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The Transformation of Gallates into Ellagates

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Herzig, Pollak & Bronneck (1908) demonstrated that aeration of an ammoniacal solution of ethyl gallate (I) yielded ellagic acid (V), and recently humic acid and a trace of hydrogen peroxide were also found (Hathway, 1957) amongst the ultimate

products of reaction. In this study, 4:5:6:4':5':6'-hexahydroxydiphenic acid was shown to be the chemical precursor of ellagic acid, and gallic acid the chemical precursor of the humic acid by-product. It is now suggested that the principal reaction may

Gallic acid (R = H)

(I), where R = Et.

(II), where R = Me.

(III), where R = β -D-glucose-1-.

Ellagic acid

(V)

Chebulinic acid
(IV)

Chebulagic acid
(VI)

have biological implications. Quantitative aspects of the transformation *in vitro* of plant metabolites of type (I)–(IV) into ellagic acid which have been explored under physiological conditions are reported in the present communication.

MATERIALS AND METHODS

Melting points were determined on a Kofler block. Specific rotations were determined for the D line of sodium, a 1 dm. microtube being used. Five pairs of readings with the solutions and five pairs with a solvent blank were taken in each case. Temperature, where not recorded, was 20–25°. Paper chromatography was carried out at $23 \pm 2^\circ$ in all-glass apparatus. Chromatograms were dried at room temp. unless otherwise stated. A Hanovia mercury arc fitted with a Wood's glass filter was used to examine chromatograms for fluorescent zones.

Ethyl and methyl gallates were prepared by Fischer–Speier ethylation and methylation of the free acid, and they were crystallized from aq. ethanol and aq. methanol respectively and dried over P_2O_5 at $120^\circ/0.01$ mm. Hg. Ethyl gallate formed rhombs, m.p. 155° ; methyl gallate formed rhombs, m.p. 198 – 199° .

Glucogallin (β -D-glucose-1-gallate) was synthesized from β -D-glucose-2:3:4:6-tetra-acetate and galloyl chloride triacetate, according to Fischer & Bergmann's method (1918). Deacetylation of the hepta-acetate, m.p. 125° , $[\alpha]_D -24^\circ$ (c, 1.0; tetrachloroethane), by Zemplén ammonolysis under H_2 , gave free glucogallin which crystallized from 80% ethanol, forming small prisms, m.p. 211 – 212° , $[\alpha]_D -24^\circ$ (c, 1.0; water).

Chebulinic acid nonahydrate, $[\alpha]_D +65^\circ$ (c, 1.0; aq. ethanol) was isolated from the dried fruit (myrobalans) of *Terminalia chebula*, and was crystallized from aq. acetone (Terminberg & Frank, 1927). The preparation ran as a single spot on a two-way chromatogram, in which n-acetic acid and butan-2-ol-acetic acid–water (14:1:5, by vol.) were used as solvent systems.

L-Ascorbic acid (Biochemicals Roche) had m.p. 188 – 190° , $[\alpha]_D +48^\circ$ (c, 1.0; methanol).

Soluble lyophilized mushroom polyphenoloxidase (L. Light and Co.) had a purpurogallin number (P.N.) of 0.15. P.N. represents the no. of milligrams of purpurogallin formed from pyrogallol in 5 min. at $20^\circ/\text{mg. dry wt. of enzyme preparation}$ (Keilin & Mann, 1938).

Acetone-dried powder of Psalliota campestris. Freshly picked mushrooms (300 g.) were sliced and put into ice-cold acetone (2 l.) and homogenized in a macerator. The homogenate was filtered on sintered glass, and the solid (20 g.) was immediately washed five times with 500 ml. portions of ice-cold acetone and dried over paraffin wax at $0^\circ/1$ mm. Hg. A Hilger Uvispek spectrophotometer was used for the P.N. determination by Keilin & Mann's (1938) method. This powder (2.8 enzyme units) had a P.N. of 0.14. One enzyme unit (E.U.) corresponds to the quantity of enzyme which produces 1 g. of purpurogallin in 5 min. at 20° .

Incubation unit. A 500 ml. Erlenmeyer flask equipped with a B24 standard ground-glass socket to receive a gas wash-bottle head (MF 28/3, Quickfit and Quartz Ltd., London) with an inlet reaching to within 5 mm. of the bottom of the flask, was immersed in a thermostat at 20° . Several units were connected in series to an air supply delivering 700–900 ml./min.

Oxidation procedure. In a typical experiment, 0.5 m-mole of finely divided substrate was dissolved in 150 ml. of buffer which had previously been equilibrated at 20° , and the unit was connected in series with the air supply. At the end of the reaction period the flask was detached from the train, and the reaction was arrested by the acidification of the reaction mixture with conc. HCl. Ellagic acid was coagulated, and filtered in a 4 G sintered-glass crucible, which was subsequently washed with hot water and ethanol successively, and dried at 100° to constant weight. When the acidified reaction mixture contained insoluble enzyme preparation, the supernatant was removed from the sediment in the centrifuge, and the sediment was boiled with a 10 ml. portion of pyridine. The pyridine extract was filtered from cellular debris, which was subsequently washed with three further 10 ml. portions of boiling pyridine. The combined pyridine extract and washings were diluted with water and acidified with conc. HCl. After coagulation, the ellagic acid precipitate was collected as previously described. This separation afforded reproducible results.

Autoxidation and enzymic-oxidation experiments involving glucogallin and chebulinic acid were performed under similar conditions, chebulinic acid being extracted from ellagic acid precipitate with a large volume of acetone. Aerobic oxidation of gallate in the presence of ascorbic acid was carried out similarly. In the attempts at coupled oxidation, methyl gallate (0.5 mm), ascorbic acid (0.5 mm) and copper ($2.5 \mu\text{g./ml.}$) were present in equilibrated phosphate buffer, pH 7.4, and the experiment was repeated in the presence of 3×10^{-2} E.U. of mushroom polyphenoloxidase.

Oxidation of ascorbic acid. Ascorbic acid (35.2 mg.) was dissolved in 200 ml. of 0.75 M- NaHCO_3 , pH 7.9, which was aerated at 20° . Samples (1 ml.) were withdrawn at 15 min. intervals, acidified to Congo red with 20% (w/v) trichloroacetic acid and titrated immediately with 0.02 mm-2,6-dichlorophenolindophenol.

Identification of the product from the autoxidations and enzymic oxidations

Paper chromatography. Spots (5 $\mu\text{l.}$) of an 0.2% solution of the product in pyridine, and marker spots (5 $\mu\text{l.}$) of 0.2% solutions of authentic ellagic acid in pyridine, and of galloflavin and isogalloflavin in methyl Cellosolve (2-methoxyethanol), were applied to starting lines 4 cm. from the lower edge of sheets of Whatman filter paper no. 1, of length 57 cm. Single-way ascending chromatography was effected with benzyl alcohol-*tert.*-butyl alcohol-propan-2-ol–water (3:1:1:1, by vol.) containing 1.8% (w/v) of formic acid (Stark, Goodban & Owens, 1951). Papers were irrigated for 20–24 hr., and dried at 60 – 70° . Single-way chromatography of the product and of authentic ellagic acid was also effected in aq. 60% (w/v) formamide buffered at pH 3.5 with formic acid (Hathway, 1956a). Ellagic acid was recognized by its distinctive soft violet fluorescence in u.v. light, changing to pale yellow on exposure to NH_3 vapour.

Derivative formation. A filtered solution of the reaction product in pyridine was regenerated by acidification with conc. HCl. The precipitate was removed, dried at 100° , and refluxed with acetic anhydride in the presence of a few drops of conc. H_2SO_4 as catalyst. Ellagic acid tetra-acetate crystallized from a large volume of acetic anhydride, forming needles, m.p. 344° (Found: C, 55.7; H, 3.3. Calc. for $\text{C}_{22}\text{H}_{14}\text{O}_{12}$: C, 56.1; H, 3.0%). A boiling soln. of the tetra-acetate in pyridine was neutralized with boiling acetic acid,

and the solution obtained was refluxed with an equal volume of 12N-HCl. The resulting ellagic acid was recovered with little loss from these operations.

Infrared spectra. Nujol (liquid paraffin) mulls were prepared from this purified specimen and from an authentic specimen of ellagic acid (Herzig *et al.* 1908) similarly purified. Infrared spectra were automatically recorded on the same sheet at 2.0 cm.⁻¹/sec., a Perkin-Elmer model 21 double-beam spectrophotometer, equipped with rock-salt optics, being used for the determinations.

Chromatographic separation of ethyl and methyl gallates from other plant phenolic compounds

Spots (5 μ l.) of 0.2% solutions of ethyl gallate, methyl gallate, protocatechuic acid and syringic acid in ethanol were applied to starting lines, 4 cm. from the lower edge of sheets of Whatman filter paper no. 2, of length 57 cm. Single-way ascending chromatography was effected with butan-1-ol-acetic acid-water, 63:10:27, by vol., upper phase only (Campbell, Work & Mellanby, 1951). Papers were irrigated for 24 hr., and sprayed with ethanolic ferric chloride reagent.

RESULTS

The present work consists of a study of the transformation *in vitro* of plant metabolites of type (I)-(IV) into ellagic acid under physiological conditions. The product of these new reactions was characterized as a crystalline tetra-acetate, and the parent compound was shown to be identical with authentic ellagic acid (Herzig *et al.* 1908) by paper chromatography and infrared measurement. Ellagic acid exhibits: a band at 1712 cm.⁻¹ due to the carboxyl-stretching vibration; aromatic bands at 1600, 1580 and 1518 cm.⁻¹; a strong band at 3200 cm.⁻¹

Fig. 1. Formation of ellagic acid at 20° by mushroom polyphenoloxidase. Each flask contained 0.75M-NaHCO₃, pH 7.9, and 0.5 m-mole of methyl gallate. ●, Without enzyme; ○, with 3×10^{-2} E.U. of enzyme; Δ, with two separate additions of 1.5×10^{-2} E.U. of enzyme. For details see Methods.

due to H-bonded hydroxyl; bands at 3570 and 3485 cm.⁻¹ due to free hydroxyl (Hathway, 1956a). Ellagic acid has a high R_F value (0.60) in benzylalcohol-*tert.*-butylalcohol-propan-2-ol-water (3:1:1:1, by vol.) containing 1.8% of formic acid, and a similar R_F value in aq. 60% (w/v) formamide, buffered to pH 3.5 with formic acid.

The aerobic oxidation of ethyl and methyl gallates was initially explored in a solution of sodium bicarbonate, pH 7.9, and in phosphate buffer, pH 8.0. Autoxidation in both media proceeded slowly, presumably since oxidation to quinone is inhibited by phenol (James, Snell & Weissberger, 1938), and was incomplete. Autoxidation was more rapid and proceeded farther in NaHCO₃ than in phosphate buffer.

Mushroom polyphenoloxidase accelerated the initial O₂ uptake in both media, and the progress curves for ellagic acid formation (Figs. 1, 2) showed

Fig. 2. Formation of ellagic acid at 20° by mushroom polyphenoloxidase. Each flask contained 0.07M-phosphate buffer, pH 8.0, and 0.5 m-mole of methyl gallate. ●, Without enzyme; ○, with 4×10^{-2} E.U. of enzyme; Δ, with two separate additions of 2×10^{-2} E.U. of enzyme.

Fig. 3. Effect of pH on ellagic acid formation from methyl gallate after aeration for 3 hr. in 0.07M-phosphate buffer. ●, Without enzyme; ○, with 2×10^{-2} E.U. of mushroom enzyme.

slow inactivation of enzyme during the reaction. Thus ellagic acid formed with the lower enzyme concentrations (Figs. 1, 2) represented incomplete oxidation of methyl gallate; and a second addition of enzyme resulted in an immediate acceleration of ellagic acid formation. A comparatively low level of enzyme activity brought the reaction in sodium bicarbonate solution to completion, and this is the first quantitative preparation of ellagic acid to be reported. Enzymic oxidation of ethyl and methyl gallates in the phosphate buffer was incomplete, since humic acid was also formed, and an optimum yield of ellagic acid of only 80% was obtained. Because of this the reaction could not be followed by manometric means, but was determined by estimating the ellagic acid formed. No ellagic acid was produced by the action of hydrogen peroxide on methyl gallate in the presence of peroxidase.

The progress curves (Figs. 1, 2) were typical of those for enzyme-catalysed reactions, but the difference in rate of formation of ellagic acid in bicarbonate and phosphate at comparable levels of enzyme activity appeared to be too great to be accounted for by a difference in pH of 0.1. For this reason the effect of pH on ellagic acid formation in phosphate buffer was investigated, both in the presence and absence of enzyme (Fig. 3), and in both cases the rate of reaction was faster at pH 8.0 than at pH 7.9. Empirical experiments involving complex-forming agents suggest that the enhanced rate in sodium bicarbonate compared with that in phosphate buffer at the same pH may be due to the fact that Cu^{2+} ion forms complexes more effectively with phosphate than with bicarbonate, for when comparable solutions of the enzyme in sodium bicarbonate and in phosphate buffer were shaken with a solution of diphenylthiocarbazone in carbon tetrachloride, and the resulting solvent phase was extracted with 0.02N-HCl to remove other metals (Caughley, Holland & Ritchie, 1939), the residual carbon tetrachloride extract obtained from the bicarbonate solution contained more copper than that from the phosphate buffer. Since mushroom polyphenoloxidase is a copper protein, the presence of a trace of Cu^{2+} ion in solution is probably essential for full catalytic influence.

Ascorbic acid had no influence on the formation of ellagic acid from methyl gallate in either the presence or the absence of enzyme in a bicarbonate buffer, pH 7.9 (Fig. 4). With phosphate buffer, however, at pH 7.4 in the presence of copper, ellagic acid was formed from methyl gallate in a 20% yield, but no formation occurred in the presence of ascorbic acid. Ascorbic acid also arrested the corresponding enzymic oxidation of gallate in this buffer, both in the presence or absence of a small addition of Cu^{2+} ion.

Aerobic oxidation of the tannin prototype,

glucogallin (III), has now been investigated in sodium bicarbonate solution, and a 50% yield of ellagic acid was achieved. By addition of enzyme this reaction went to completion (Fig. 5). Glucose was detected chromatographically in the reaction medium.

Fig. 4. Formation of ellagic acid in the presence of ascorbic acid. The reaction flask contained 0.75M- NaHCO_3 , pH 7.9, 0.5 m-mole of methyl gallate and 0.2 m-mole of ascorbic acid. O, Without enzyme; Δ , with 3×10^{-2} E.U. of mushroom enzyme (see ordinate on right). Corresponding results obtained in the absence of ascorbic acid are shown by the broken curves. The control (●) shows destruction of ascorbic acid under these conditions (see ordinate on left). For details see Methods.

Fig. 5. Transformation of glucogallin into ellagic acid by mushroom polyphenoloxidase. Flasks contained 0.75M- NaHCO_3 , pH 7.9, and 0.1 m-mole of glucogallin. ●, Without enzyme; O, with 10^{-2} E.U. of enzyme.

Table 1. *Chromatographic behaviour of plant metabolites and their reactions with ferric iron*

Solvent system: butan-1-ol-acetic acid-water (63:10:27, by vol.; upper phase).

Plant metabolite	R_F	Ferric reaction
Methyl gallate	0.84	Violet
Protocatechuic acid	0.87	Green
Syringic acid	0.90	Wine-red
Ethyl gallate	0.90	Violet

Table 2. *Distribution of gallates in dicotyledonous plants*

Gallate or gallotannin	Genus	Family	Reference
(III)	<i>Rheum</i>	Polygonaceae	Gilson (1903)
(II)	<i>Caesalpinia</i>	Leguminosae	Nierenstein (1905)
(II)	<i>Geranium</i>	Geraniaceae	Huzikawa & Ina (1940); Fujikawa, Nakamura & Asami (1942)
(II)	<i>Euphorbia</i>	Euphorbiaceae	Huzii, Huzikawa & Kudo (1937)
(II)	<i>Koeleruteria</i>	Sapindaceae	Carlson, Olynky, Duncan & Smolin (1951)
(III) } (IV) }	<i>Terminalia</i>	Combretaceae	Fridolin (1884 <i>a, b</i>); Hathway (1956 <i>b</i>)
(I)	<i>Arbutus</i>	Ericaceae	Sosa (1950)

Aerobic oxidation of the gallotannin chebulinic acid (Fridolin, 1884*a, b*) (IV) has also been similarly investigated, and 0.6 mole of ellagic acid was obtained, indicating that the 1-galloyl residue of the tannin was hydrolytically oxidized to ellagic acid. Addition of mushroom polyphenoloxidase did not affect the rate of reaction, since the initial preponderance of tannin inactivated the enzyme.

Table 1 records R_F values and ferric reactions for ethyl and methyl gallates, and for protocatechuic and syringic acids. These substances were located at the same site on a two-dimensional paper chromatogram in which *N*-acetic acid and butan-2-ol-acetic acid-water (14:1:5, by vol.) were used as solvent systems (King, 1956). They were separated on a single-way paper chromatogram irrigated with the upper phase of the butan-1-ol-acetic acid-water (63:10:27, by vol.) solvent system which has been successfully used by Bradfield & Flood (1952). Methyl gallate has now been detected by these means in one of the phenolic fractions derived from myrobalans, the dried fruit of *Terminalia chebula*.

DISCUSSION

Since ellagic acid is widely distributed among Dicotyledonae (Bate-Smith, 1956; Hathway, 1956*a*; Wehmer & Hadders, 1932), the present transformation of gallates into ellagate suggests that the incidence of ellagic acid may at least in part be related to the concomitant occurrence of gallate precursors. In Table 2 a list is given of the families and genera of plants from the leaves of which gallates, of type (I)–(IV), have been isolated. The families which contain the gallates cited in this table belong to the same orders (Willis, 1931) as those in which ellagic acid most consistently occurs. From a pioneer study of the distribution of polyphenols in the green leaves of more than a thousand dicotyledonous species, Dr E. C. Bate-Smith (private communication) has found that the occurrence of gallic acid is comparatively scarce, the free acid occurring only in extracts of those

plants which contain gallates or gallotannins. The fact that free gallic acid is not transformed into ellagate by aerobic oxidation does not therefore conflict with the possible formation of the ellagic acid of some Dicotyledonae from gallate precursors. The close structural relationship between chebulinic and chebulagic acids, (IV) and (VI), the unique gallotannin and ellagitannin respectively of myrobalans (Schmidt, 1956; Schmidt & Mayer, 1956), suggests that the ellagitannin is formed from chebulinic acid by the oxidative elimination of two hydrogen atoms from adjacent galloyl residues, attached to C₃ and C₆ of the β -D-glucose residue. The hexahydroxydiphenyl residue of chebulagic acid, the ellagitannin of the *Terminalia*, may therefore originate from the galloyl residues of the trigallate, chebulinic acid. The present demonstration of oxidative removal of the galloyl residue of chebulinic acid explains why a neutral aqueous extract of myrobalans deposits ellagic acid on standing. Free ellagic acid may therefore be formed in the fruit of *Terminalia* after abscission by the aerobic oxidation of methyl gallate, glucogallin and chebulinic acid, as well as by hydrolysis of the ellagitannin, chebulagic acid (VI).

The transformation of gallate into ellagate and the polymerization through quinone of catechin into phlobatannin (Hathway & Seakins, 1955, 1957*a, b*) involves the same enzyme system, and this may be relevant to the tannins of the closely related (Fagaceae) oak (*Quercus pedunculata* and *Q. sessiliflora*) and chestnut (*Castanea sativa*). The barks contain phlobatannins, built up from catechin and gallocatechin units, and considerable quantities of ellagitannins, which give glucose, gallic acid, ellagic acid, dehydrodigallic acid and other unidentified phenolics on hydrolysis (Mayer, 1956), whereas chestnut heartwood contains only ellagitannins (Hathway, 1956*c*). These preliminary results pose many questions, and the distribution of tannin within the plant is now being studied in this Laboratory together with the chemical investigation of oak-bark tannins.

SUMMARY

1. The aerobic oxidation of ethyl and methyl gallates to ellagic acid in aqueous sodium bicarbonate solution, pH 7.9, and phosphate buffer, pH 8.0, is reported. Autoxidation is incomplete, and mushroom polyphenoloxidase accelerates the initial uptake of oxygen in these media.

2. Enzymic oxidation in sodium bicarbonate solution gives a quantitative yield of ellagic acid, whereas in phosphate buffer a humic acid by-product is also formed.

3. Ascorbic acid interrupts aerobic oxidations of methyl gallate in phosphate buffer, pH 7.4, whereas these oxidations are unaffected by ascorbic acid in sodium bicarbonate solution.

4. Aerobic oxidation of the tannin prototype, glucogallin, and of the gallotannin, chebulinic acid, gives ellagic acid.

5. Since ellagic acid most consistently occurs in those orders of the Dicotyledonae which contain gallates and gallotannins, the possibility that in some of these the gallate metabolites may behave as ellagic acid precursors is considered.

6. The fact that polyphenoloxidase is involved in the transformation of gallate into ellagate and in the polymerization through quinone of catechin into phlobatannin may be related to the simultaneous occurrence of ellagitannins and phlobatannins.

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The Biosynthesis of Sucrose

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Although sucrose is the most widespread and generally the most abundant sugar present in plants, the mechanism of sucrose synthesis by the plant has, until recently, remained obscure.

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Numerous experiments have been carried out on the formation of sucrose in intact plant tissues when these were supplied with hexoses, e.g. Nelson & Auchincloss (1933), Nurmia (1935), Leonard (1939). Calvin & Benson (1949) allowed the alga *Chlorella* to photosynthesize in the presence of $^{14}\text{CO}_2$ and found that the first labelled free carbohydrate to appear