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Minor Constituents of Quebracho Tannin Extract

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Previous papers in this series (White, 1949; Kirby, Knowles & White, 1951; White, Kirby & Knowles, 1952) have shown that each so-called 'vegetable tannin' is a complex mixture of many substances—far more so than has been generally realized. The two most important tannin extracts, 'Mimosa' or 'Wattle' extract from the bark of *Acacia mollissima* and 'Quebracho' extract from the heartwood of *Schinopsis Lorentzii* Engl., were selected for detailed study of this complexity. Two-dimensional paper chromatography enabled characterization of the numerous substances of unknown nature present in each extract and provided, for the first time, a method of assessing the efficiency of fractionation procedures applied to them. Using this approach each extract was split into fractions by solvent extraction or by countercurrent distribution between water and organic solvents (Kirby, Knowles & White, 1952, 1953). The components present in any fraction and the degree of component overlapping from one fraction to the next were determined by two-way paper chromatography, the presence of the components being detected by their fluorescence in ultraviolet light or by reaction with appropriate spray reagents. In the specific case of Quebracho tannin extract the material could be

separated readily into six fractions (Q_{111}, Q_{112}, Q_{121}, Q_{122}, Q_{21}, Q_{22}) and the further investigation of fraction Q_{111} forms part of this communication.

A particular feature of Quebracho tannin extract is its content of brilliant yellow-fluorescing material. This was formerly ascribed to the presence of fisetin, and it was suggested that Quebracho tannin extract was composed largely of a tannin formed by condensation of a hypothetical 'quebracho-catechin' related structurally to fisetin in a manner similar to the catechin–quercetin relationship (Freudenberg & Maitland, 1934). Earlier in this series it was shown that in fact a number of yellow-fluorescing substances, all presumably flavonoid, were present in Quebracho extract and that the tanning capacity of the extract is due not to one but to many polyhydric phenols. The nature of these polyphenolic tannins is under investigation and the greater proportion of them seem to have molecular weights of the order of 800–1000 (Kirby & White, 1954). The yellow-fluorescing substances are simpler, and it was considered advisable to concentrate initially on determining the nature of these, and any other low molecular weight minor components of the extract, in view of the possibility that this might indicate the type and range of structures likely to be present in the more complicated polyhydric phenols which form the actual tannins. The yellow-fluorescing components are most highly concentrated in fraction Q_{111} but are present to some extent in all six main fractions since the scheme of fractionation was designed to separate the tannins present rather than the minor substances. This present communication therefore has to consider substances in relation to the extract as a whole as well as those present in the particular fraction Q_{111}.

EXPERIMENTAL

General. All evaporations were carried out under reduced pressure unless otherwise stated. Melting points are uncorrected. C and H and —C.CH_3 determinations are all by Messrs Weiler and Strauss, Oxford.

Fluorescent spots on chromatograms were detected by using a Hanovia Hg arc with a Wood's glass filter.

Hydrolysis of whole Quebracho extract

Quebracho extract (700 g.) was mixed with ethanol (2 l.) and 10% (w/v) HCl (2 l.), allowed to stand overnight and then refluxed on a water bath for 8 hr. Next day, about 1 l. of ethanol was distilled off, water (750 ml.) added and the mixture extracted 10 times by shaking with an ether–ethyl acetate (9:1, by vol.) mixture (1 l. each time). The extract was dehydrated with Na_2SO_4 and taken to dryness to yield 24 g. of a dark brown resin. This was dissolved in ethyl acetate (150 ml.), ether (200 ml.) added and a black tar filtered off. The insoluble matter was washed with ethyl acetate–ether (1:2, by vol.) and ether (150 ml.) added to the filtrate. The dark precipitate which formed was again filtered off and washed with ethyl acetate–ether (4:9, by vol.). The filtrate was then extracted as follows to give four fractions: 1, 10 × 100 ml. of 5% (w/v) $NaHCO_3$; 2, 6 × 100 ml. of 4% (w/v) $Na_4B_4O_7$; 3, 4 × 100 ml. of 4% (w/v) $Na_4B_4O_7$; 4, unextracted residue.

Fraction 1. The $NaHCO_3$ extract was immediately acidified to Congo Red paper and extracted thoroughly with ether. This extract was dehydrated with Na_2SO_4 and taken to dryness to give fraction 1*a*, while the aqueous layer was thoroughly extracted with ethyl acetate, the extract dehydrated with Na_2SO_4 and taken to dryness to give fraction 1*b*.

Fraction 1*a* was dissolved in 5 ml. acetic acid and poured on to a column of cellulose powder (Whatman's Ashless) prepared in 5% (w/v) aqueous acetic acid. The column was developed with this solvent and each sub-fraction collected was chromatographed on Whatman no. 2 paper with 6·7% (w/v) aqueous acetic acid as solvent. The papers were sprayed with bis-diazotized benzidine, which showed up the presence of three substances of R_F values 0·66, 0·57 and 0·43.

Sub-fraction 1*a* (1) was taken to dryness, yielding 0·24 g. of material containing primarily the substance of R_F 0·66. As yet it remains unidentified.

Sub-fractions 1*a* (2–5) were bulked and concentrated, whereupon 0·63 g. of pure gallic acid crystallized out, representing the substance of R_F 0·43. The remaining substance of R_F 0·57 was identical with the ethyl gallate of fraction 2*b* which is mentioned later. Fraction 1*b* was chromatographed similarly with 5% (w/v) aqueous acetic acid on a cellulose powder column but only gallic acid was

Table 1. *Chromatographic separation of fraction* 1*a on cellulose powder*

The R_F values were determined on Whatman no. 2 paper using 6·7% (w/v) acetic acid as solvent. Figures in brackets denote that a faint spot was detected.

Sub-fraction	Eluting solvent (%, w/v)	Volume of eluent (ml.)	R_F		
1*a*/1	5 Acetic acid	25	0·66	(0·57)	(0·43)
1*a*/2	5 Acetic acid	25	0·66	0·57	0·43
1*a*/3	5 Acetic acid	25	0·66	0·57	0·43
1*a*/4	5 Acetic acid	50	0·66	(0·57)	0·43
1*a*/5	5 Acetic acid	50	(0·66)	(0·57)	(0·43)
1*a*/6	5 Acetic acid	50	—	—	—
1*a*/7	5 Acetic acid	50	—	—	—
1*a*/8	20 Acetic acid	10	0·05	—	—
1*a*/9	20 Acetic acid	100	0·05	—	—
1*a*/10	Methanol	100	—	—	—

present, 0·27 g. being recovered in crystalline form on concentrating the eluates.

Fraction 2. This was acidified with 10% (w/v) HCl and ethyl acetate added (200 ml.). This threw down 0·58 g. of a yellow precipitate forming fraction 2*c* and identified as fisetin. After crystallizing from *iso*propanol only a single fluorescent spot in the position of fisetin was observed on two-way paper chromatography using *tert.*-butanol–acetic acid–water (14:25:61, by vol.) as the first-way solvent, and *sec.*-butanol–acetic acid–water (70:5:25, by vol.) as the second-way solvent. The substance also had the characteristic ultraviolet spectra of fisetin both in ethanol and in aqueous alkaline solution.

Table 2. *Ultraviolet absorption spectra of fisetin and fraction 2c*

	Synthetic fisetin		Fraction 2*c*	
Solvent	$\lambda_{max.}$	log $E^{1\%}_{1cm.}$	$\lambda_{max.}$	log $E^{1\%}_{1cm.}$
Ethanol	254	2·76	252	2·70
	316	2·62	318	2·66
	365	2·94	365	2·97
0·013 N-NaOH	250	2·70	250	2·72
	342	3·03	340	3·00

The ethyl acetate was separated off and the aqueous layer further extracted with ethyl acetate until nothing more was being removed. The combined ethyl acetate extracts were dehydrated with Na_2SO_4, concentrated to 100 ml. and poured into 1 l. light petroleum (60–80°). The precipitate forming fraction 2*a* was filtered off, washed with light petroleum (60–80°) dried and weighed (7·5 g.). It was chromatographed two-dimensionally on Whatman no. 2 paper in the same way as fraction 2*c* and was found to contain three yellow-fluorescing components 3:7:4′-trihydroxyflavone, 3:7:3′:4′-tetrahydroxyflavone (fisetin), and 3:7:3′:4′:5′-pentahydroxyflavone (robinetin), together with a number of non-fluorescent polyphenolic substances which remain unidentified but which can be separated by chromatography on powdered silica. The identity of the three flavones was established by the fact that they had the same R_F values as synthetic specimens when chromatographed two-dimensionally on paper using 10% (w/v) acetic acid followed by *n*-butanol–acetic acid–water (4:5:1, by vol.) as one solvent-pair and *tert.*-butanol–acetic acid–water (14:25:61, by vol.), followed by *sec.*-butanol–acetic acid–water (70:5:25, by vol.) as an alternative solvent pair. Further evidence for the identity of the trihydroxyflavone came from chromatographing with water-saturated *n*-butanol on paper buffered with 0·1 M borate and 0·1 M-Na_2HPO_4 and on unbuffered papers (Swain, 1953). Since the R_F values were identical on all three papers, the substance clearly cannot have two hydroxyl groups situated on adjacent carbon atoms.

Fraction 2*b* weighed 1·0 g. and was obtained by taking the filtrate from the precipitates of 2*a* to dryness. It was observed by paper chromatography to contain a substance reacting yellow with bis-diazotized benzidine together with a smaller amount of a substance which stained purple with the same reagent. The fraction was dissolved in 5% (w/v) aqueous acetic acid and chromatographed on a column of Whatman's 'Ashless Cellulose Powder' (1 cm. × 15 cm.). It was eluted with 5% (w/v) aqueous acetic acid and collected in 10 ml. fractions tested with bis-diazotized benzidine. The yellow-reacting substance present was eluted in 40 ml. of solvent which was taken to dryness. The product was dissolved in a little ethyl ether, and crystallized after filtration by adding light petroleum (40–60°). It was recrystallized from 50 ml. of toluene and separated as colourless needles, yield 0·1 g. It had m.p. 157–8° and gave a crystalline acetate m.p. 136–7° when acetylated with pyridine and acetic anhydride. It was identified as ethyl gallate, m.p. 157–8°, and no depression was observed in mixed melts of the original substance with ethyl gallate or of the acetate with triacetyl ethyl gallate, m.p. 136–7°. Both ethyl gallate and the isolated material travelled to the same position on two-way chromatography on Whatman no. 2 paper with 6% acetic acid followed by *sec.*-butanol–acetic acid–water (14:1:5). The two substances had identical u.v. absorption spectra in alcohol and in 5% aqueous NaOH solution, the alkali shift being a general lowering of absorption instead of the combined shift and increase of the maximum normally given by phenols. The substance is an artifact, owing to the use of 50% ethanol during the hydrolysis of the tannin extract, but the occurrence of more than 10% of the liberated gallic acid in this form under these conditions of hydrolysis is surprising (Found: C, 56·4; H, 5·0; O.CH_2.CH_3, 24·6. $C_9H_{10}O_5$ requires C, 54·5; H, 5·0; O.CH_2.CH_3, 22·7%.)

Fraction 3. This remains to be investigated.

Fraction 4. The final organic layer was dehydrated with Na_2SO_4, filtered, and taken to dryness, 15 ml. of ether being added to the residue. A dark brown solid separated overnight and was filtered off (35 mg.), dissolved in *iso*propanol, treated with charcoal, filtered and then concentrated to 6 ml. Pale yellow needles separated (27 mg.) and were recrystallized from *iso*propanol. This 4′-methoxyfisetin had m.p. 288° (decomp.). (Found: OMe, 9·35. $C_{16}H_{12}O_6$ requires OMe, 10·3%.) It showed only one fluorescent spot when chromatographed two-dimensionally on paper as for subfraction 2*a*. Similar chromatography after demethylation with HI showed the presence of fisetin as well as traces of the original substance.

Synthesis of 4′-methoxyfisetin

(*a*) *Benzoyl* iso*vanillic acid.* Benzoyl *iso*vanillin (75 g.) was dissolved in acetic acid (1 l.) and CrO_3 (20 g.) in water (15 ml.) and acetic acid (250 ml.) added gradually with cooling to maintain a temperature of 20°. Some crystals separated at the end of the addition and the mixture was kept in a refrigerator overnight before filtering off the white needles. They were washed with acetic acid, then water, dried, and boiled with benzene to remove unchanged aldehyde. The acid was then recrystallized from *iso*propanol and had m.p. 178°.

(*b*) *Benzoyl* iso*vanillic anhydride.* Benzoyl *iso*vanillic acid (25 g.) was mixed with $SOCl_2$ (25 ml.) and a few drops of pyridine and refluxed 1 hr. After the distilling off of excess $SOCl_2$ the residue crystallized. It was dissolved in benzene, taken to dryness, then recrystallized from benzene. Benzoyl *iso*vanillic acid (16 g.) was mixed with this acid chloride (17 g.) in ether (250 ml.) and pyridine (50 ml.) added. The mixture was kept overnight in the refrigerator and after the addition of ice the precipitate was filtered off, washed with water, 10% (w/v) HCl, 10% (w/v) Na_2CO_3 and again with water (all at 0°) and dried *in vacuo* over P_2O_5. The anhydride crystallized from benzene with m.p. 130°.

(*c*) *4′-Methoxyfisetin.* ω-Benzoyloxyresacetophenone (2·1 g.), benzoyl *iso*vanillic anhydride (16·8 g.) and triethylamine (5·35 ml.) were heated together for 2 hr. at 155–160° and then 2 hr. at 160–170°. The product was boiled with ethanol (150 ml.) in which it was not completely soluble, and then KOH (20 g.) in water (30 ml.) was added and the mixture boiled for 1 hr. The ethanol was removed under reduced pressure, water added (100 ml.) and the solution decanted off from a dark resin. On saturating the solution with CO_2 the flavone separated. It was filtered off, boiled with ethanol (250 ml.) and the extract filtered and concentrated to 30–40 ml., when a brown precipitate formed and was filtered off. The filtrate was concentrated to 15 ml., and on cooling *4′-methoxyfisetin* crystallized as pale yellow needles. It was recrystallized twice from *iso*propanol and had m.p. 288° (decomp.). (Found: C, 63·8; H, 3·8. $C_{16}H_{12}O_6$ requires C, 64·0; H, 4·0%.) This product had the same chrome-yellow fluorescence and R_F value on two-way paper chromatography as the substance from fraction 4 of hydrolysed Quebracho extract, and the natural and synthetic compounds had identical ultraviolet spectra in ethanol.

Synthesis of 3:7:4′-trihydroxyflavone. 3:7:4′-Trihydroxyflavone was synthesized from ω-methoxyresacetophenone, anisic anhydride and potassium anisate by the general method of flavone synthesis of Allan & Robinson (1924).

Synthesis of 3:7:3′:4′:5′-pentahydroxyflavone (robinetin). Robinetin was synthesized by the method of Charlesworth & Robinson (1933) by condensing ω-methoxyresacetophenone with trimethyl gallic anhydride and sodium trimethyl gallate, followed by hydrolysis and demethylation.

Hydrolysis of Quebracho extract fractions

Each fraction (Q_{111}, Q_{112}, Q_{121}, Q_{122}, Q_{21}, Q_{22}) was dissolved (0·1 g.) in 2-methoxyethanol (0·5 ml.) and 10% (w/v) HCl (0·5 ml.) and heated in a sealed tube on a boiling-water bath for 3 hr. The resultant solutions were all dark red and were each spotted directly on to Whatman no. 2 paper for two-way paper chromatography. The first-way solvent was *tert.*-butanol–acetic acid–water (14:25:61, by vol.) and the second-way solvent *sec.*-butanol–acetic acid–water (70:5:25, by vol.). The only yellow fluorescent spots found after these hydrolyses were 3:7:4′-trihydroxyflavone, 4′-methoxyfisetin, fisetin and robinetin, the latter in Q_{111}, Q_{21} and Q_{22} only.

The papers were also sprayed with aniline hydrogen phthalate to detect sugars, dried, and then dipped in ferric chloride–potassium ferricyanide reagent to detect phenolic substances. Gallic acid and glucose were observed in all six cases, although the former was absent from Q_{112}, Q_{121}, and Q_{122} before hydrolysis, while glucose has not been observed in Q_{122} and Q_{22} before hydrolysis. Fisetin appeared in Q_{112}, Q_{121}, Q_{122} and Q_{22} from which fractions it was practically absent before hydrolysis, and so did 3:7:4′-trihydroxyflavone and 4′-methoxyfisetin. The hydrolysis produced robinetin in Q_{22}, where it was previously absent.

Separation of Quebracho extract sugars from Q_{111}

Kirby & White (1954) described the further sub-fractionation of the main Quebracho extract fraction Q_{111} by counter-current distribution between ethyl methyl ketone and water. A sample (0·45 g.) of the material isolated from the final tube of this distribution (i.e. the water-soluble end), was dissolved in water (1 ml.) and placed on a column of Whatman's 'Ashless Cellulose Powder' (1·5 in. × 17 in.) which had been well washed with water-saturated *n*-butanol containing a trace of ammonia. The same solvent was used for elution, 15 ml. fractions being collected. Each fraction was examined by single way chromatography on Whatman no. 1 paper, all three of the solvent systems *n*-butanol–ethanol–water (40:11:19, by vol.), water-saturated phenol; and *tert.*-butanol–ethyl methyl ketone–water (20:60:15, by vol.) being separately used. The sugars were detected using aniline hydrogen phthalate as the spray reagent.

Separation of ribose and fucose. The combined fractions 15–25 were taken to dryness, dissolved in the minimum of water and placed on a column of cellulose powder (2·5 cm. × 30 cm.) that had been well washed with water-saturated ethyl methyl ketone. The same solvent was used for elution, 5 ml. fractions being collected.

Table 3. *Chromatographic separation of sugars of fraction* Q_{111} *by elution from cellulose powder with water-saturated* n*-butanol containing ammonia*

Sub-fraction no.	Sugar present	Yield (g.)	$[\alpha]_D$ (°)
1– 5	Hydroxymethylfurfural	0·02	—
6–14	Rhamnose	0·08	+13·7
15–17	Rhamnose, ribose	0·009	—
18–20	Ribose, fucose	0·015	—
21–25	Ribose, fucose, xylose	0·024	—
26–30	Xylose	0·030	+ 9·6
31–32	Xylose, arabinose	0·014	—
33–49	Arabinose	0·102	+74
50–57	Arabinose, glucose	0·043	—
58–67	Glucose, galactose (faint)	0·037	—
68–84	Glucose, galactose		

Table 4. *Chromatographic separation of ribose and fucose by elution from cellulose powder with water-saturated ethyl methyl ketone*

Conditions as in text.

Fraction no.	Sugar present	Yield (mg.)	$[\alpha]_D$ (°)
51– 63	Rhamnose	—	—
64– 91	Ribose	5	—
92– 99	Ribose + fucose	—	—
100–146	Fucose	27	−40·7
147–	Xylose	—	—

Table 5. *Chromatographic separation of sugars of fraction* Q_{111} *by elution from cellulose powder with water–ethyl methyl ketone–*tert.*-butanol* (15:60:25, *by vol.*)

Fraction no.	Sugars present
9–10	Rhamnose, hydroxymethylfurfural
11–12	Rhamnose
13–15	Rhamnose, ribose, fucose
16–17	Ribose, fucose, xylose
18–22	Ribose, xylose, arabinose
23–29	Xylose, arabinose
30–31	Arabinose
32–37	Arabinose, glucose
38–59	Glucose
60–	Galactose (600 ml. collected)

Separation of glucose and galactose. The separation detailed in Table 3 was repeated using water–ethyl methyl ketone–*tert.*-butanol (15:60:25, by vol.) mixture as the eluting solvent instead of water-saturated *n*-butanol containing ammonia. Successive fractions (25 ml.) were examined as before, glucose and galactose being separated by this solvent.

Although the fractions on which the optical rotations were determined were not completely pure the indications are that L-rhamnose (+10°), D-xylose (+19°), L-arabinose (+74°) and L-fucose (−76·4°) are present in Quebracho extract.

p-*Toluenesulphonhydrazones of glucose, rhamnose, xylose, arabinose, fucose, and ribose.* The derivatives of the above isolated sugars were made as described by Easterly, Hough & Jones (1951) except that a little ether was added to induce crystallization of the rhamnose and xylose derivatives, and a little water to affect solution of the glucose. The hydrazones had the following m.p.'s: glucose, 179°; ribose, 164°; fucose, 169°; arabinose, 153°; xylose, 149°. Authentic derivatives for comparison were made in the same way except that glucose (1 g.) was dissolved in 1 ml. water and the *p*-toluenesulphonhydrazine (2·5 g. in 9 ml. ethanol) added to the solution. The derivative crystallized quickly. No crystalline derivative was obtained from the galactose fractions. *Rhamnose* p-*toluenesulphonhydrazone* has apparently not been described previously. It has m.p. 137° (decomp.).

Isolation of anthraquinone from Q_{111}

A sample of Quebracho fraction Q_{111} (15 g.) was dissolved in acetone (15 ml.) and ether (45 ml.) added. The solution was poured on to a column of chromatographic silica gel (200 g.: L. Light and Co., 100–200 mesh) suspended in acetone–ethyl ether (1:9, by vol.). The column was eluted exhaustively with the same solvent (1:9) and the eluate concentrated under reduced pressure. During concentration 28 mg. of crystalline material separated out. This was filtered off, dried, sublimed twice (180–220°/0·1 mm.) to give 18·2 mg. pale yellow needles, m.p. 272–274°. These were recrystallized from ethanol and still had m.p. 272–274°.

The product gave a positive oxanthrol test and no depression was observed in a mixed melting point determination with authentic anthraquinone. (Found: C, 80·1; H, 4·2. Calc. for $C_{14}H_8O_2$: C, 80·7; H, 3·9%.)

A portion of the material (10 mg.) was reductively acetylated with acetic anhydride, sodium acetate and Zn dust and the product crystallized from benzene had m.p. 260°, undepressed by admixture with an authentic specimen of dihydroanthraquinone diacetate. Both specimens had an unusually intense blue fluorescence in solution in organic solvents. A similar fluorescence can be produced by reductive acetylation of whole Quebracho extract. Further attempts to isolate anthraquinone from Q_{111} have been unsuccessful but the characteristic blue fluorescence of the diacetate is readily demonstrable by reductive acetylation of the fraction. Chromatography on paper with *n*-butanol–acetic acid–water (4:1:5, by vol.) followed by spraying with 20% (w/v) aqueous NaOH solution saturated with sodium dithionite gave a rapidly fading pink spot at R_F 0·95 in the position characteristic of authentic anthraquinone specimens.

RESULTS

Inter-relationship of fluorescent components

A reasonable knowledge of the nature and inter-relationships of the yellow-fluorescing components in Quebracho extract has now been obtained by hydrolysing the extract and its fractions with hydrochloric acid. Two-dimensional paper chromatography of the extract and its fractions before and after hydrolysis, using the solvents given in the Experimental section, demonstrates that the extract contains at least sixteen yellow fluorescent components. Their position on the reference chromatogram is shown in Fig. 1. Hydrolysis with acid causes all but four of these fluorescent spots to disappear and a list of the fluorescent substances present in the extract and its fractions both before and after hydrolysis is given in Table 6. Simultaneously with the disappearance of fluorescent spots 5–16, the intensity of fluorescence of spots 1–4 increases, or these substances appear after hydrolysis in fractions where they were previously absent. Similarly, glucose and gallic acid appear for the first time or increase in amount as a result of hydrolysis. These facts are demonstrated by first examining the chromatograms under ultraviolet light to detect the fluorescent spots—then spraying the papers with aniline hydrogen phthalate to locate carbohydrates, and finally dipping the papers in ferric chloride–ferricyanide reagent to detect phenolic substances (White *et al.* 1952).

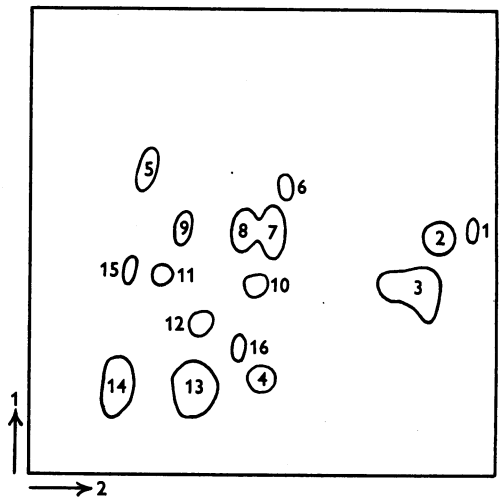


Fig. 1. Two-dimensional paper chromatogram of yellow-fluorescing components of Quebracho tannin extract. Direction 1: *tert.*-butanol–acetic acid–water (14:25:61, by vol.). Direction 2: *sec.*-butanol–acetic acid–water (70:5:25, by vol.).

The manner in which the fluorescent spots 1, 2, 3, 4, glucose and gallic acid appear after hydrolysis in Quebracho extract fractions from which they were previously absent is clear from Table 6. It is difficult to avoid the conclusion that spots 1, 2, 3 and 4 are the primary flavonoid fluorescent components of Quebracho extract and that substances 5–16 are derived from them by combination with sugars, gallic acid and possibly other undetected substances which are released on acid hydrolysis. This would be in accord with the many observations of the occurrence of flavonoid glycosides in other plants.

Nature of the primary fluorescent components

The fluorescent spot 3 is fisetin (II), first isolated from Quebracho extract by Perkin & Gunnell (1896). In view of the manner in which the hydroxyl group content of flavonoid substances governs their distance of travel on chromatographing on paper (Bate-Smith & Westall, 1950) it seemed reasonable from the observed R_F values (see Fig. 1) that fluorescent spot 1 might be 3:7:4′-trihydroxyflavone (I) and that fluorescent spot 4 might be robinetin (III). Both substances were synthesized and their identity with spots 1 and 4 established by two-dimensional paper chromatography as already described. There appears to have been no previous report of 3:7:4′-trihydroxyflavone as a natural product.

Spot 2 had a chrome-yellow fluorescence as opposed to the green-yellow shown by fisetin and robinetin. It was ultimately isolated in crystalline form (see below) and found to contain a methoxyl group. Two-dimensional chromatography on paper after demethylating with HI showed that demethylation formed fisetin and left traces of the original unchanged material. Since this original material had a chrome-yellow fluorescence (Kuhn & Low, 1944*a*) it seemed likely that it was 4′-methoxyfisetin (IV, see p. 588). This flavone does not appear to have been recorded previously and was synthesized by condensing benzoyl *iso*vanillic anhydride with ω-benzoyl-hydroxyresacetophenone in the presence of triethylamine and debenzoylating with alkali (Kuhn & Low, 1944*b*). This synthetic product had the same fluorescence and R_F value on paper chromatography as spot 2 and the same ultraviolet absorption spectrum and melting point as the isolated natural product. There is no doubt of the identity of the latter, which may, to some extent, account for the small methoxyl content of Quebracho extract.

The identification of 3:7:4′-trihydroxyflavone and robinetin rests on the chromatographic and synthetic evidence since both are present in Quebracho extract in much smaller quantity than fisetin and its 4′-methoxy derivative and they have not been

Table 6. *Glucose, gallic acid and yellow-fluorescent-component content of Quebracho extract fractions before and after acid hydrolysis*

Fraction	Before hydrolysis: Glucose	Before hydrolysis: Gallic acid	Before hydrolysis: Fluorescent components 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	After hydrolysis: Glucose	Gallic acid	Fluorescent components 1	2	3	4	5–16
Q_{111}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−
Q_{112}	+	−	−	−	±	−	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
Q_{121}	+	−	−	−	±	−	−	+	+	+	+	+	+	−	+	+	−	+	+	+	±	+	+	−	−
Q_{122}	−	−	−	−	±	−	−	+	+	+	+	+	+	−	+	+	−	−	+	+	±	+	+	−	−
Q_{21}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	±	−
Q_{22}	−	+	−	±	±	−	+	+	+	+	+	+	+	−	+	+	−	−	+	+	+	+	+	±	−

± Components very faint.

isolated in crystalline form so far although they have been highly concentrated in the following work.

In an attempt to isolate or concentrate these four primary fluorescent substances and any other recognizable simple components of the extract a large quantity of Quebracho extract (700 g.) was hydrolysed with ethanol–10 % (w/v) aqueous HCl mixture (1 : 1, by vol.). Details of the hydrolysis and of the subsequent procedure which resulted in the isolation of seven fractions (1*a*, 1*b*, 2*a*, 2*b*, 2*c*, 3 and 4) containing the acid-resistant low molecular weight components of Quebracho extract are given in the Experimental section.

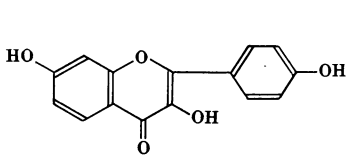
Fraction 1*a* yielded 0·63 g. of crystalline gallic acid and 0·24 g. of a phenolic substance which is so far unidentified. Paper chromatography also showed up the presence of the ethyl gallate of fraction 2*b*. Fraction 1*b* yielded only gallic acid (0·27 g.).

Fraction 3 remains for detailed investigation.

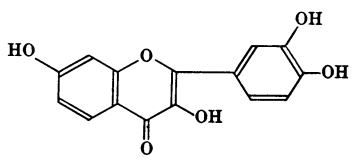
Fraction 4 yielded crystalline 4′-methoxyfisetin (35 mg.) as described in the Experimental section.

Sugars

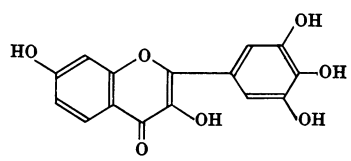
A detailed examination of the substances present in Quebracho fraction Q_{111} and particularly of the sugars has been commenced. The contents of the final tube in a 10-tube countercurrent distribution of Q_{111} between ethyl methyl ketone and 1 % (w/v) acetic acid (Kirby & White, 1954) were examined by chromatography on cellulose powder with water-saturated *n*-butanol as described in the Experimental section using the method of Hough, Jones & Wadman (1949) to follow the separation of the sugars. Rhamnose, ribose, fucose, xylose, arabinose, glucose, galactose and a component since identified as 5-hydroxymethylfurfural (H. C. G. King, personal communication) were found to be present. Rhamnose, xylose and arabinose were completely separated by this method but the separations of ribose from fucose and glucose from galactose were poor. Ribose and fucose were however separated by using water-saturated ethyl methyl ketone as an eluent instead of the water-saturated *n*-butanol, and glucose and galactose were separated by eluting with *tert.*-butanol–ethyl methyl ketone–water (25:60:15, by vol.). The amount of galactose recovered was too small to allow formation of a crystalline derivative but the remaining six sugars were all crystallized as the *p*-toluenesulphonhydrazones (Easterly *et al.* 1951; Freudenberg & Blummel, 1924). The configuration of the sugars appears to be L-rhamnose, D-xylose, L-arabinose and L-fucose. They may conceivably arise by autohydrolysis of other components present in Quebracho heartwood during the original water extraction of the tannin.



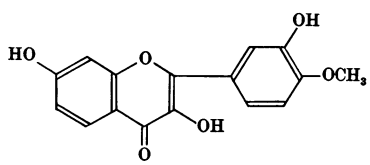
I



II



III



IV

Fraction 2*a* (7·5 g.) was observed by paper chromatography to be a complex mixture containing 3:7:4′-trihydroxyflavone, fisetin, and robinetin together with a number of non-fluorescent polyphenolic substances which have still to be separated and identified. Fraction 2*c* contained little other than fisetin, of which 0·58 g. was recovered in crystalline form and identified by paper chromatography, and comparison with synthetic material.

Fraction 2*b* (1 g.) contained primarily a substance staining yellow with bisdiazotized benzidine on paper chromatograms. It was isolated in crystalline form as described in the Experimental section and ultimately identified as ethyl gallate. It is an artifact produced from liberated gallic acid during the course of the acid hydrolysis as a consequence of using 1:1 water–ethanol as a solvent for the hydrolysis, but the occurrence of some 10 % of the isolated gallic acid in this form is somewhat surprising in view of the amount of water present and the method of isolation.

Presence of anthraquinone

In addition to these sugars, Q_{111} contains the more organic-solvent soluble and lower molecular weight components of Quebracho extract, e.g. the flavonoid fluorescent substances. A reasonable separation of some of these components can be obtained by chromatographing Q_{111} with various eluting organic solvents on columns of dry chromatographic quality silica gel. In particular, fisetin and gallic acid can be obtained crystalline by eluting first with ether–acetone or ether–ethyl acetate (both 9:1, by vol.). On one particular occasion a third crystalline compound separated from the fisetin eluate as pale yellow needles, m.p. 272–274° (some sublimation). It sublimed readily under reduced pressure, gave a positive oxanthrol test and by reductive acetylation was converted into the strongly blue fluorescing dihydroanthraquinone diacetate which showed no m.p. depression on mixing with an authentic sample. There is little doubt that the initial compound was anthraquinone which has not been reported previously as occurring naturally, although β-methylanthraquinone occurs in teak wood (Kafuku & Sebe, 1932). Fractionation of further samples of Q_{111} and of Quebracho extract has failed to yield anthraquinone in crystalline form but evidence of its presence is given by the production of an intense blue fluorescence when the extract is reductively acetylated with Zn dust and acetic anhydride, and also by paper chromatography. It may be relevant that Nierenstein (1907) reported that by treating Quebracho extract with potassium persulphate and distilling the product with Zn dust he was able to isolate anthracene.

DISCUSSION

As a result of these investigations the nature of a number of the minor components of Quebracho tannin extract is now known. The striking yellow fluorescence of the extract is due to the presence of at least 16 flavonoid substances of which 4 appear to be the primary parent compounds. These four are 3:7:4′-trihydroxyflavone, 3:7:3′:4′-tetrahydroxyflavone (fisetin), 3:7:3′:4′:5′-pentahydroxyflavone (robinetin), and 3:7:3′-trihydroxy-4′-methoxyflavone (4′-methoxyfisetin). The relationship between these primary fluorescent substances, all having the same carbon skeleton and varying in regular sequence in their phenolic hydroxyl group content, may be significant in relation to the structure of the Quebracho tannins which have yet to be investigated, although it must be emphasized that the fluorescent substances are not tannins and form only a very small part of the extract. It is interesting to recall the similar mono-, di-, and tri-hydroxyphenol relationship in the simple aldehyde oxidation products of lignin—i.e. *p*-hydroxybenzaldehyde, vanillin, syringic aldehyde (Leopold, 1952), and in the anthocyanins (Mayer & Cook, 1943). Three of these related fluorescent compounds (not the 4′-methoxyfisetin) are also present in 'Mimosa' tannin extract from the bark of *Acacia mollissima*.

The remaining twelve of the sixteen yellow fluorescent substances seem from hydrolysis experiments to be gallic acid and sugar derivatives of the four primary fluorescent compounds. The tannin extract also contains gallic acid and seven monosaccharides in the free condition, the total sugar content (*ca.* 3%) being such that these substances clearly do not have the structural significance that they possess in the case of the hydrolysable tannins.

The significance of the single isolation of anthraquinone is doubtful although the reductive acetylation test indicates it to be present also in 'Mimosa' extract and in other tannin extracts. It is clear, however, that if ideas as to the structure of the condensed tannins are to be based on the nature of the flavanoid substances present in the extracts (e.g. Freudenberg & Maitland, 1934) the suggestions should be made with caution until sufficient single pure substances possessing the capacity to tan have been isolated from condensed extract and a better idea of the real function of tannins in plants has been obtained.

SUMMARY

1. Previous work on the general fractionation of Quebracho extract and the method of characterizing the components of the extract is summarized.

2. The minor components of the extract have been examined in some detail. The characteristic yellow fluorescence of the extract under ultraviolet light is due to the presence of at least sixteen substances, of which twelve appear to be glycosyl and galloyl derivatives of the remaining four.

3. The four primary fluorescent substances are 3:7:4′-trihydroxyflavone; 3:7:3′:4′-tetrahydroxyflavone (fisetin); 3:7:3′:4′:5′-pentahydroxyflavone (robinetin); and 3:7:3′-trihydroxy-4′-methoxyflavone (4′-methoxyfisetin), recalling the corresponding mono-, di- and tri-hydroxyphenol relationship of the aldehydic oxidation products of lignin or the corresponding sequence in anthocyanins. The possible significance of this in relation to the structure of tannins is commented on.

4. Gallic acid, glucose, xylose, arabinose, fucose, rhamnose, ribose, galactose and 5-hydroxymethylfurfural are present in the extract in the free condition. Gallic acid and glucose increase in amount on hydrolysis of the extract and its fractions. Ethyl gallate was isolated as an artifact arising during hydrolysis.

5. Anthraquinone has been isolated from one batch of extract and other samples (and other extracts) show evidence of its presence.

6. The fact that these findings add to the complexity of the problem of determining the composition and the natural function of tannin extracts is emphasized.

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A Hexose-1-Phosphatase in Silkworm Blood*

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A number of phosphatases have been found in insects; however, little is known concerning their substrate specificity and the part they play in intermediary metabolism. Drilhon (1943) showed that the gut of certain species of Coleoptera, Neuroptera, Orthoptera and Lepidoptera contains an alkaline phosphatase, while an acid phosphatase is present in the Malpighian tubules. The presence of an acid phosphatase has also been observed in the honey-bee, housefly, and cockroach (Rockstein & Levine, 1951). Day (1949), applying histological methods, studied the distribution of alkaline phosphatase in several insects. With β-glycerophosphate as substrate, activity was found in the alimentary tract, in muscle, and in storage, nervous, and reproductive tissue. The silk glands of the silkworm (*Bombyx mori* L.) contain a strong phosphomonoesterase (maximum activity, pH 4·2–5·0) and a feebler alkaline phosphatase (pH maximum, 7·5–8·5). An alkaline pyrophosphatase is also present (Denucé, 1952). A specific adenosinetriphosphatase (ATP-ase) is present in the mitochondria of the housefly (Sacktor, 1953). This enzyme is activated by magnesium and inhibited by azide but not fluoride. A fluoride-sensitive ATP-ase is present in the 'soluble' fraction of the housefly preparation.

The present paper is concerned with some of the properties of a specific hexose-1-phosphatase which has been found in silkworm blood.

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EXPERIMENTAL

Silkworm-blood preparation

Silkworm-blood preparations were obtained from pure-line strains of white, yellow, and zebra varieties of *B. mori* reared at this laboratory. Fifth-instar larvae within 2–3 days of pupation were used throughout the study. The larvae were bled into a test tube embedded in crushed ice. Blood collected in this manner can be stored for at least 3 months at −28° without loss of hexose-1-phosphatase activity. In the experiments to be described here 0·5ml. of a 1:10 (v/v) dilution of blood in water was used routinely. This represents, on the average, 250 μg. total N in non-dialysed or 75 μg. total N in dialysed blood.

Materials

All materials used were reagent grade. Glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, ribose 5-phosphate, hexose diphosphate and adenosine