.) by the ascending method in water. The dried developed papers were examined in u.v. light in the presence of NH_3 fumes. (+)-Catechin appeared as a dull-blue-fluorescent band. The band corresponding to the marker was cut into small fragments and eluted with ethanol. The eluate was filtered, concentrated and applied as a band to a second chromatogram (ten sheets) and developed in solvent A. The appropriate band was eluted with ethanol. The eluate was filtered and evaporated to dryness *in vacuo* at 40° in a stream of O_2 -free N_2 . The residue was taken up in 15ml. of warm water and left in the refrigerator. The colourless crystals that separated were recrystallized from water and dried over P_2O_5 at 60° *in vacuo* for 16hr. They had m.p. 176° ; $[\alpha]_D^{25}$ 17.8° (in acetone) and the yield was 3.44g.

Penta-acetyl-(+)-catechin. A small quantity of the isolated (+)-catechin (100mg.) in 1ml. of redistilled pyridine was treated with 2ml. of acetic anhydride in the dark for 24hr. at 28° . The solution was then concentrated to dryness under vacuum. The brown gummy residue was dissolved in acetone and about 50ml. of cold water added. The crystals that separated were removed by centrifuging and after recrystallization twice from ethanol had m.p. $131\text{--}132^\circ$; the yield was 74.6mg.

3',4',5,7-Tetramethyl-(+)-catechin. A small quantity of the isolated (+)-catechin (100mg.) in 50ml. of acetone was mixed with 140mg. of dimethyl sulphate, and anhydrous K_2CO_3 (4g.) was added. The mixture was heated under reflux for 1–2hr. in a boiling-water bath in a stream of O_2 -free N_2 . The solution was then cooled and filtered. The solid was washed with warm acetone. The filtrate and washings were concentrated *in vacuo*. A solution of the residue in water was left in the refrigerator overnight, then acidified with 2N-HCl and extracted with ether (4 $\times$ 50ml.). The extract was washed free from acid and dried with anhydrous Na_2SO_4 . Evaporation of the solvent left 3',4',5,7-tetramethyl-(+)-catechin. After recrystallization from aqueous ethanol twice, the compound gave needles, m.p. $143\text{--}144^\circ$; the yield was 43.7mg.

Alkaline degradation of (+)-catechin. (+)-Catechin (10mg.) was fused with 400mg. of dry KOH (Zaprometov, 1964) in the presence of O_2 -free N_2 . The degradation products were obtained by acidification with 4ml. of 2N- H_2SO_4 to pH 1 followed by ethyl acetate extraction. The ethyl acetate extract was concentrated to dryness under

reduced pressure in O_2 -free N_2 . The residue in 3ml. of ethanol was applied as a band on Whatman no. 3MM paper (pre-washed in solvent D) and separated in solvent D. Authentic protocatechuic acid and phloroglucinol were applied on each side of the band. Bands of the developed chromatogram corresponding to protocatechuic acid (R_F 0.68), and phloroglucinol (R_F 0.37), were cut into small fragments and eluted with ethanol. The identity of the two eluted compounds was confirmed by co-chromatography in solvents A, B, D, E and F, and by determination of the u.v. spectra (Table 1).

Determination of (+)-catechin, protocatechuic acid and phloroglucinol. This was as described by Griffiths (1964) except that measurements were made at $660\text{m}\mu$ in a Hilger spectrophotometer.

Measurement of radioactivity. Radioactive spots on chromatograms were located by radioautographs on X-ray film (Kodirex, Kodak Ltd.) with exposure for 3–4 weeks, or by a Packard 7200 scanner.

Radioactivity of phloroglucinol and protocatechuic acid obtained by alkaline degradation was measured by plating duplicate 1ml. samples of the ethanolic eluates obtained from the band chromatograms on aluminium planchets at infinite thinness (less than $200\mu\text{g./cm}^2$). Counts were made by the windowless Geiger-Müller tube of the gas-flow detector model D-47 with model 181A decade scaler (Nuclear-Chicago Corp.).

Radioactivity of catechin was determined by adding an aqueous 0.1% solution to 10ml. of phosphor. The phosphor was prepared by adding 6g. of 2,5-diphenyloxazole and 0.12g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene to 1l. of xylene. This solution (2 vol.) was then mixed with 1 vol. of Triton X-100 (Lennig Chemicals Ltd.) and stirred until homogeneous (Patterson & Greene, 1965). Counts were made in an automatic scintillation counter (Nuclear-Chicago Corp.).

Ultraviolet spectra. These were determined in ethanol in a Bausch and Lomb Spectronic 505 recording spectrophotometer.

Infrared spectra. These were determined in KBr disks in a Jasco i.r. spectrophotometer (model IR-S; Japan Spectroscopic Co. Ltd.).

RESULTS

Ethanol-soluble polyphenols present in fresh leaf material of U. gambir. A freeze-dried preparation of freshly harvested leaves was homogenized in cold ethanol at 0° for 10min. The filtered extract was examined by two-dimensional chromatography on Whatman no. 1 paper in solvents A and B. Five compounds reacting with Barton's reagent and diazotized *p*-nitroaniline were detected (Fig. 1). The compound corresponding to spot 1, which was present in large amounts, was identified as (+)-catechin (see below), and spot 2, which was present in trace amounts only, was tentatively identified by co-chromatography as (–)-epicatechin. Spots 3, 4 and 5, which were present in traces, were not identified. The catechin was isolated by band chromatography and its identity confirmed by the determination of the mixed m.p. and by co-

Fig. 1. Diagram showing the positions of the ethanol-soluble polyphenols of *U. gambir*. Spot 1, (+)-catechin; spot 2, (-)-epicatechin; spots 3-5, unidentified compounds.

Table 1. Properties of phloroglucinol, protocatechuic acid and (+)-catechin

The R_F values were obtained by ascending chromatography on Whatman no. 1 paper at a room temperature of 26°; the length of the solvent run was 40 cm.

Solvent	R_F values		
	Phloroglucinol	Protocatechuic acid	(+)-Catechin
A	0.73	0.81	0.62
B	0.59	0.83	0.43
C	—	—	0.63
D	0.37	0.68	—
E	0.48	0.87	0.27
F	0.18	0.52	0.66
G	—	—	0.49
$\lambda_{\max.}$ (m μ)	270	260, 290	282

chromatography with an authentic specimen of (+)-catechin in solvents A, B, C, E, F and G (Table 1). Comparison of the i.r. and u.v. spectra (Table 1) of the recrystallized compound and its tetramethyl and penta-acetyl derivatives with those of the reference compounds showed that these were identical.

(+)-Catechin content of leaves of *U. gambir*. Since the polyphenol content of leaves has been shown to vary with their physiological age (Griffiths, 1958), the mean (+)-catechin content of young, mature and old leaves of *U. gambir* was determined. The young leaves were detached from the tip of a vigorously growing shoot; the old leaves were of leathery appearance and were obtained from the

Table 2. Content of catechin in leaves of different ages

Position and state of leaves	No. of leaf samples	Catechin content [% of wet wt. of leaves (mean $\pm$ s.d.)]
Tip, young	16	5.0 ($\pm$ 0.2)
Intermediate, mature	16	6.2 ($\pm$ 0.5)
Basal, old	16	9.4 ($\pm$ 0.5)

basal end of the shoot. The results (Table 2) agree with those reported by Zaprometov (1963), who found that, in developing leaves of the tea plant, the amount of free catechins increased from the bud to the third leaf.

Incorporation of ¹⁴C-labelled compounds. ¹⁴CO₂ and sodium [1-¹⁴C]acetate were administered to both young and old shoots of *U. gambir*. One or two shoots were used in each series of experiments. Examination of ethanolic extracts by two-dimensional chromatography and radioautography showed considerable activity in the spot corresponding to (+)-catechin (spot 1; Fig. 1). Two additional areas on the chromatograms caused darkening of the X-ray film but did not give a blue colour with Barton's reagent.

Labelled (+)-catechin from the leaf material was recrystallized to constant specific activity (Table 3). Its chromatographic and radiochemical purity was confirmed by examination in three solvents, A, B and C, when the radioactivity was shown to coincide with the area occupied by catechin. No other radioactive areas were observed. The distribution of radioactivity in the degradation products of catechin is shown in Table 4.

DISCUSSION

Earlier studies on the polyphenolic constituents of *U. gambir* were carried out with either 'cube gambir', a dried aqueous extract of the compressed leaves and twigs (Perkin & Yoshitake, 1902), or oven-dried leaf material (Hathway & Seakins, 1957). In both of these cases, and more especially in the former, considerable post-mortem change would be expected as a result of both auto-oxidative and enzymic action. It was important therefore to establish also the nature of the polyphenols normally present in the fresh leaf.

Chromatographic examination of fresh freeze-dried leaf material showed that (+)-catechin is the only major flavonoid present, although traces of (-)-epicatechin and three other unidentified polyphenols were present. The high (+)-catechin content in leaves of *U. gambir* (namely up to 9.4% of the fresh weight), together with a high rate of ¹⁴C incorporation, makes this plant a highly

Table 3. *Specific activities of (+)-catechin formed from sodium [1-¹⁴C]acetate and from ¹⁴CO₂ by U. gambir*

Expt. no.	Age of shoots	Wt. of shoots (g. wet wt.)	Catechin isolated (mg.)	Total radioactivity administered (mc)	Specific activity of catechin (mc/mole)	Incorporation (%)
From sodium [1- ¹⁴ C]acetate						
1	Young	10	272	0.5	42	6.4
2	Old	7.5	258	0.5	29	4.5
From ¹⁴ CO ₂						
3	Young	12.5	481	1.0	16.3	2.1
4	Old	10	673	1.0	4.7	0.9

Table 4. *Distribution of radioactivity in the (+)-catechin formed by U. gambir*Distribution of radioactivity on molar basis (%)
[(+)-catechin=100%]

Expt. no.	Ring A (phloroglucinol)	Ring B+C (protocatechuic acid)	C-3+C-4 (by difference)
From sodium [1- ¹⁴ C]acetate			
1	95.3	4.5	0.2
2	94.1	5.5	0.4
From ¹⁴ CO ₂			
3	50.1	42.5	7.4
4	49.9	41.5	8.6

suitable material for studies on the biosynthesis of (+)-catechin. That a high turnover rate may be characteristic of catechin metabolism in plant tissues is supported by other observations including that of Hillis & Isoi (1965), who found that in *Eucalyptus* species the incorporation of ¹⁴C from [¹⁴C]phenylalanine and [¹⁴C]tyrosine is considerably higher into (+)-catechin than into the other flavonoids present. Likewise Zaprometov (1962) has shown that, in tea shoots, ¹⁴C-labelled precursors including shikimic acid give rise to higher specific activities in the simple catechins than in any of the other flavonoids present in tea leaf. However, Hasegawa & Yasue (1962) found that radioactive (+)-catechin was not transformed into quercetin or taxifolin in *Chamaecyparis pisifera*. Similarly Hillis & Isoi (1965), although noting the rapid conversion of labelled (+)-catechin into unidentified products in *Eucalyptus*, found no conversion into other flavonoids.

The higher incorporation of both ¹⁴CO₂ and [¹⁴C]acetate in Expts. 1 and 3 compared with Expts. 2 and 4 may reflect the difference in physiological age, since Zaprometov (1963) reported that in the tea plant the rate of biosynthesis is greater in young shoots than in old.

The finding that the administration of ¹⁴CO₂ to *U. gambir* leads to extensive incorporation of ¹⁴C into both rings A and B of the catechin molecule, whereas administration of [¹⁴C]acetate leads to incorporation of ¹⁴C almost wholly into the A ring, supports the hypothesis of Birch & Donovan (1953) that the A ring of the flavonoid molecule is derived from a polyoxoacyl chain formed from three molecules of acetate. Previous evidence for this hypothesis was provided by Watkin *et al.* (1957) in their studies on the biosynthesis of quercetin in buckwheat and by Grisebach (1957), who has described the pattern of incorporation of labelled acetate into cyanidin in red cabbage. In both cases, as in the present work, the distribution of ¹⁴C in the products of alkaline hydrolysis indicated that the [¹⁴C]acetate had been incorporated mainly into the A ring.

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