5. The heartwood tannins of *A. mollissima* consist of a gradation over the molecular-weight range 290–1650 in which the yield of anthocyanidins decreases with increasing average molecular weight.

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Condensed Tannins

5. THE OXIDATIVE CONDENSATION OF (+)-CATECHIN

By S. R. EVELYN, E. A. MAIHS and D. G. ROUX
Leather Industries Research Institute, Rhodes University, Grahamstown, South Africa

Post-mortem oxidation, whether atmospheric or enzymic, affects the composition of polyphenols present in vegetable extracts and is invariably associated with the well-known 'browning' phenomenon. (+)-Catechin and (−)-*epi*catechin from the leaves of *Uncaria gambir* and heartwood of *Acacia catechu* respectively are known to be associated with dark-coloured condensed tannins in gambier and Burma-cutch extracts. Some of the tannins appear to be present in the original plant material (Hathway & Seakins, 1957b), but others may originate during manufacture of the extracts (Howes, 1953). Atmospheric oxidation or autoxidation of (+)-catechin, for example, results in a product which possesses marked tanning properties, as shown by its reaction with collagen monolayers (Ellis & Pankhurst, 1954).

Hathway & Seakins (1955) and Hathway (1958) recognized two different types of autoxidation polymers, namely those obtained at low pH (< 2), which are of the Freudenberg & Maitland (1934) type, and those obtained within a higher pH range (2–8), which occur by polymerization through quinones (Hathway & Seakins, 1957b).

In previous work the tacit assumption was made that polymerization does, in fact, occur under the above conditions. This aspect is examined in the present study.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Acetyl values are by Miss E. Paulus of this Laboratory. Infrared-absorption curves are by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria.

Conditions of oxidation of (+)-*catechin*. (+)-Catechin was isolated from cube gambier (obtained from the leaves of *Uncaria gambir*) by the method of Perkin & Yoshitake (1902), m.p. 175°. Atmospheric oxidation of (+)-catechin

dissolved in water (2·5 g./100 ml.) was allowed to proceed at 80° in small beakers at pH 4·6. Water evaporating during heating was replenished at frequent intervals and the solutions were maintained as close as possible to their original volumes. Heating was discontinued at different intervals (Table 1), and the water immediately removed under a partial vacuum in a current of carbon dioxide.

Molecular-weight estimation. The dry oxidized products (2 g.) were acetylated with a mixture of acetic anhydride (6 ml.) and sodium acetate (1 g.) and poured into iced water. The water-insoluble acetylated derivative was redissolved in a minimum of acetic acid, and again precipitated with water after filtering. The molecular weight of the product was determined by using a modified Ray ebulliometer with benzene as solvent (Roux & Evelyn, 1958a–c). The acetyl content of the product was determined by the trans-esterification method of Matchett & Levine (1941), thus enabling the calculation of the average molecular weight of the oxidation mixtures (Table 1), as shown by Evelyn (1954), to be made.

Quantitative estimation of (+)-catechin and phloroglucinol in oxidation products. Paper-chromatographic examination of the oxidation mixtures derived from (+)-catechin after 20 hr. oxidation (see below) showed that the product in each instance contained much unchanged catechin, and also phloroglucinol in low concentration. These were estimated by two-dimensional quantitative paper chromatography in conjunction with the spray reagents bisdiazotized benzidine and ammoniacal silver nitrate [for (+)-catechin only], and a simple densitometer (Roux & Maihs, 1960). The standard curve for (+)-catechin was determined over the range 20–100 μg., and the oxidation products were applied and the catechin was estimated within the same concentration range. The contribution made by the estimated percentage of (+)-catechin to the average molecular weight of the oxidation product could be subtracted to give the average molecular weight of the residual tannin mixture (Table 1). The standard curve for phloroglucinol was determined over the range 0·5–5 μg. and the oxidized products were applied over the range 0·5–6 mg. for the estimation of phloroglucinol present. Phloroglucinol was present to the extent of 0·09–0·12% when (+)-catechin was oxidized for 120 hr.

Examination of the oxidation reaction by paper chromatography. Four two-dimensional chromatograms of the oxidation products at each level of oxidation were run with water-saturated butan-2-ol for the first direction and 2% acetic acid for the second direction. The chromatograms were sprayed with each of the following reagents: (a) vanillin–toluene-p-sulphonic acid in ethanol (Roberts, Cartwright & Wood, 1956); (b) bisdiazotized benzidine (Lindstedt, 1950); (c) ferric ammonium sulphate (a 3% aqueous solution prepared and used immediately) and (d) ammoniacal silver nitrate.

The components present among the oxidation products, together with their colour reactions with each reagent, are shown in Fig. 1.

Alkali fusion of (+)-catechin and of tannins derived from catechin by oxidation. Oxidized catechin (128 hr. oxidation) (0·2 g.) in methanol was streaked on a Whatman no. 3 18¼ in. × 22½ in. paper-chromatographic sheet and the sheet developed in 2% acetic acid. After drying, tannins which remain at the origin were cut out in a strip and eluted with 70% ethanol. The brown solid recovered

(0·1 g.) was dried under vacuum and the dried product (3 mg.) carefully fused under anhydrous conditions which eliminate oxidation and side reactions (Roux, 1958a). The fusion product was divided into phenolic and phenolic–carboxylic acid fractions by the usual bicarbonate technique, and each fraction examined by paper chromatography with butan-1-ol–acetic acid–water (4:1:5, by vol.) as irrigant. (+)-Catechin (3 mg.) was subjected to microfusion under identical conditions. Phloroglucinol and protocatechuic acid were the predominant degradation products in each instance, and from visual comparison there appeared little difference in the yields of these substances from both the catechin and its polymer (condensate) resulting from oxidation.

Alkali fusion and molecular weight of tannins from cube gambier. Cube gambier was dissolved in an acetone–water (1:1, v/v) mixture and the solution filtered. The filtrate was dried and treated like the oxidized catechin. Alkali fusion

Table 1. *Estimation of average molecular weight of oxidation mixture of (+)-catechin, of percentage of (+)-catechin in oxidation mixture, and of the average molecular weight of the tannins formed*

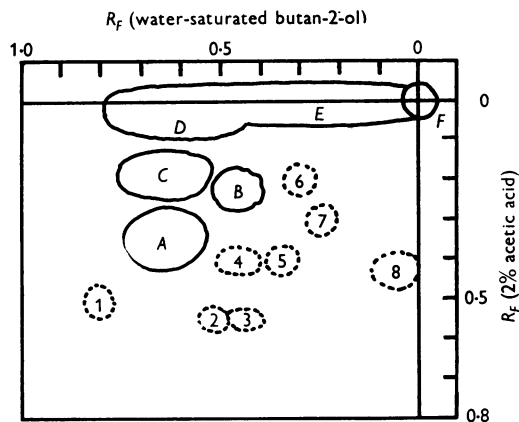| Heating period (hr.)	Average mol.wt. of mixture	Catechin (%)	Average mol.wt. of tannins
0	290	100	Nil
4	314	80	410
8	307	92	503
8·5	355	80	615
20	354	74	536
72	525	40	682
128	795	21	929

Fig. 1. Two-dimensional chromatogram of oxidation product of (+)-catechin indicating relative positions of major (unbroken lines) and minor (broken lines) components. Component A is (+)-catechin, B is (probably) (+)-epicatechin, and 1 is phloroglucinol. With spray-reagents the following colours are developed by the oxidation products of (+)-catechin: vanillin, A, B, 1 (carmine), C (red), D, E, F (weak red); benzidine, A–F (ochre), 1 (purple); ferric alum, A, B (green), C (brown), D, E, F (weak brown); silver nitrate, A, B (brown). C–F (purple–brown).

gave phloroglucinol and protocatechuic acid as the main degradation products in about the same yields as before. The tannins (0·8 g.) were acetylated with acetic anhydride (4 ml.) in pyridine (5 ml.) for 2 hr. at 50°, and poured into water, giving a dark-brown product (Found: $CO \cdot CH_3$, 32·4%). The molecular weight of the acetylated tannins with acetone as solvent was 2238, giving a calculated average molecular weight of 1530 for this fraction (R_F 0 in 2% acetic acid) of gambier tannins.

Acid-induced condensation of (+)-catechin. (+)-Catechin (1·0 g.) was heated with N-hydrochloric acid (25 ml.) and after complete solution was boiled slowly. The solution turned milky after 3·5 min. and a buff-coloured precipitate settled out after 4–6 min. This was separated from the hot solution by suction and the boiling continued for two further periods of 5 min. each, when the above procedure was repeated. The combined precipitates were washed with cold water and dried (0·7 g.). Alkali fusion under anhydrous conditions afforded phloroglucinol and protocatechuic acid as before. Paper chromatography of the condensed material showed that a high proportion of tannins, which give an ochre colour with the benzidine reagent, was present near the origin. The acid-induced condensate (0·7 g.) was acetylated with acetic anhydride (5 ml.) and anhydrous sodium acetate (0·5 g.) and poured into water. The washed product (0·9 g.), which was obviously highly condensed, was insoluble in acetone, benzene and other solvents and therefore unsuitable for molecular-weight estimations (Found: $CO \cdot CH_3$, 34·2, 34·7%). The acetylated condensate was re-acetylated with acetic anhydride (3 ml.) in pyridine (5 ml.) (Found: $CO \cdot CH_3$, 35·0, 34·9%) and compared with penta-acetyl-(+)-catechin, m.p. 131° (Found: $CO \cdot CH_3$, 42·1%. Calc. for $C_{25}H_{24}O_{11} : CO \cdot CH_3$, 43·0%).

Infrared-absorption spectra of various catechin tannins. The zero-R_F (in water) fractions of tannins formed on autoxidation of catechin (128 hr. oxidation; mol.wt. > 929), and of tannins present in gambier extract (mol.wt. 1530) and also tannins formed by acid-induced condensation of catechin, were all dried at 110° *in vacuo* for 2–3 hr. The tannins (0·6 mg.) were milled with potassium bromide (250 mg.), and disks made from the mixtures were examined in a Perkin–Elmer Infra-cord infrared spectrophotometer. Curves of the autoxidation polymers accurately follow similar inflexions obtained from polymeric tannins from gambier extract over the range 2–15 μ, but at a lower level of absorption (Fig. 2). Acid-induced tannins, by comparison, showed much-reduced absorption over the band 3345–3335 cm.$^{-1}$, attributable to hydroxyl groups, and showed no significant absorption over the range 1680–1660 cm.$^{-1}$. In the latter range the autoxidation polymers and gambier tannins show significant weak and strong shoulders respectively, which may be due to carbonyl stretching vibrations of o-quinones. Differences between the acid-induced polymers and others also occur at 1135 cm.$^{-1}$, but over the remainder of the range examined the three curves are remarkably similar. Strong absorption bands present in (+)-catechin at 1145 and 1050–1030 cm.$^{-1}$ are absent in all three 'tannins' and that at 827 cm.$^{-1}$ in catechin is greatly reduced in the tannins.

DISCUSSION

At the natural pH (4·6) of aqueous solutions of (+)-catechin oxygen is apparently responsible for the slow conversion of (+)-catechin into tannins at elevated temperatures (80°), since heating for 2 hr. at 105° in an autoclave under pressure, with the exclusion of air, produces only epimerization to (+)-*epi*catechin, and no tannins result (Roux, 1955). In support of the differentiation made by Hathway & Seakins (1955) and by Hathway (1958) it is found that tannins produced by oxidation in the higher pH range (2–8) apparently differ in properties from those formed by acid-induced condensation at lower pH (< 2). The latter are buff-coloured and apparently only partly soluble in
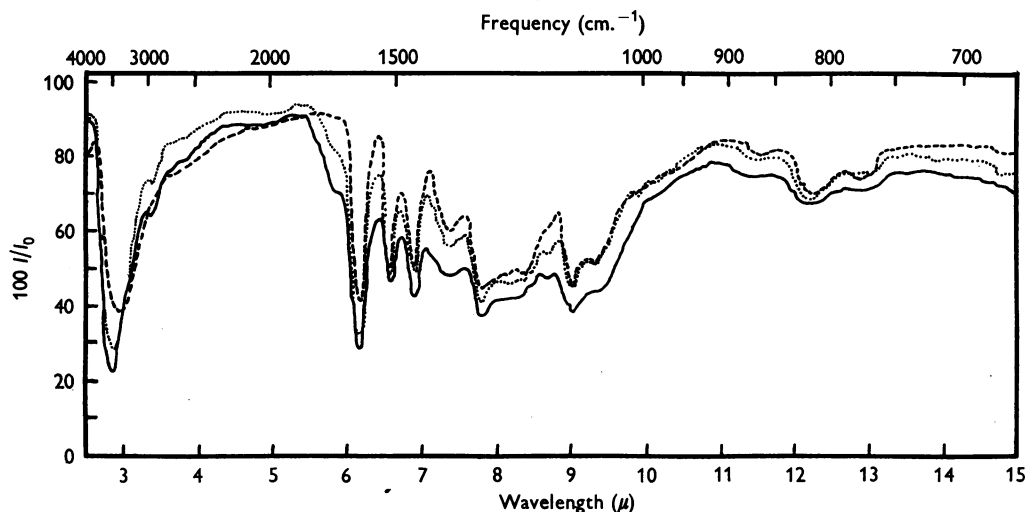

Fig. 2. Infrared-absorption curves of gambier tannins (R_F 0 in water) (———), of autoxidation products of (+)-catechin (R_F 0 in water) (.....), and of 'tannins' from hydrochloric acid-condensation of (+)-catechin (- - - -).

water and in those organic solvents which readily dissolve the chocolate-brown oxidation products of (+)-catechin.

Roberts & Wood (1951) had shown that during enzymic oxidation of green-tea catechins a marked decrease in concentration of catechins in the high-R_F region, and a corresponding increase in concentration in the diffuse area near the origin, occur on paper chromatograms. The latter area was considered to represent condensation products of the catechins, and Kursanov, Dzhemukhadze & Zaprometov (1947) showed that the fermentation process is accompanied by some increase in average molecular weight. In all other studies an assumption has been made that oxidation results in polymerization or condensation of catechin to form tannins. Table 1 shows that the average molecular weight of the mixture formed from the oxidation of (+)-catechin increases linearly with time and that condensation does apparently occur, although also accompanied by degradation. The percentage of phloroglucinol present as degradation product has been shown to be small (about 0·1 %) after prolonged (128 hr.) oxidation. This may indicate either that the degree of degradation is small or that the degradation products readily participate in condensation and therefore remain at low concentration. Estimation of the residual catechin in each oxidation level enabled calculation to be made of the average molecular weight of the tannins formed (Table 1). These tannins must have varying molecular weights which increase with time over the range 400–920.

The relative stability of (+)-catechin to oxidative condensation at elevated temperatures is demonstrated. After heating for 128 hr., for example, 21 % of the (+)-catechin still survives (Table 1), the reduction in percentage of catechin with time apparently following an asymptotic curve. A number of intermediate substances are formed in addition to phloroglucinol and (+)-epi-catechin, which Freudenberg & Purrman (1923) showed to be the main product of epimerization. Of the intermediates (Fig. 1), the mobile component C (R_F 0·67 in water-saturated butan-2-ol, and 0·18 in 2 % acetic acid), formed on prolonged (60–120 hr.) oxidation, appears to be most significant, as its colour reactions are similar to those of the tannins in the low-R_F trail. The brown colour of these products with ferric alum, compared with the bright green of (+)-catechin and (−)-epi-catechin (Fig. 1), suggests that some of the o-dihydroxy function has been altered, possibly by quinone formation, as suggested by Hathway & Seakins (1955, 1957a, b) and by Hathway (1958). No conclusions may be drawn from the improved method of degradation with alkali, except that phloroglucinol and protocatechuic acid are the main degradation products resulting from (+)-catechin, oxidized catechin tannins and catechin tannins present in gambier extract and from the acid-induced condensate of (+)-catechin. In all these tannins high percentages of catechol groups with the same mode of linkage as in catechin apparently survive.

Kursanov et al. (1947) have shown that enzymic oxidation of ethyl acetate-soluble catechin and catechin gallates of average molecular weight 352–455 from green tea forms the intensely coloured ethyl acetate-insoluble tannins of black tea. The latter are water-soluble and have an average molecular weight of 614–782. The tea tannins are similar in colour, solubility properties and range of average molecular weight to tannins formed from (+)-catechin by atmospheric oxidation (cf. Table 1). Some tannins (R_F 0) present in gambier extract are similarly very dark brown and have a high average molecular weight (1530). By analogy with findings for wattle and quebracho tannins (Roux & Evelyn, 1958a, c), this probably represents the most highly condensed fraction of the gambier tannins. The gambier tannins and autoxidation products of (+)-catechin examined are soluble in methanol and also in acetone–water (1:1, v/v). They have similar infrared-absorption curves over the range 2–15 μ (Fig. 2), with weak bands at 1670 cm.$^{-1}$ which may be attributable to the presence of o-quinones in low concentration. This suggests a close similarity between their origin and mode of formation, for example by means of enzymic oxidation or autoxidation of (+)-catechin.

By comparison acid-induced polymers of (+)-catechin are mainly insoluble in methanol and in acetone–water, are light in colour and appear from their solubility behaviour to be highly condensed. Condensation under the conditions used also results in a reduction of total hydroxyl function, as evidenced by strongly reduced absorption at 3345–3335 cm.$^{-1}$, and also by the decreased degree of substitution possible with acetyl groups. Freudenberg & Maitland (1934), however, attributed the decrease in degree of acetylation of the acid-induced condensate of (+)-catechin to incomplete substitution resulting from the partial solubility of these 'tannins' in the acetylating medium, and this explanation must be partly correct.

Roux (1958b) and Roux & Evelyn (1958a, c) have shown the parallel relationship between the increase in average molecular weight of tannins and their chemical affinity for any given substrate. It is suggested that the increased tanning action of the oxidation product of catechins is due to two factors: the increase in average molecular weight, and the formation of quinonoid structures (Roberts, 1952) well known for their tanning action. Oxi-

dative browning occurs to a limited extent in wattle (*Acacia mollissima*) and quebracho (*Schinopsis* spp.) extracts, which contain mainly tannins differing from the autoxidation polymers of catechins (Hathway & Seakins, 1957b).

SUMMARY

1. The autoxidation of (+)-catechin in aqueous solution is relatively slow and is accompanied by an increase in the average molecular weight of the mixture formed.

2. The percentage of (+)-catechin remaining in the oxidation product is estimated and the average molecular weight of the tannins calculated.

3. The autoxidation reaction appears to be complex. Degradation accompanies the condensation reaction and the percentage of phloroglucinol formed is low.

4. The oxidation is accompanied by a 'browning' reaction; the product is soluble in methanol and acetone–water, and apparently similar to that which accompanies (+)-catechin in commercial gambier extract.

5. Tannins present in cube gambier from the leaves of *Uncaria gambir* are shown to be polymeric.

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The Oxidation of Fatty Acids by a Particulate Fraction from Desert-Locust (*Schistocerca gregaria*) Thorax Tissues

By H. MEYER,* B. PREISS AND SH. BAUER
Department of Biological Chemistry, The Hebrew University, Jerusalem, Israel

The oxidation of fatty acids in the insect body has not been the subject of the same amount of research work as has been done with preparations from mammalian tissues. Krogh & Weis-Fogh (1951) and Weis-Fogh (1952) investigated changes in the respiratory quotients of *Schistocerca* at rest and in flight and found that the R.Q. was 0·75 in the flying insect, which pointed to an extensive mobilization of fat reserves, in comparison with the R.Q.

0·82 observed in the resting insect; they suggested that this value resulted from the predominant oxidation of carbohydrates by the insect at rest. Preparations from the flight muscles of the cockroach (*Periplaneta americana*) (Barron & Tahmisian, 1948) and locust-thorax mitochondria (Rees, 1954) were able to oxidize acetate, but butyrate and octanoate were not attacked. McShan, Kramer & Schlegel (1954) reported slight oxidation of octanoate by whole homogenates obtained from woodroach (*Leucophaea maderae*) thorax muscles. No

* Deceased.