The Leaf Tannin of Willow-Herb

[Chamaenerion angustifolium (L.) Scop.]

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1. The leaf tannin of willow-herb [Chamaenerion angustifolium (L.) Scop.] has been isolated and separated into two fractions of differing solubility. 2. The tannin contains a penta-O-galloyl-β-D-glucose core to which further galloyl groups are depsidically bound. 3. The unfractionated tannin contains an average of 10-5 galloyl groups/glucose molecule; the soluble fraction has on average 7-6 galloyl groups/glucose molecule and the less soluble fraction has 12-4. 4. The tannin is a mixture of molecules ranging at least from hepta- to trideca-galloyl-β-D-glucose. 5. The tannin forms complexes with proteins and the fact that it is a hydrolysable gallotannin has a bearing on the release of nitrogen from the protein of the dead leaf.

It has been suggested (Handley, 1954, 1961) that when the leaves of many plants die the residual proteins form complexes with protein-precipitating substances, which may be tannins, present in the leaves. It is considered that the formation of such complexes will render the protein resistant to decomposition when the dead leaf reaches the soil. The resistance to decomposition appears to be such that under some conditions, e.g. in base-deficient soils, the leaves, and especially the protein complex contained in them, remain for many years as a largely undecomposed layer of organic material on the surface of the mineral soil. Such conditions are generally associated with markedly sub-optimum supplies of mineral nitrogen for plant growth.

There are, however, some species of plants whose dead leaves contain protein complexes that do not seem to give rise to organic layers on the surface of base-poor mineral soils. Willow-herb (Chamaenerion angustifolium) is an example of such a plant. The leaves of this plant contain substances that yield precipitates with gelatin solution and an intense blue-black colour with solutions of ferric salts.

It has also been observed (Handley, 1961) that the protein-precipitating materials from the leaves of different plant species form complexes with protein that apparently vary in their resistance to decomposition and in the provision of mineral nitrogen for plant growth. The investigations of Gustavson (1956) suggest that collagen tanned with hydrolysable tannins is less resistant to the action of trypsin than collagen tanned with condensed tannins. Comparative tests (Handley, 1961) on aqueous extracts of fresh leaves of Calluna vulgaris, Circaea lutetiana and Chamaenerion angustifolium suggested that Calluna leaves may contain condensed tannin whereas those of Circaea and Chamaenerion may contain hydrolysable tannins. Brief chemical examination of the protein-precipitating constituents from the leaves of these species (Brown, Love & Handley, 1962) has confirmed this.

Hydrolysable tannins of known structure consist of a polyhydric alcohol esterified with gallic acid or derivatives of gallic acid. The work of Haworth and his collaborators (Haworth, 1961) has shown that a subdivision of the gallotannins can be made on the basis of the esterified core present, e.g. Chinese, Sicilian-sumach and Stagshorn-sumach tannins (Armitage et al. 1961) have a penta-O-galloyl-β-D-glucose core further esterified with gallic acid, whereas Turkish tannin (Armitage, Haslam, Haworth & Searle, 1962) has a mixture of 3,4,6- and 2,3,6-tri-O-galloyl-β-D-glucose as its core and Tara tannin (Haslam, Haworth & Keen, 1962) has a core of 3,4,5-tri-O-galloylquinic acid. In the present paper we describe investigations on the structure of the hydrolysable tannin isolated from Chamaenerion angustifolium.

EXPERIMENTAL

Paper chromatography. Whatman no. 4 paper was used and chromatograms were developed at 25±2°.

(a) Phenols were chromatographed in two dimensions with solvent systems composed of (i) 2% (v/v) acetic acid and (ii) butan-1-ol-acetic acid–water (4:1:5, by vol., supernatant layer). Phenols were revealed with a spray composed of FeCl₃ and K₂Fe(CN)₆ (Kirby, Knowles & White, 1953).

(b) Carbohydrates were chromatographed with the solvent system (ii) above and detected by sprays of AgNO₃ (Trevelyan, Procter & Harrison, 1950) or aniline hydrogen phthalate (Gordon, Thornberg & Wercum, 1956).
(c) Methylated phenolic acids were chromatographed two-dimensionally in solvent systems (i) and (ii) above. Phenols with a free para-position were detected by spraying with 0.1% 2,6-dibromobenzoquinone 4-chloroimide in methanol (Gibbs reagent), followed by NaHCO₃ solution.

**Extraction of willow-herb tannin.** Leaves (1 kg.) of willow-herb (Chamaenerion angustifolium), collected in October, were kept in the dark under water-acetone (7:3, v/v) (21 l) at room temperature for 7 days. Saturation of the supernatant liquid with NaCl gave two liquid layers. The upper layer was removed, diluted with twice its volume of ethanol and treated with ether until precipitation was complete. The precipitate (7 g. of salt–tannin mixture) was collected by centrifugation and frequently washed with ether. Dialysis in aqueous solution against running distilled water was carried out until Cl⁻ ion and phenols could no longer be detected in the diffusate. The dialysis residue consisted of a solution with suspended solid. Water was added to dissolve the solid and the resulting solution was freeze-dried to yield the tannin (3.1 g.) as a greyish-brown solid, [α]D +10.7 ± 0.2° [c 1.0 in acetone–water (1:1, v/v)] (Found in material dried at room temperature for 24 hr.: C, 53-05; H, 3.2-3.3%; at room temperature for 48 hr.: C, 53-4; H, 3.3-4.4%; at room temperature for 5 days: C, 53-5, 53-4; H, 3-4, 3-5; N, 0-0; residue, 0-0%).

Paper chromatography in solvent systems (i) and (ii) and application of the FeCl₃-K₂Fe(CN)₅ spray revealed only the tannin as a streak from the origin in solvent system (i), immobile in solvent system (ii). The tannin was unaffected by a spray consisting of conc. HCl–10% (w/v) vanillin in methanol (1:2, v/v) (Mayer & Baumi, 1956). Phenolic compounds and free saccharides with discrete Rf values were absent.

Treatment of an aqueous solution of the tannin with an aqueous solution of gelatin yielded a precipitate. The mother liquor was shown to be free of tannin (absence of phenolic streak on paper chromatography) and extraction of the precipitate with acetone–water (3:2, v/v) regenerated some free tannin. Passage of an aqueous solution of the tannin through a short column of hide powder caused complete retention of the tannin by the hide powder, the eluate being free of phenolic compounds. The same result was obtained when an aqueous solution of the tannin was shaken with hide powder.

**Fractionation of willow-herb tannin.** In a second experiment the solid in the dialysis residue was separated from the solution.

The solution was freeze-dried to yield the first tannin fraction (A) (0.8 g.) as a greyish-brown solid, [α]D +12.8 ± 0.2°, +13.0 ± 0.2° [c 0.25 in acetone–water (1:1, v/v)] (Found in material dried at 150°/0.2 mm. for 30 hr.: C, 53-6; H, 3-45; N, 0-0; Cl, 0-0; residue, 0-0%). The solid (2.1 g.) from the dialysis residue was dissolved in water and freeze-dried to yield the second tannin fraction (B) as a greyish-brown powder that was dried at 150°/0.2 mm. for 30 hr., [α]D +0.0 ± 0.2° [c 0.25 in acetone–water (1:1, v/v)]. Both fractions resembled the unfractiioned tannin in their behaviour on chromatography.

**Determination of glucose in willow-herb tannin.** A typical run was as follows. Tannin (10 mg.) inaq. 0.5N-NaOH (5 ml.) was maintained at 40° for 24 hr. The solution was then acidified with dilute HCl and passed down an Amberlite IRA-400 ion-exchange column (30 cm. x 1.5 cm.) with distilled water as eluent. Fractions (5 ml.) were collected and each fraction was treated with a 0.2% solution of anthrone in AnalaR conc. H₂SO₄ (10-0 ml.). The mixtures were stirred with a glass rod and after 20 min. the E₅₂₀ values were measured. Standard glucose solutions of concentrations both above and below those expected for the tannin fractions were treated in the same way. From these a calibration curve was established from which the glucose concentrations in the fractions could be determined. The sum of these was taken as the glucose content of the tannin (usually glucose was only found in the first two or three fractions). Results are shown in Table 1.

**Determination of gallic acid in willow-herb tannin.** (a) By measurement of E₂₇₀. Tannin (30 mg.) was hydrolysed at room temperature with aq. 0.5N-NaOH in an atmosphere of N₂ for 48 hr. The hydrolysate was diluted to 100 ml. and samples of this solution were diluted suitably in acid and the E₂₇₀ values measured. Results are shown in Table 1.

(b) By titration. A standard neutralization curve was established by titrating a mixture of gallic acid (53.0 mg.) and 0.1 N HCl (1 ml.) with aq. 0.02 N-KOH. Neutralization was followed with the aid of a pH-meter (glass electrode) and an atmosphere of N₂ was used in this and all subsequent titrations. No distinction between the mineral and organic acid was attempted, but the end point for both the acid groups was found to be at pH 6.85 and the end point for the titration of the first phenolic group was found to be at pH 10.15. A typical determination of the gallic acid content of the tannin was as follows: tannin (82.75 mg.) was treated with 0.441 N-KOH (2.50 ml.). The mixture was kept at room temperature in an atmosphere of N₂ for 2–3 days and then the pH was adjusted to 6.85 with an autotitrator and the acid consumed recorded. After being corrected for the volume of acid consumed by the alkali, the titres were compared with those obtained from a similar weight of gallic acid treated similarly, whence the weight of gallic acid liberated from the known weight of tannin was found. Results are shown in Table 1.

**Acid hydrolysis of willow-herb tannin.** The second tannin fraction (B) (1.0 g.) was heated at 100° with aq. 1% (v/v) H₂SO₄ (300 ml.) under an atmosphere of N₂ for 16 hr. A portion (2 ml.) of the resulting solution was treated with excess of BaCO₃ and the mixture was centrifuged. The supernatant liquid, which gave a positive Molisch reaction, was subjected to paper chromatography in solvent system (ii) and a sugar was detected with aniline hydrogen phthalate. The Rf value, 0.18, was that of d-glucose and the identity was proved by chromatography with both an internal and an external reference of authentic d-glucose.

Paper chromatography of the solution revealed the presence of gallic acid, but not of ellagic acid. The remainder of the solution was concentrated by rotary evaporation (35°/12 mm.) and chromatographed on a cellulose column (50 cm. x 6.5 cm.) in water. Evaporation of the eluate (200 ml.) and crystallization of the residue from water gave gallic acid as needles, m.p. and mixed m.p. 248–252° (decomp.), identical with authentic material on paper chromatograms.

The first tannin fraction (A) was treated similarly and d-glucose and gallic acid were identified on paper chromatograms.

**Alkaline hydrolysis of willow-herb tannin.** Unfractionated tannin (30 mg.) in 0.5N Ba(OH)₂ solution (15 ml.) was kept at room temperature for 5 days. The solution was neutralized to pH 7.0 (narrow-range indicator paper) by adding 0.5N-NaOH.
H₂SO₄. The BaSO₄ was removed by centrifugation. Paper chromatography of the supernatant liquid in solvent system (ii) and spraying with aniline hydrogene phthalate showed D-glucose (R₂0-19) as the only carbohydrate present, identified by comparison with both an external and an internal standard of D-glucose.

**Methodology of willow-herb tannin.** Unfractionated willow-herb tannin (1-14g.) was dissolved in a mixture of 0-5N-acetate buffer, pH 6-0 (10ml.), and methanol (100ml.) that had previously been deoxygenated by being boiled under N₂; the mixture was boiled under reflux for 5 days with a very slow stream of N₂ passing through it. Removal of the solvents at 30°C, solution of the residue in water (100ml.) and continual extraction with ethyl acetate gave an oily solid that when analysed by paper chromatography in solvent systems (i) and (ii) revealed the pattern shown in Table 2.

**Results and Discussion.**

Analyses for carbon, hydrogen, gallic acid and glucose and the [α]₀ and values of the unfractionated tannin and of the two fractions of willow-herb tannin are shown in Table 1 with the corresponding theoretical values for two polygalloylgalactoses and the values for several gallotannins investigated by Haworth and his co-workers (Armitage et al. 1961). The gallic acid content of the tannin was determined, after alkaline hydrolysis, by measurement of E₂₇₅ [(a), Table 1] and by titration of the gallic acid liberated on alkaline hydrolysis [(b), Table 1], and the glucose was estimated after removal of the gallic acid on a column of Amberlite IRA-400 by the method in which anthrone is used (Park & Johnson, 1949) or the glucose content was calculated from the optical rotation of the tannin by use of the relationship discussed below.

The amounts of glucose and gallic acid, which relate to the amounts of each substance liberated on hydrolysis, suggested that the unfractionated tannin had a structure containing an average of 10-4
Gallic acid was determined: (a) by measurement of $E_{270}$; (b) by titration. Experimental details are given in the text. References: 1, this paper; 2, Armitage et al. (1961). The values for decagalloylglucose and octagalloylglucose are theoretical.

<table>
<thead>
<tr>
<th>Tannin</th>
<th>C (%)</th>
<th>H (%)</th>
<th>Glucose (a)</th>
<th>[α]D Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Willow-herb (unfractionated)</td>
<td>53.35</td>
<td>3.3</td>
<td>10.25*</td>
<td>+10.7°</td>
</tr>
<tr>
<td>Decagalloylglucose (C$<em>{76}$H$</em>{52}$O$_{38}$)</td>
<td>53.7</td>
<td>3.05</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>Willow-herb (fraction A)</td>
<td>53.6</td>
<td>3.45</td>
<td>98.4</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>97.2</td>
<td>98.6</td>
</tr>
<tr>
<td>Octagalloylglucose (C$<em>{85}$H$</em>{62}$O$_{38}$)</td>
<td>53.4</td>
<td>3.2</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td>Willow-herb (fraction B)</td>
<td>53.2</td>
<td>4.0</td>
<td>111</td>
<td>113</td>
</tr>
<tr>
<td>Chinese</td>
<td>53.5</td>
<td>3.75</td>
<td>102</td>
<td>99.8</td>
</tr>
<tr>
<td>Sicilian sumach</td>
<td>53.3</td>
<td>4.0</td>
<td>102</td>
<td>99.4</td>
</tr>
<tr>
<td>Stagshorn sumach</td>
<td>53.3</td>
<td>4.0</td>
<td>97.8</td>
<td>12.4</td>
</tr>
</tbody>
</table>

* Calculated from the optical rotation.

Galloyl groups/glucose molecule, that the more soluble fraction (A) contained an average of 7.6 galloyl groups/glucose molecule, and that the less soluble fraction (B) contained an average of 12.4 galloyl groups/glucose molecule, a value considerably greater than that for the more soluble fraction or for the galloittannins investigated by Armitage et al. (1961). This range of molecular weights, resulting from structures containing 7 or less galloyl groups/glucose molecule to those containing 13 or more, accounts for the behaviour of the tannin on paper chromatography and contrasts with the large but discrete spot found, for example, for Chinese tannin, which probably contains structures ranging less widely from penta- to octa-galloyl-D-glucose (E. Haslam, personal communication).

It was considered important to show that, despite the analytical differences, the two tannin fractions of differing solubility contained essentially the same type of molecule. It was with this object in mind that a general method for identification of the core of hydrolysable tannins was established by correlating the optical rotations of known galloyl-glucose and galloittannins with their glucose contents (Brown & Brown, 1965). A plot of specific rotation, $[α]_D$, for acetone or aqueous-acetone solutions of a series of penta-O-galloyl-β-D-glucoses against the weight per cent of glucose (G) gives a good straight line whose equation is $[α]_D = 0.76 G + 2.9$. The values of $[α]_D$ and G for the two tannin fractions obtained from willow-herb fall on this line, so that both fractions can be assigned a penta-O-galloyl-β-D-glucose core [(I), where $R^1 = R^2 = R^3 = R^4 = R^5 = \text{galloyl (II)}$].

The arrangement of galloyl groups in the tannin was further elucidated by alkaline hydrolysis of the methylated tannin, which yielded only 3,4-di-O-methyl- and 3,4,5-tri-O-methyl-gallic acid, both of which were isolated and identified, showing that only depsidically meta-linked galloyl groups (e.g. III and IV) could be present, as in the galloittannins investigated by Armitage et al. (1961). The molecular ratio (1:1 of di- to 1:0 of tri-methyl) of these two methylated acids indicated that the unfractionated tannin contained an average of 10.6 galloyl groups/glucose molecule. It was hoped to obtain further evidence of the extent of the depside linkages between gallic acid residues in the tannin by methanolysis under the conditions described by Armitage et al. (1961). However, this did not prove to be as successful as had been hoped, for after the tannin had been boiled in aqueous methanol at pH 6.0 under an atmosphere of nitrogen for 5 days a considerable amount of tannin was unchanged (see Table 2). When the methanolysis was interrupted after 2 days, methyl m-digallate was detected by paper chromatography (Table 2), indicating the presence of at least a trigalloyl chain (IV) in some of the galloittannin molecules.

The average composition of willow-herb tannin has been determined by three independent methods, leading to the following concordant values for the number of galloyl groups/glucose molecule: 10.4 (from $[α]_D$ of the tannin); 10.6 (from hydrolysis of methylated tannin); 10.6 (from analysis of fractions A and B). The tannin thus contains on average 10.5 galloyl units to each glucose molecule. However, the fractionation shows that molecules ranging at least from hepta- to trideca-galloyl-β-D-glucose, i.e. molecules in which a penta-O-galloyl-β-D-glucose core [(I), where $R^1 = R^2 = R^3 = R^4 = R^5 = \text{galloyl (II)}$] is depsidically attached to 2–8 other
Table 2. Chromatography of methanolysis products from willow-herb tannin

Experimental details are given in the text.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Substance</th>
<th>After 2 days</th>
<th>After 5 days</th>
<th>( R_f )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gallic acid</td>
<td>Intense</td>
<td>Intense</td>
<td>0.37</td>
</tr>
<tr>
<td>2</td>
<td>Methyl gallate</td>
<td>Intense</td>
<td>Intense</td>
<td>0.52</td>
</tr>
<tr>
<td>3</td>
<td>Methyl ( m )-digallate</td>
<td>Weak</td>
<td>Absent</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>Unknown</td>
<td>Weak</td>
<td>Weak</td>
<td>0.38</td>
</tr>
<tr>
<td>5</td>
<td>Pentagalloyl-( \beta )-d-glucose</td>
<td>Weak</td>
<td>Weak</td>
<td>approx. 0.00</td>
</tr>
<tr>
<td>6</td>
<td>Unknown</td>
<td>Weak</td>
<td>Weak</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>Tannin</td>
<td>Intense</td>
<td>Intense</td>
<td>0.00-0.65</td>
</tr>
</tbody>
</table>

The finding that leaves of willow-herb contain a hydrolysable gallotannin confirms the suggestion made by Handley (1954, 1961), who examined the leaf tannin–protein complexes of several species to determine the relative ease with which they are decomposed in biological systems. Handley (1954) found that the complex formed from gelatin and extracts from fresh leaves of common heather (Calluna vulgaris) was solubilized more slowly by fungi than the complex formed from gelatin and extracts from fresh leaves of willow-herb. Subsequently Handley (1961) showed that the nitrogen absorbed by birch seedlings grown in sand culture was greater when the willow-herb-tannin–casein complex was the source of nitrogen than when the source was the Calluna-tannin–casein complex. The establishment of willow-herb tannin as a gallotannin provides a possible explanation for this in chemical terms, since this tannin will be readily broken down by esterases of various types, in contrast with the condensed tannin of Calluna vulgaris, which contains no simple ester links susceptible to ready hydrolysis (Brown & Love, 1961). Decomposition of the tannin part of a tannin–protein complex might be expected to make enzyme-catalysed proteolysis easier.

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