PHYTOCHEMICAL CHANGES INITIATED BY INSECTS

PART I. PRELIMINARY WORK ON LEAVES AND "BEAN GALLS" OF *SALIX FRAGILIS* L.

BY STEPHEN B. CHALLEN

*From the Department of Pharmacognosy, School of Pharmacy, University of London, Brunswick Square, London, W.C.*1

Received May 1, 1959

The occurrence of leaf galls and the relation between the host plant and the causative insect are discussed. The leaves, galls, and leaves freed from galls of *Salix fragilis* have been examined by one-way and two-way paper chromatography and a separation of the major constituents achieved on powdered cellulose columns. The galls and leaves freed from galls were compared by band chromatography and the results suggest that one phytochemical change initiated by the sawfly is an accumulation of catechins, leucoanthocyanins and a ninhydrin positive substance.

PESTS of cultivated plants have attracted much work in recent years, but less attention has been given to the biological relation between plant and pest. Gall formation which is initiated by insects and mites presents an opportunity to investigate a relation of this kind, especially the chemical changes which may occur. Gall formation is common among higher plants. The galls arise only from meristematic tissues which have received an irritating stimulus and it is this which initiates abnormal growth[1]. An insect supplies the initial stimulus but the host carries the process forward. The commonest insect galls are produced by the *Cynipidae* (gall wasps) and *Tenthredinidae* (sawflies). Gall formation by gall wasps follows larval emergence while with sawflies the gall is already formed before larval emergence. The adult sawfly seems to supply the necessary stimulus during oviposition and the possibility of the stimulus being a chemical one cannot be ruled out[2].

In the Salicaceae, galls are common and frequently sawflies are responsible. The galls formed on leaves may involve inward rolling of the margin, deformity of the lower surface, or abnormal growth within the leaf blade which shows on both surfaces. This latter type of gall is found on *Salix fragilis* as well as other willow species[3], it is known as the "bean gall" and is caused by *Pontania proxima.*

To study the chemical changes initiated by insects in leaves, *Salix fragilis* was first chosen. Information on the constituents of willow leaf galls is scanty. The colouring matters of various insect galls including those of *Salix sp.* have been studied and the pigments eriophyesin and pontanin have been isolated[4]. These are reputed to be glucosides of purpurogallin but as they were isolated from air dried galls they could be artefacts[5]. A hypothetical scheme[6] for which there appears to be no real biochemical evidence has been put forward for the formation of purpurogallin. A comprehensive investigation of the normal leaf

constituents of commonly occurring British willows has not been made although some work on specific glycosides, especially in barks, has been published[7–13]. The presence of leucoanthocyanins has been recorded in the leaves of two species of *Salix*[14]. The constituents of *S. fragilis* leaves have been reported[15] as gallic acid, catechin and quercetin.

Experimental Methods

Material was collected from trees growing near Leatherhead, Surrey, in September. Three types of samples, leaves, galls, and leaves freed from galls were prepared for chemical analysis by two methods. Half of each type of sample was dried at 90°, then powdered. The other half was treated as follows. The material was chopped fine and macerated in 70 per cent ethanol for 2 weeks. The tissue was then filtered, lightly pressed and washed with small quantities of the solvent. The mixed filtrates were finally adjusted to a standard volume with further solvent. Extracts of leaves and of leaves freed from galls contained 20 leaves per 100 ml. and extracts of galls, 40 galls per 100 ml.

Chromatography of Dried Material

Using the method recommended for leucoanthocyanins[16], but with suitable quantities of reagents, powdered leaf, galls, and leaf freed from gall were separately heated with 2 N hydrochloric acid at 100° for 20 minutes and filtered while hot. The filtrate of each was gently shaken with *n*-amyl alcohol and drops of the alcoholic extract applied to the starting line of a paper chromatogram until a distinct pink to brown spot was produced after drying. The ascending technique was used with Whatman No. 1 paper and a single phase mixture (Forestal solvent)[17], water : acetic acid : hydrochloric acid, 10 : 30 : 3, which was run for 20 hours. Chromatograms were examined under ultra-violet light. Chromatographic comparison was made with pure samples of quercetin, myricetin and caffeic acid, while cornflowers were used as a reference source of pelargonidin and cyanidin.

A separation of the major constituents was tried using a glass tube ($\frac{3}{4}$ in. × 10 in.) packed with dry powdered cellulose. Samples of powdered leaf, gall, and leaf freed from gall 5 g., were separately boiled for 30 minutes with 100 ml. of distilled water, filtered and the extraction repeated with a further 100 ml. of the solvent. The mixed filtrates of each were cooled and treated with 20 per cent w/v lead acetate solution until no further precipitation occurred. The lead complex was filtered off and suspended in 100 ml. of absolute ethanol and the lead precipitated by hydrogen sulphide. The lead sulphide was removed by filtration and the ethanol removed from the filtrate on a boiling water bath, the residue taken up in 2 ml. distilled water and applied to the top of the cellulose column. This was developed with distilled water, without reduced pressure, until the faster moving components reached the bottom of the column. It was then extruded and the bands distinguished by ultra-violet light. Tests were made for the major constituents using suitable reagents[18].

Chromatography of Extracts

Extracts of leaf, gall, and leaf freed from gall were examined by two-way chromatography with Whatman No. 1 paper and the ascending technique. Using a micrometer pipette, 0·08 ml. of extract was applied near one corner of the chromatogram, dried and the alcoholic phase of the mixture, *n*-butanol: acetic acid: water, 4:1:5 run for 20 hours in one direction. After drying the chromatogram in a current of hot air the second solvent, distilled water, was run for 3 to 4 hours at right angles.

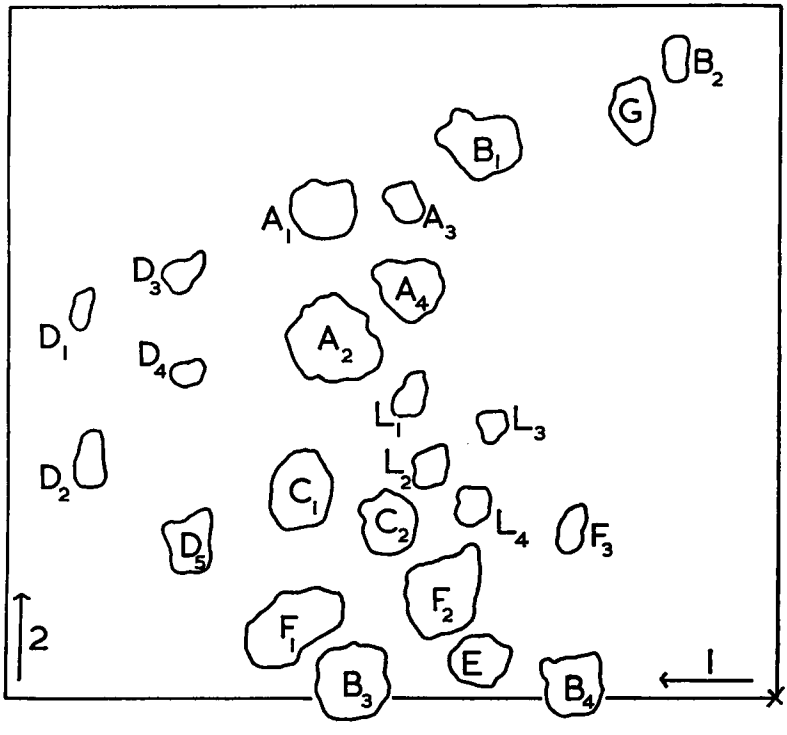

FIG. 1. Two-way chromatogram showing the separation of constituents present in extracts of leaves, galls, and leaves freed from galls. (see Table I for key to spots).

1 = *n*-butanol:acetic acid:water, 4:1:5.

2 = distilled water.

Chromatograms were dried and examined in visible light, then in ultra-violet light before and after exposure of the papers to the vapour of ammonia. Replicate chromatograms were done and spots revealed by spraying the papers with suitable chemical reagents[19,20].

Band chromatography on paper sheets was tried with the object of separating constituents of the extracts of gall, and leaf freed from gall and also to enable semi-quantitative estimations to be made. For both extracts 1 ml. was applied to the length of a starting line measuring 12 in. on a sheet of Whatman 3 MM paper and after drying, a further

TABLE I

EXTRACTS OF LEAVES, GALLS, AND LEAVES FREED FROM GALLS, COLOUR TESTS APPLIED TO TWO-WAY CHROMATOGRAMS

Key to spots in Fig. 1	Visible light	Ultra-violet light	NH_3/Ultra-violet light	*a* $NaNO_2$ then NaOH	*b* D.S.A.	*c* Vanillin	*d* Ferric alum/NH_3	*e* Ferric-ferricyanide	Remarks
A_1, A_2 A_3, A_4	None	Blue	Green	Pink	Orange	None	Green to black	Blue	Chlorogenic acids
B_1, B_2 B_3, B_4	None	Violet	Intense violet	None	None	None	None	None	Non-phenolic
C_1, C_2	None	None	None	None	Yellow	Red	Green to blue	Blue	Catechins
D_1, D_2 D_3, D_4 D^5	None	Blue	Blue/green	None	None	None	None	Blue	Minor phenolic constituents
E*	Red	—	—	—	—	—	—	—	Anthocyanin
F_1, F_2	Yellow	Brown	Yellow	Yellow	Orange	None	Brown to yellow	Blue	Flavonoid
F_3	None	None	Yellow	Yellow	None	None	None	None	Glycosides
L_1, L_2 L_3, L_4	None	None	None	None	Yellow	Red	Green	Blue	Leucocyanidins

* Pigment E detectable in gall only. (See footnote on page *228 T.*)

NOTES:—

a = $NaNO_2$ 1 per cent w/v in 10 per cent acetic acid followed by N NaOH.
b = diazotised sulphanilic acid reagent B.P.
c = equal volumes of hydrochloric acid B.P. and a mixture of vanillin 2 per cent w/v + acetyaldehyde 1 per cent v/v in 95 per cent ethanol.
d = ferric alum 0·2 per cent w/v in water, followed by ammonia vapour.
e = ferric chloride 0·3 per cent w/v in water and potassium ferricyanide 0·3 per cent w/v in water, equal volumes freshly mixed.

1 ml. was similarly applied. The solvent, distilled water, was run for 5 to 6 hours, the paper dried and the positions of the bands revealed by ultra-violet light. Each band was cut out, eluted with 10 ml. of cold absolute ethanol and the solvent removed on a boiling water bath. The fractions so obtained were analysed by two-way chromatography to check the components of each band. Replicate chromatograms were sprayed using the vanillin and ninhydrin reagents.

RESULTS

Dried Material

With leaf, gall, and leaf freed from gall one-way chromatograms showed similar results. There were two spots which were bluish under ultra-violet light, one of which coincided with caffeic acid. Two further spots were bright yellow, one of which coincided with quercetin. Another spot, which was red in visible light coincided with cyanidin. The cellulose columns produced a separation of the constituents into three distinct bands. The first band was of constituents which are non-mobile in water and showed a deep violet fluorescence. The second band gave a velvety brown fluorescence and an aqueous extract of it produced a red colour with vanillin reagent, a red colour when boiled with 2 N hydrochloric acid and a crimson colour with the magnesium:hydrochloric acid test. The third and largest band gave a strong blue fluorescence and an aqueous extract of it produced a green colour with ferric ammonium sulphate reagent.

TABLE II

BAND CHROMATOGRAPHY ON PAPER SHEETS OF EXTRACTS OF GALLS, AND LEAVES FREED FROM GALLS

Bands from top of sheet	Run in distilled water 5 to 6 hr.	Run in alcoholic phase of *n*-butanol/acetic acid/water, 4:1:5, 20 hr.
	Band components	
1	B_1, B_2, G	D_1, D_2
2	A_1 to A_4	D_3, D_4, D_5
3	Narrow bands	F_1
4	Minor	A_1, A_2, B_2
5	Constituents	A_3, A_4, F_2
6	D_5 and vanillin positive constituents	B_3
7	F_2	B_4, F_3
8	F_1	G
9	B_3, B_4	B_2

Extracts

With leaf, gall, and leaf freed from gall two-way chromatograms showed similar results except that substances C_1 and C_2, L_1 to L_4 and G (see Fig. 1) were clearly visible only on chromatograms prepared from gall extracts, which alone showed the presence of the red pigment E. The results of colour tests applied to chromatograms are shown in Table I and Figure 1. Additional colour tests were applied to chromatograms and the results obtained were as follows. After spraying with 0·1 per cent

w/v ninhydrin in 10 per cent acetic acid and heating for a few minutes in a current of hot air, the spot G having a violet colour was revealed. This substance has an R_F value of 0·7 (approx.) in phenol saturated with water and does not coincide with either aspartic acid or glutamic acid. Red spots were not produced when chromatograms were sprayed with 2 N sulphuric acid, and then heated in a current of hot air and pink spots were not produced when potassium cyanide 1 per cent w/v in water was used as a spray reagent. Band chromatograms of gall extracts showed wider and more strongly coloured vanillin and ninhydrin positive bands than corresponding chromatograms of extracts prepared from leaf freed from gall. Except for these differences chromatograms were similar and the components of each band are given in Table II. Nine bands were produced but the faster moving bands 1 and 2 were wavy in outline owing to interfering substances. The vanillin positive constituents formed a single band (6) which also contained substance D_5. A different solvent, the alcoholic phase of the mixture *n*-butanol : acetic acid : water, 4 : 1 : 5 can be used as running solvent for 20 hours. This gives uniform separation of the constituents again into 9 bands distinguishable by ultra-violet light. The vanillin positive constituents C_1 and C_2 overlapped bands 3 and 4 and the other vanillin positive constituents overlapped bands 6 to 8 and gave a red colour when boiled with 2 N hydrochloric acid.

Discussion

Dried Material

For leaf, gall, and leaf freed from gall, the results of one-way chromatography and of tests on fractions from cellulose columns indicate that two types of flavonols are present, one of which is based on quercetin. Leucoanthocyanins are present and are probably based on cyanidin and these constituents account in part for the red colour obtained by the vanillin reaction. No further conclusions could be made from this preliminary work and hence the necessity for more detailed chromatographic analysis.

Extracts

From the results of colour tests which were applied to two-way chromatograms and the pattern of spots which were revealed it is suggested that there are no significant qualitative chemical differences between the extracts of leaf, gall, and leaf freed from gall. It is probable that the group of substances A_1 to A_4 are chlorogenic acids; that the pair of vanillin positive constituents C_1 and C_4 are (+)-catechin and (−)-epicatechin respectively and the other group of vanillin positive constituents are leucocyanidins. The substances F_1 and F_2 are flavonoid glycosides but no further conclusions can be made about their identity except that they are based on different aglycones. The red gall pigment E* is possibly an anthocyanin but it should be isolated from fresh galls rather than dried material and separated from leucoanthocyanins to make precise identification possible. The absence of gallic acid and gallocatechins, as

* This pigment has now been shown to be present in young normal leaves.

suggested by the negative potassium cyanide test, does not lend support to Nierenstein's scheme[6] for the chemical relation between the plant and the sawfly, as gallic acid features as an intermediate in this scheme. As the material was obtained from trees in September, failure to detect salicin and saligenin by the sulphuric acid test cannot be taken as proof that these are absent at earlier stages in growth. The results of band chromatography indicate that the method effects a semi-quantitative separation. As the bands of vanillin positive constituents and the ninhydrin positive substance G are wider and more distinct on chromatograms of gall extracts then corresponding chromatograms of extracts prepared from leaf freed from gall, it is assumed that a greater accumulation of catechins, leucoanthocyanins and the ninhydrin positive substance G occurs in the gall than in the leaf.

Acknowledgement.—I wish to acknowledge the advice of Dr. E. A. H. Roberts (Indian Tea Association Research Laboratory) and the receipt from him of three marker substances.

REFERENCES

1. Swanton, *British Plant Galls*, Methuen, London, 1921.
2. Imms, *Insect Natural History*, 2nd Edn., Collins, London, 1956, p. 170.
3. Carleton, *J. Linn. Soc.* (*Zoo*), 1939, **40**, 575.
4. Nierenstein and Swanton, *Biochem. J.*, 1944, **38**, 373.
5. Paech and Tracey, *Moderne Methoden der Pflanzenanalyse*, Springer-Verlag, Berlin, 1955, **3**, 352.
6. Nierenstein, *The Natural Organic Tannins*, Churchill, London, 1934, p. 127.
7. Rabate, *Bull. Soc. Chim. biol.*, 1935, **17**, 439.
8. Fujikawa and Nakajima, *J. Pharm. Soc. Japan*, 1948, **68**, 175.
9. Bridel and Beguin, *Bull. Soc. Chim. biol.*, 1926, **8**, 901.
10. Wattiez, *ibid.*, 1931, **13**, 658.
11. Sakai, Tpsurumi, Eno and Inukai, *Bull. Inst. Phys. Chem. Res.* (*Tokio*), 1943, **22**, 868.
12. Zemplen, Bognar and Fzekeoy, *Ber.*, 1943, **76B**, 386.
13. Kariyone, *J. Pharm. Soc. Japan*, 1942, **62**, 514.
14. Bate-Smith and Metcalfe, *J. Linn. Soc* (*Bot*), 1957, **55**, 669.
15. Wehmer, *Die Pflanzenstoffe*, 2nd Edn., Jena, 1929, **1**, 200.
16. Bate-Smith, *Biochem. J.*, 1954, **58**, 122.
17. Harbourne, *J. Chromatog.*, 1958, **1**, 473.
18. Paech and Tracey, *Moderne Methoden der Pflanzenanalyse*, Springer-Verlag, Berlin, 1955, **3**, 471.
19. Roberts and Wood, *Biochem. J.*, 1951, **49**, 414.
20. Hillis and Swain, *J. Sci. F. & Agric.*, 1959, **10**, 135.

After Dr. Challen presented the paper there was a DISCUSSION.