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Chromatographic separation and examination of carbohydrate and phenolic components of the non-tannin fraction of black wattle (*Acacia mearnsii*) bark extract

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ABSTRACT

Commercial black wattle extract, from which most of the tannin had been removed with organic solvents (so being enriched with "non-tannins") and low-molecular-weight constituents by dialysis, was fractionated by chromatography on polyvinylpolypyrrolidone (PVPP). Steric-exclusion chromatography (SEC) then yielded a polysaccharide–protein conjugate, two polysaccharide fractions and two minor fractions containing both carbohydrate and phenolic components. Examination of each by SEC, gas–liquid chromatographic analysis of the carbohydrate and thin-layer chromatography of the fractions and derivatives thereof demonstrated the main structural features of the carbohydrate components and an association between carbohydrate and phenolic moieties in the minor fractions (as shown also for a dialysate). Chromatographic methods also afforded information on the behaviour of the non-dialysable "non-tannins" on fractionation by use of solvents, lead precipitation and the standard hide-powder technique.

INTRODUCTION

Extract from the bark of black wattle (*Acacia mearnsii*), which is widely used in the manufacture of leather, contains a high proportion of tannins, which are concentrated by extraction into acetone [1]. This tannin fraction has been shown [2–4] to consist of a mixture of condensed tannins having molecular structures based on *ar*-hydroxylated flavan-3,4-diol nuclei; a molecular weight range from 350 to *ca.* 3000 has been reported [5]. Some phenolic components of lower molecular weight, such as the phloroglucinol derivative fisetin, have also been isolated [6].

The acetone-insoluble fraction of wattle bark extract has been found [2,7] to contain sugars, mainly sucrose, with traces of glucose and fructose, and the cyclitol (+)-pinitol, which are removed, with some tannin, by further fractionation with 95% ethanol and by dialysis. Gummy polysaccharides of high molecular weight constitute a major component of the material remaining after such treatment, and protein is present in significant proportions, but the so-called "non-tannin" fraction also contains phenolic components [8] in high proportion (*ca.* 40%). The polysaccharides are known to associate strongly with tannins, owing to occlusion and/or adsorptive effects [6]. Similar strong interaction of starch with condensed tannins in the hot-water

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extract from the bark of Ponderosa pine has been reported [9]. No evidence has been obtained for covalent bonding between carbohydrate and tannin in these cases, although a polymer believed to be a covalently bonded flavologlycan, flavolan chains of various degrees of polymerization being linked to a galacturonoglycan chain of high molecular weight, has been isolated from mangrove leaves [10].

The incomplete separation of carbohydrate and tannin components of wattle bark extract, due to this strong interaction, presents problems in some of the commercial applications of this material. The presence of carbohydrate is beneficial in leather manufacture, tempering the astringency of the phenolic tanning solution, but is deleterious in the preparation of adhesives formed by condensation of the phenolic components with, for example, methanal. It is, therefore, of industrial interest to examine more closely the association of carbohydrate and tannin in fractions derived from wattle bark extract.

Adsorbents of the polyamide type have proved invaluable in both column [10,11] and planar [11,12] chromatographic separations of tannins and highly hydroxylated flavonoids, and paper chromatography [3,13] and steric-exclusion chromatography (SEC) are useful in fractionating both condensed tannins [11] and carbohydrates [14]. This paper describes the use of these methods, individually and in combination, in the isolation of the components of the "non-tannin" fraction of wattle bark extract, and the investigation of these components and of fractions obtained by classical methods of purification and analysis.

EXPERIMENTAL

Chromatographic materials and methods

Preparative-scale fractionation of the non-tannin fraction of the wattle bark extract was performed by chromatography on a glass column (22 cm × 3.5 cm I.D.) packed with polyvinylpolypyrrolidone (PVPP) (Sigma, St. Louis, MO, USA). Elution with water until no further carbohydrate was detected (phenol–sulphuric acid colorimetric assay [15]) in the column effluent was followed by elution with redistilled formamide and then with 8 *M* aqueous urea [10]. The portion eluted with water was further fractionated by preparative SEC on a glass column (84 cm × 3.3 cm I.D.) packed with the agarose gel Sepharose 4B (Pharmacia, Uppsala, Sweden), which was eluted with water at a flow-rate of 40 ml/h. The same column, eluted with 0.5 *M* pyridinium acetate buffer solution (pH 5.0) at a flow-rate of 50 ml/h, was used to purify the fraction obtained from the PVPP column by elution with formamide.

Analytical SEC was performed with 1 *M* sodium chloride as the eluent in all instances. Three different columns were used: (i) acrylic plastic, 60 cm × 9 mm I.D. (Pharmacia column K 9/60), packed with Sepharose 4B, eluted at 15 ml/h; (ii) borosilicate glass, 90 cm × 15 mm I.D. (Pharmacia column K 15/90), packed with Sephacryl S-300 Superfine (Pharmacia; allyldextran cross-linked with N,N′-methylenebisacrylamide), eluted at 25–30 ml/h; and (iii) glass, 50 cm × 15 mm I.D., packed with Bio-Gel P-10, 100–200 mesh (Bio-Rad Labs., Richmond, CA, USA; polyacrylamide cross-linked with N,N′-methylenebisacrylamide), eluted at 20 ml/h. Fractions (1–1.5 ml) were monitored for protein or tannin by UV spectrophotometry (220 or 280 nm) and for carbohydrate by the phenol–sulphuric acid assay [15]. Estimates of relative, average molecular weights ($\bar{M}_w$) of carbohydrate components from SEC elution volumes were based on calibrations with characterized dextran fractions (Pharmacia).

Thin-layer chromatography (TLC) was performed on plastic strips (Polygram, 20 cm × 5 cm) precoated with Polyamide-6 UV_{254} (0.1-mm layer) (Macherey-Nagel, Düren, Germany). The strips, cut to lengths of 8–10 cm, were developed with formamide. Phenolic components were detected on these plates by their UV absorbance. Spray reagents used for detection were (a) freshly mixed iron(III) chloride–potassium hexacyanoferrate(III) (1:1, v/v), both 0.1 *M*; (b) 3 *M* sulphuric acid, followed by heating at 110°C for 5 min.

For TLC of acetylated derivatives, Kieselguhr plates were used, prepared by coating glass plates (10 cm × 5 cm) with Kieselguhr G (particle diameter 10 μm; E. Merck, Darmstadt, Germany), layer thickness *ca.* 0.5 mm. The plates were developed with chloroform–acetic acid (1:1, v/v) and the detection reagent was *p*-anisaldehyde–sulphuric acid–ethanol (1:1:18, v/v).

Paper chromatography of hydrolysates was carried out by the descending method on Whatman No. 1 paper, with the following solvent systems (all v/v/v): (A) ethyl acetate–pyridine–water (8:2:1); (B) 1-butanol–acetic acid–water (2:1:1); and (C) upper phase of 1-butanol–ethanol–water (4:1:5) (for methylated sugars). Spots were revealed by spraying with 3% (w/v) *p*-anisidinium chloride in aqueous 1-butanol, or ammoniacal silver nitrate (5%, w/v), followed by heating at 110°C for 5–10 min.

Gas–liquid chromatography (GLC) of sugars in hydrolysates, as the alditol acetates [16], was performed on a Carlo Erba 4200 or (for capillary columns only) a Carlo Erba GC 6000 Vega Series 2 instrument, each with a flame ionization detector coupled to a Spectra-Physics SP4290 integrator. Another Carlo Erba 4200 gas chromatograph was coupled through a jet separator to a VG Micromass 16F mass spectrometer. The columns used were (1) a glass column, 2 m × 3 mm I.D., packed with 3% OV-225 on Chromosorb W HP (80–100 mesh); (2) a glass capillary (surface-coated open tubular, SCOT) column, 30 m × 0.35 mm I.D., coated with OV-225; and (3) a fused-silica capillary column, 30 m × 0.32 mm I.D., with OV-225 (0.25 μm thickness) as a bonded phase (Durabond DB-225; J & W Scientific, Folsom, CA, USA). The carrier gas was helium in all instances.

Peracetylated alditols were analysed on column 1, isothermally at 210°C, and on column 2, with cold on-column injection and temperature programming from 100 to 250°C at 4°C/min. Partially methylated alditol acetates were analysed on column 1, isothermally at 175°C and column 3 at 195°C; for GLC–mass spectrometry (MS) column 2, with temperature programming from 100 to 230°C at 5°C/min, or column 3, isothermally at 210°C, were used. For quantitative analyses of partially methylated alditol acetates, the molar response factors calculated by Sweet *et al.* [17], were applied to the peak areas obtained in GLC. Empirically determined molar response factors were used in GLC analysis of mixtures of peracetylated alditols, to which *myo*-inositol was added as an internal standard.

Analytical methods

GLC of the alditol acetates derived from carbohydrate components was preceded by hydrolysis in 2 *M* trifluoroacetic acid at 100°C, under nitrogen in a sealed tube, for 6 h for neutral carbohydrates and for 18 h for those containing acid-resistant aldobiouronic acid linkages and for methylated derivatives. In order to compensate for losses due to decomposition of the free sugars under the hydrolytic conditions used (pentoses being especially labile [18,19]), correction factors [20], obtained by

analysis of standard mixtures of sugars heated under the same conditions prior to derivatization, were applied in the GLC analyses of sugar mixtures in hydrolysates. For samples containing tannin these factors were not valid, as destruction of the free sugars was greatly increased. An attempt was made to apply appropriate response factors, obtained by analysis of standard sugar mixtures to which free wattle bark tannin was added, in a proportion similar to that in the sample, before heating under the conditions used in acid hydrolysis, but the reproducibility of these factors was poor. Precise quantitative analysis of sugar ratios in this manner was, therefore, impossible for fractions containing both carbohydrate and tannin.

In methylation analysis of carbohydrate components samples were methylated by the Hakomori procedure [21], as modified by Phillips and Fraser [22], followed by several treatments by Purdie and Irvine's method [23]. Those containing uronic acid, which was determined spectrophotometrically on the polysaccharide by Blumenkrantz and Asboe-Hansen's method [24], were further examined by carboxylate reduction with lithium aluminium deuteride [25] and by base-catalysed β-elimination [26] of portions of the per-O-methylated derivatives. All methylated samples were hydrolysed as described above, prior to GLC–MS of the derived alditol acetates. Use of 2,3,4-tri-O-methylgalactose as an internal standard showed a very low recovery of partially methylated alditol acetates in the presence of tannin, so that the precision of methylation analysis must also be adversely affected for samples containing tannin together with carbohydrate.

Tannin was determined by UV spectrophotometry, at 280 nm as recommended by Roux [27] or, in the absence of protein, at 220 nm. The latter wavelength was preferred in monitoring eluates from columns, owing to the greater sensitivity resulting from the higher molar absorptivity. The sensitive, specific spectrophotometric method using *p*-nitobenzenediazonium tetrafluoroborate (Brentamine Fast Red 2G; Sigma), which has been applied successfully in the determination of polyphenols in seaweeds [28,29], was also used in the analysis of samples of low tannin content ($\leqslant 10\%$ by weight). In all of these determinations, a sample of wattle bark tannin isolated from the acetone-soluble fraction of the extract (see Fig. 1) served as a calibrant. The same sample was used to establish correction curves applied to determinations of carbohydrate by the phenol–sulphuric acid method in the presence of tannin, which interferes, giving an appreciable colour response with the reagents [30]. This response was found to be lower in 1 *M* sodium chloride than in water.

The nitrogen content of some fractions obtained from the wattle bark extract indicated the presence of protein in significant proportions. A sample of such a fraction (see below) was hydrolysed under the conditions (6 *M* hydrochloric acid, 110°C, 24 h) required for cleavage of peptide linkages and the hydrolysate was injected into an amino acid analyser (Model 420; Waters Assoc., Milford, MA, USA). Hydroxyproline, an important amino acid in many glycosylated proteins from plant sources [31,32], was determined independently by the Leach spectrophotometric method [33].

Isolation of "non-tannin" fraction from wattle bark extract

The sample of commercial wattle bark extract (donated by Wattle Industry Centre, Pietermaritzburg, South Africa) that was used in this study had been prepared by hot-water extraction of stripped bark from *Acacia mearnsii*. Isolation of carbohydrate-rich material was carried out at room temperature by steeping the

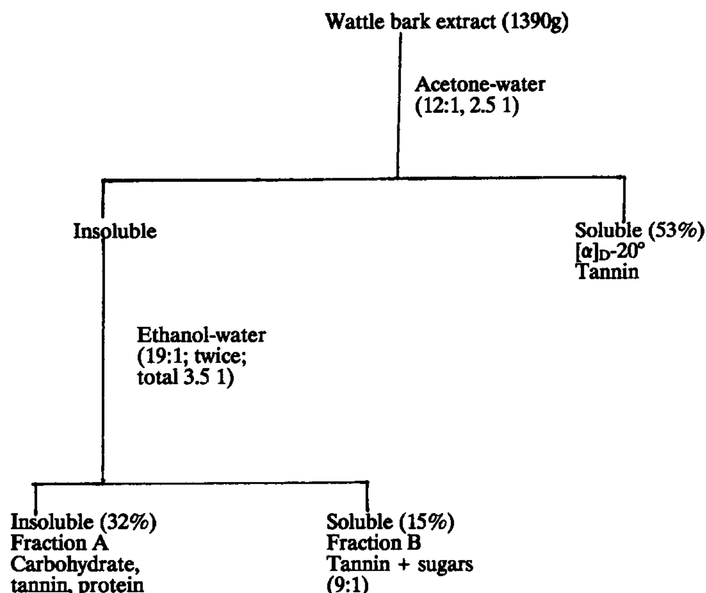


Fig. 1. Solvent fractionation of wattle bark extract.

brown, glassy material in acetone (containing some water), recovering the insoluble fraction by filtration and repeating the extraction twice with 95% ethanol. The yields and composition of the various fractions are shown in Fig. 1. The residue, fraction A, was dried *in vacuo* at 40°C and a portion was hydrolysed for GLC analysis of the sugar constituents (Table I). The ethanol-soluble fraction, B, was found (paper chromatography) to contain the sugars sucrose, glucose and fructose, together with tannin.

Dialysis of a sample (20 g) of A in Spectra/Por 1 tubing (Spectrum Medical Industries, Los Angeles, CA, USA; 32 mm diameter, molecular-weight cut-off 6000–8000) against distilled water (2 l, changed twice) for 6 days resulted in passage of a high proportion (70% by weight) into the pooled dialysate, D1. The tannin content of the retentate R1 was, however, not greatly diminished (see Table I). A larger sample of A was dialysed in preparative tubing (120 mm diameter, molecular-weight cut-off 12 000–14 000) in three stages (Fig. 2), yielding retentate R2 and final dialysate D2, which was not pooled with those removed at earlier stages of dialysis. An appreciable proportion (14% by weight) of the sample was irreversibly adsorbed by the dialysis membrane, the porosity of which was decreased by interaction with the tannin. The sugar ratios in D1, D2, R1 and R2 were determined (Table I), and the retentates R1 and R2 were used in subsequent experiments as "non-tannin" fractions of the wattle bark extract.

Fractionation of R1 on PVPP and agarose columns

The bulk of R1 was fractionated by chromatography on PVPP followed by sub-fractionation on Sepharose 4B, as described above. The yields and composition

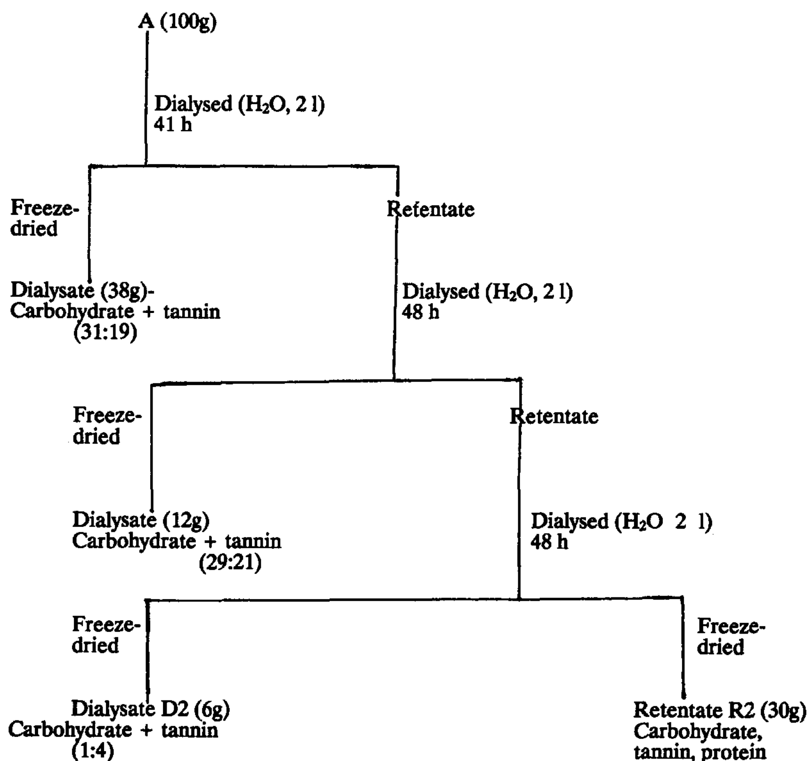


Fig. 2. Preparative-scale dialysis of fraction A.

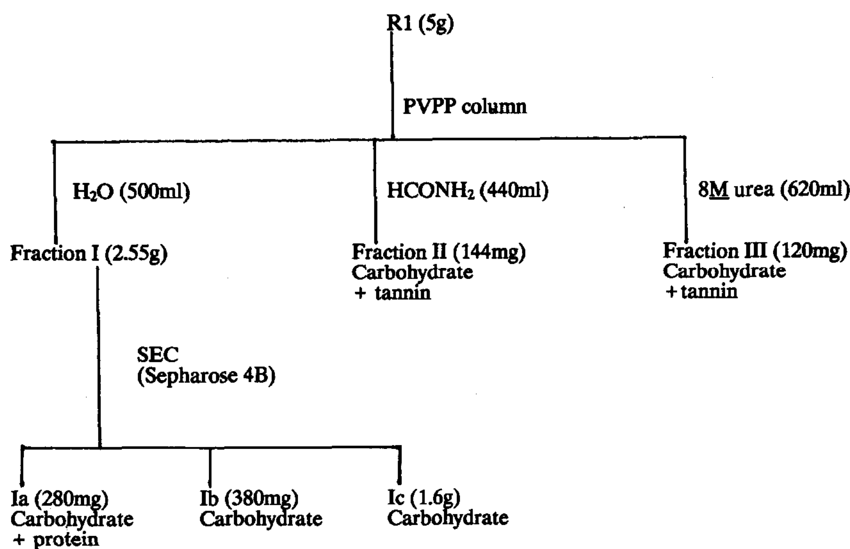


Fig. 3. Fractionation of R1 on PVPP and agarose columns.

of the fractions obtained are shown in Fig. 3. A high proportion (over 40%) of R1, including nearly all of the tannin, was retained on the PVPP column. Fractions Ia, Ib and Ic were recovered by freeze-drying the aqueous eluate from the agarose column, after pooling of fractions corresponding to different zones in the chromatogram (see Fig. 4). The formamide eluate from the PVPP column was concentrated by vacuum distillation (b.p. 60°C at 1 mmHg) and the remaining solvent removed by continuous extraction, first with diethyl ether and then with ethyl acetate; the residue was purified by SEC as described and the eluate freeze-dried to yield fraction II. The aqueous urea eluate, after concentration by freeze-drying, was fractionated with ethanol (2 volumes), which gave a brown precipitate that was dissolved in water and freeze-dried, yielding fraction III.

All fractions were examined by GLC analysis of the sugar components (Table I), analytical SEC, methylation analysis and TLC. Portions were acetylated, fractions Ia–Ic by the method of Carson and Maclay [34], with formamide as a dispersing agent, and fractions II and III with acetic anhydride–sodium acetate, for TLC on Kieselguhr. Polyamide plates were used in TLC of the tannin-containing fractions II and III, and products of partial acid hydrolysis (2 *M* trifluoroacetic acid, 100°C, 1 h) of these and of de-O-acetylation (sodium methoxide, 20°C, 16 h) of their acetylated derivatives. A portion of the proteinaceous fraction Ia was analysed for amino acids as described.

Fractionation of D2 and R2 by precipitation with lead

A portion (3 g) of freeze-dried dialysate D2, dissolved in water (50 ml), was treated at pH 5.0 with 1 *M* lead acetate solution until precipitation was complete. After centrifugation, lead was removed from both the centrifugate and precipitate by reaction with 1 *M* oxalic acid (neutralized by addition of $NaHCO_3$) and the solutions thus obtained were freeze-dried to give fractions C (centrifugate; 1.2 g) and P (precipitate; 1.6 g). The latter consisted almost entirely of tannin, with only a trace of carbohydrate, but C contained carbohydrate and tannin, in a mass ration (9:11) comparable with that in fractions II and III from the PVPP column (Table I). Fraction C was examined by GLC, SEC, TLC and methylation analysis as described for II and III.

A sample (10 g) of R2, dissolved in water (200 ml), was similarly fractionated by precipitation with 1 *M* lead acetate solution (30 ml), the uptake of Pb^{2+} being monitored by back-titration of the excess with standard 0.05 *M* EDTA, with xylenol orange as indicator. After centrifugation and removal of Pb^{2+} from the centrifugate and precipitate by leaching with oxalate as described, the two fractions were purified by addition of 1 *M* barium acetate to remove excess of oxalate, treatment with H^+-form cation-exchange resin to remove Ba^{2+} and freeze-drying. The precipitated fraction R2 P (80% of R2) contained 35% of carbohydrate in addition to tannin, whereas the centrifugate fraction R2 C contained only 4% of phenolics, consisting almost entirely of carbohydrate; protein was totally removed from R2 by this treatment. The two fractions were examined by SEC and TLC, and the distribution of neutral and acidic sugars in each was investigated by paper chromatography of hydrolysates.

The behaviour of R2 on treatment with lead was compared, by model experiments conducted under the same conditions, with that of pure tannin, L-arabinose, an arabinan isolated from apple juice [35], a neutral arabinogalactan of low molecular

weight ($\bar{M}_w$ 3600) obtained from the gum of *Acacia difformis* by Smith degradation [36] and the acidic polysaccharide isolated from the gum exudate of *A. mearnsii* [37,38].

Fractionation of R2 with aqueous acetone

A portion (5 g) of R2 was dissolved in water and acetone (3 volumes) was added, precipitating about 50% of the sample. The acetone-insoluble and acetone-soluble fractions, recovered by freeze-drying, were examined by SEC and TLC and hydrolysates by paper chromatography. The phenolic content of the acetone-insoluble fraction (R2 I) was below 5%, the main component being polysaccharide, with some protein. The acetone-soluble portion (R2 S) was sub-fractionated by further treatment with aqueous acetone, which gave another insoluble fraction (54% of R2 S), containing 13% of tannin, and a soluble fraction (58% tannin). The former gave no precipitate with lead acetate, but most of the latter was precipitated.

Fractionation of R2 and A by adsorption on hide powder

Samples of R2 and A were shaken in the conventional way with hide powder (donated by the Leather Industries Research Institute, Grahamstown, South Africa), which had been lightly chrome-tanned [39]. The "non-tannins", recovered by freeze-drying the supernatant solution, constituted 53% by weight of R2, 64% of A. The non-tannins from A gave no precipitate with Pb^{2+}, but a small gelatinous, colourless precipitate was formed on addition of lead acetate to R2 non-tannins. Both reacted positively with the iron(III)–hexacyanoferrate(III) reagent and spectrophotometric assay [28,29] indicated the presence of 4–5% of phenolics in each. The molecular-weight distribution of the carbohydrate in these fractions was determined by SEC on Sephacryl S-300.

Tests with gelatin reagent and surfactant

A solution containing gelatin (1%, w/v) in 2 *M* sodium chloride, in an acetate buffer (pH 4.7), was added dropwise to solutions (0.1%, w/v) of each of the various fractions obtained from wattle bark extract. Dense precipitates were given immediately by the pure tannin, A, R1, R2, D1, D2 and R2 S; precipitation occurred more slowly with II, III, C and R2 P, and with R2 C only a slight turbidity was observed. As expected, there was no reaction with the tannin-free fractions Ia, Ib and Ic, and there was none with the non-tannins from hide-powder fractionation of A and R2.

The inhibition of interaction between tannin and protein by surfactants such as cetyltrimethylammonium bromide (Cetavlon) has been reported [40,41]. The presence of cetavlon (1%, w/v) was found to suppress precipitation of tannin, R2 S, II and C by the gelatin reagent. A sample of II that had been left for 24 h in the Cetavlon solution was re-examined by TLC, to ascertain whether association in the tannin–carbohydrate conjugate was affected by competitive interaction of tannin with the surfactant.

RESULTS AND DISCUSSION

Isolation and chromatographic fractionation of R1

It is evident (Table I) that despite repeated solvent fractionation (Fig. 1) and

TABLE I

ANALYTICAL DATA FOR FRACTIONS FROM WATTLE BARK EXTRACT

Fraction	$[\alpha]_D$ (°)	Composition (%, w/w)			Sugar proportions[b] (mol%)						
		Carbo hydrate	Tannin	Protein[a]	Uronic acid	Ara	Glc	Gal	Rha	Xyl	Man
A	+10	54	41	5	7	46	28	7	4	4	4
R1	+14	56	35	9	8	47	21	7	10	3	4
R2	+15	55	33	12	10	50	13	12	9	3	3
D1	0	58	42	–	–	39	39	9	2	5	6
D2	−7	20	80	–	–	44	36	5	7	3	5
Ia	+51	65	–	35	15	50	3	20	10	1	1
Ib	+84	100	–	–	15	36	16	16	12	tr	5
Ic	+22	100	–	–	18	42	15	7	10	4	4
II	+3	40	60	–	–	76	13	5	2	2	2
III	−2	46	54	–	–	26	51	5	11	2	5
C		45	55	–	–	25	52	5	7	4	7

[a] Calculated from nitrogen content.

[b] For fractions containing tannin, ratios of neutral sugars serve only to indicate the distribution pattern in the carbohydrate component, as replicate analysis showed standard deviations of the order of 20–60%, owing to variable destruction of sugars.

prolonged dialysis (Fig. 2), an appreciable proportion of tannin persists in the so-called non-tannin fraction from wattle bark extract. Dialysis had a minimal effect on the carbohydrate: tannin ratio of the retentates, although it should be noted that adsorption of phenolic material by the cellulose membrane, which was considerably darkened and hardened during the dialysis process, must have influenced progressively its permeability to the various components of the extract. Carbohydrate and phenolic components were found in both retentates and dialysates, only protein being totally retained. The material recovered from the dialysates consisted of carbohydrate of relatively low molecular weight ($\bar{M}_w \leqslant 30\,000$), co-eluted with some phenolic components at the total (maximum) volume, V_t, in SEC on the analytical Sepharose 4B column. Much of the phenolic fraction was retarded by adsorptive interaction with the gel matrix and was eluted after V_t, with pronounced tailing; some was irreversibly adsorbed. This behaviour is illustrated in Fig. 4 for the phenolic components of retentate R1. There was some overlap with the major carbohydrate component, $\bar{M}_w$ 70 000, which was, however, well separated from a minor fraction containing polysaccharide of high molecular weight together with protein, eluted at the void volume, V_0, of the column.

The polysaccharide fraction of R1 was separated from the phenolic material by elution with water from the PVPP column, which retained all the phenolic components under these conditions (Fig. 3). The fraction (I) recovered from the aqueous eluate consisted mainly of carbohydrate with some protein (6% by weight). On sub-

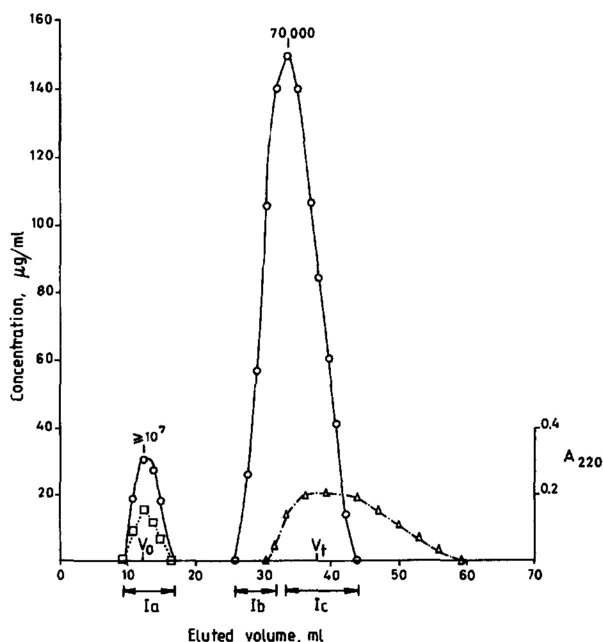


Fig. 4. Analytical SEC of retentate R1 on Sepharose 4B. Conditions as given in the text. Absorbance at 220 nm (A_{220}) plotted for protein only; concentrations plotted for other components. Zones from which fractions Ia, Ib and Ic were isolated on preparative SEC are indicated. ○ = Carbohydrate; △ = tannin; □ = protein.

fractionation on the preparative column of Sepharose 4B, three polysaccharide fractions, Ia–Ic, were isolated. All of the protein appeared in fraction Ia, together with the polysaccharide ($\bar{M}_w \geq 10^7$) eluted at V_0 from the analytical column (Fig. 4). The major polysaccharide component was resolved on the larger column into discrete fractions, Ib ($\bar{M}_w$ 150 000) and Ic ($\bar{M}_w$ 50 000). The sugar constituents of Ia–Ic (Table I) were, in general, typical of acidic arabinogalactans from plant sources [42], and their composition differed from that of the gum exudate of *Acacia mearnsii* [37,38] only in the presence of glucosyl residues in significant proportion in Ib and Ic.

Polysaccharides Ia–Ic also differed from the gum polysaccharide of *A. mearnsii* in their positive specific rotations (Table I), that of the gum being negative ($[\alpha]_D - 33°$) [38]. The modes of glycosidic linkage in Ia–Ic, determined by methylation analysis, were generally similar in all three and constituted another point of resemblance between these and the gum polysaccharide. Terminal arabinofuranosyl (Ara*f*), rhamnopyranosyl (Rha*p*) and galactopyranosyl (Gal*p*) residues, → 5)-linked Ara*f*, and → 3)-, → 4)- and → 6)-linked Gal*p* were structural features common to bark polysaccharides and gum, though some Rha*p* was present as → 2,4)-linked branch-points, which was unusual. The glucopyranosyl residues (Glc*p*) were → 3)- and → 4)-linked, a characteristic of cell-wall polysaccharides, not gums [42]. Fraction Ic differed from the others in containing terminal Glc*p* and → 3)-linked Ara*f* in significant proportions.

GLC–MS after reduction of the carboxylate ester groups in methylated Ia–Ic with lithium aluminium deuteride [25] showed, from the presence in the hydrolysates of 2,3-di-O-methylglucose and 2-O-methylglucose deuterated at C-6, that the glucuronic acid (GlcA) residues occurred both as → 4)-linked chain units and as → 3,4)-linked branch-points, in equal proportions. There was no evidence for terminal GlcA, found in the gum [43,44]. A significant increase in the proportion of 2,3,4,6-tetra-O-methylgalactose, with concomitant disappearance of 2,3,4- and 2,3,6-tri-O-methylgalactose, in the hydrolysate after portions of methylated Ia–Ic had been submitted to base-catalysed β-elimination [26] showed the attachment of GlcA to Gal at O-6 or O-4. The former mode of linkage was also indicated by the detection of the aldobiouronic acid GlcAβ1–6Gal on paper chromatography (solvent B) of the products of partial acid hydrolysis (0.5 *M* trifluoroacetic acid, 100°C, 6 h) of Ia–Ic. All Rha was lost by base-catalysed β-elimination of methylated Ia–Ic, which suggested the location of these residues immediately exterior to → 4)-linked GlcA, as in many *Acacia* gum polysaccharides [26,42]. There was also loss of Glc in this reaction, but this may have been due to alkaline degradation ("peeling") [45] from the reducing end of a glucan component, rather than an indication of the location of Glc exterior to GlcA in the acidic polysaccharides.

The main amino acid constituents of the protein component of Ia were aspartic acid (15% of total amino acids), glutamic acid (12%), serine (11%), glycine (10%) and alanine (10%). Hydroxyproline was present in very low proportion (<1%). In this respect, therefore, the protein in Ia resembles that associated with the seed galactomannans guar and locust bean gum [46], rather than that in some *Acacia* gums [18,47].

Elution of the PVPP column with formamide and then with 8 *M* aqueous urea yielded two further fractions, II and III, in low yields (each 2–3% of starting material; see Fig. 3). These contained both carbohydrate and phenolic components, the latter

preponderating. Carbohydrate and phenolic components were co-eluted at V_t on SEC on the analytical Sepharose 4B column. When the Sephacryl S-300 column was used, II and III were eluted at K_{av} 0.90 and 0.87, respectively; in both instances carbohydrate and tannin were co-eluted, each giving a single, sharp peak, and there was no broad, tailing peak extending beyond V_t, as given by free tannin. This suggested very strong association, perhaps covalent linkage, between carbohydrate and phenolic moieties in II and III. The carbohydrate was evidently of relatively low molecular weight, but it was impossible to estimate actual values as the relationship between V_e and $\bar{M}_w$ must obviously be different from that for dextrans, used to calibrate the column, and the retarding effect of the phenolic component will be superimposed on the normal SEC mechanism.

The carbohydrate components of II and III differed from Ia–Ic in containing no uronic acid. Arabinose and glucose were the main neutral sugar constituents, Ara preponderating in II, whereas Glc appeared to be the major constituent of III (but analyses of III were particularly seriously affected by variable destruction of sugars when heated in the presence of tannin and therefore must be interpreted with caution). Methylation analysis showed the same glycosidic linkages as were found in Ia–Ic, with terminal and → 5)-linked Ara*f*, → 3) and → 4)-linked Glc*p* preponderant. A high proportion of terminal groups, without a corresponding number of sugar residues present as branch points, suggests attachment of short chains of sugar residues, or single units, to non-carbohydrate components and may thus be indicative of covalent linkage between carbohydrate and phenolic moieties.

Further evidence for very strong association between carbohydrate and tannin in II and III was afforded from their behaviour on TLC, on both Kieselguhr and polyamide plates. Acetylated samples moved as discrete spots ($R_F \approx 0.75$) on the Kieselguhr plates, in contrast to acetylated Ib, Ic and tannin, which streaked (methylated Ia remained at the origin). Acetylated tannin gave a characteristic blood-red colour with the anisaldehyde–sulphuric acid spray reagent, but no such colour was produced by acetylated II or III, which turned dark grey with this reagent, as did the acetylated polysaccharide fractions. On the polyamide plates free tannin streaked from the origin to $R_F \approx 0.6$, but II and III moved as compact spots ($R_F \approx 0.9$). With the Fe^{3+}–$Fe(CN)_6^{3-}$ spray reagent (a) the tannin streak gave the characteristic Prussian blue colour, whereas the spots due to II and III did not react unless the plate was subsequently sprayed with 3 *M* sulphuric acid (b) and heated as described. The blue coloration produced under these conditions was confined to the area of the spots (as indicated by examination under UV), suggesting release of free tannin *in situ* by the action of the acid spray. Spraying with b alone turned the spots brown, and the tannin streak pink. On polyamide TLC of samples of II and III that had been submitted to partial acid hydrolysis the streaking shown by free tannin was observed and the Prussian blue coloration was produced by spray reagent a. The acid spray gave a corresponding pink streak, together with a brown streak closer to the solvent front, which was ascribed to free carbohydrate. The same behaviour was shown on similar TLC of the products of de-O-acetylation of acetylated II and III in base. These results indicate production of free tannin and carbohydrate from II and III only after cleavage of bonds (of whatever type) by treatment with acid or base, and supplement the evidence from SEC for complexation between carbohydrate and phenolic moieties in these minor fractions from R1.

Fractionation by precipitation with lead

The precipitated fraction, P, obtained on treatment of dialysate D2 with lead acetate behaved as free tannin on polyamide TLC, whereas C, the fraction isolated from the centrifugate, behaved as described for fractions II and III, only the products of partial acid hydrolysis, and of de-O-acetylation of acetylated C, showing the presence of free tannin. The carbohydrate portion of C, which was co-eluted with the phenolic component at V_t on SEC on the analytical Sepharose 4B column, appeared to resemble fraction III with respect to sugar ratios (Table I), but the reproducibility of the analytical results was as poor as that for III. Methylation analyses showed the distribution of glycosidic linkages found in II and III.

In contrast to the behaviour of D2, treatment of R2 with lead acetate resulted in the coprecipitation of a high proportion of carbohydrate (over 50% of the total carbohydrate) with tannin, which was also precipitated to a greater extent than that in D2. The uptake of Pb^{2+} by R2 corresponded to 1 mol per 880 g; for pure tannin the uptake of Pb^{2+} under similar conditions was 1 mol per 520 g. The precipitated fraction, R2 P, $[\alpha]_D$ +45°, was found, by paper chromatography (solvents A and B) of the hydrolysate, to contain glucuronic acid in appreciable proportion, together with the neutral sugar constituents of R2, whereas the centrifugate fraction, R2 C, which had $[\alpha]_D$ −36°, consisted almost entirely of the neutral sugars, with only a trace of glucuronic acid.

SEC of R2 P on Sepharose 4B gave a peak for carbohydrate of $\bar{M}_w$ 70 000 but most of the sample was co-eluted with tannin at V_t. Use of the Bio-Gel P-10 column resulted in the co-elution of tannin and carbohydrate, mainly at V_0, although in a model experiment tannin that had not been treated with lead was eluted from the column, as expected, in a broad peak at and after V_t. Owing to adsorptive interaction with the polysaccharide gel matrix, the recovery of tannin was low (*ca.* 20%) in the model experiment, but over 80% of that in the lead precipitate was recovered from the column. The best resolution of the carbohydrate components was achieved by SEC on Sephacryl S-300 (Fig. 5A), but again there was overlap with the tannin, which was eluted (recovery *ca.* 70%), in a sharp peak at K_{av} 0.54, within the range at which carbohydrate emerged (K_{av} 0.29–0.84). In a model experiment tannin not subjected to lead precipitation was eluted in a broad peak in the region of V_t, with low recovery from this column as from Bio-Gel P-10. These results indicate that tannin removed from lead precipitates by leaching with oxalic acid undergoes self-condensation or other chemical change, reducing its affinity for polyacrylamide and allyldextran gels.

No such effect was observed on SEC of R2 C on Sephacryl S-300 (Fig. 5B), the phenolic components (which may not be tannins) being eluted in the region of V_t, with only slight overlap with the carbohydrate components of lowest molecular weight. The carbohydrate in this fraction had a broader molecular weight distribution than that in R2 P (Fig. 5A), which suggests a certain optimum size range for precipitation with tannin.

Fractions R2 P and R2 C behaved similarly on polyamide TLC. The phenolic components were detected as streaks, turning blue when sprayed with reagent a; this was very faint for R2 C. Further spraying with acidic reagent b, after reagent a, had no effect (*cf.*, II and III).

In model experiments designed to investigate the factors governing coprecipitation of carbohydrate with tannin on treatment with lead, it was found that arabi-

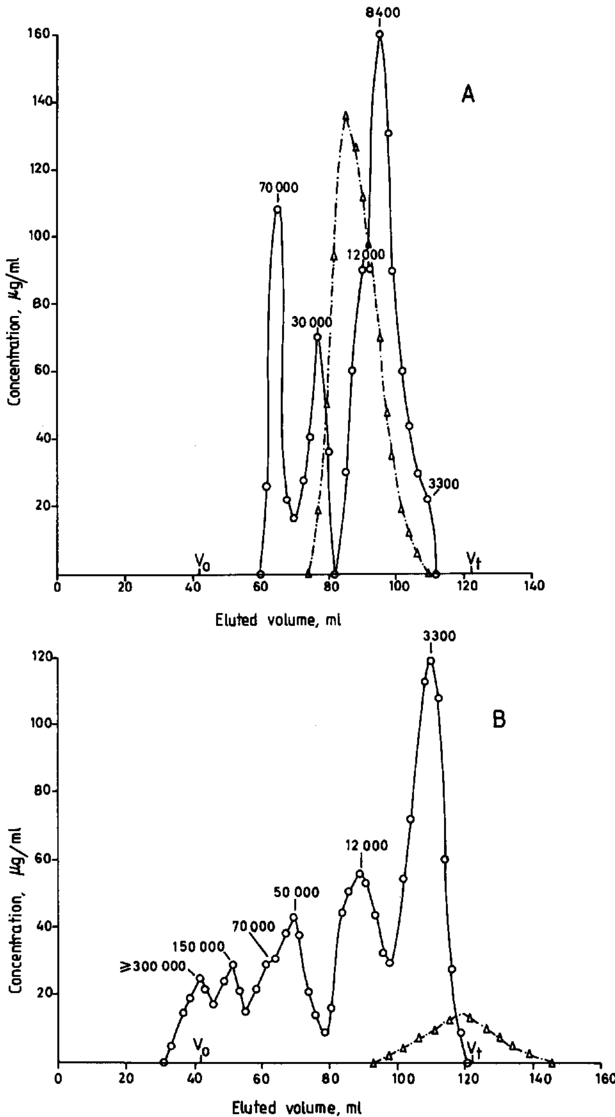


Fig. 5. SEC on Sephacryl S-300 of (A) lead-precipitated fraction (R2 P) of R2 and (B) fraction R2 C from centrifugate. Conditions as given in the text. (A) ○ = Carbohydrate; △ = tannin. (B) ○ = Carbohydrate; △ = phenolics.

nose, the apple juice arabinan, and the Smith-degraded arabinogalactan from *Acacia difformis* gum did not coprecipitate. However, the acidic arabinogalactan from *A. mearnsii* gum, which was 10% precipitated by lead alone, was 90% precipitated in the presence of an equal weight of tannin. The gum contains components in the molecular-weight range of the carbohydrate fraction of R2, whereas the other carbohydrates tested were of much lower molecular weight (below 10 000). The fact that the

carbohydrate in dialysate D2, which was of low molecular weight, did not coprecipitate is also significant here. Further, D2 and the other carbohydrates that did not coprecipitate contained no uronic acid, but the *A. mearnsii* gum polysaccharide is similar to R2 with respect to uronic acid content. It has been noted that the uronic acid in R2 was concentrated into R2 P. Thus, both uronic acid content and molecular size appear to be important in determining whether or not carbohydrates will coprecipitate with tannin in the presence of lead.

In these model experiments, the behaviour of tannin on SEC on Bio-Gel P-10 and Sephacryl S-300 was altered after recovery from the lead precipitate, as has been described for R2, whether the tannin was precipitated alone or together with the *A. mearnsii* gum polysaccharide. This demonstrated that the change in the tannin was a consequence of the chemical processes involved rather than any interaction with the carbohydrate.

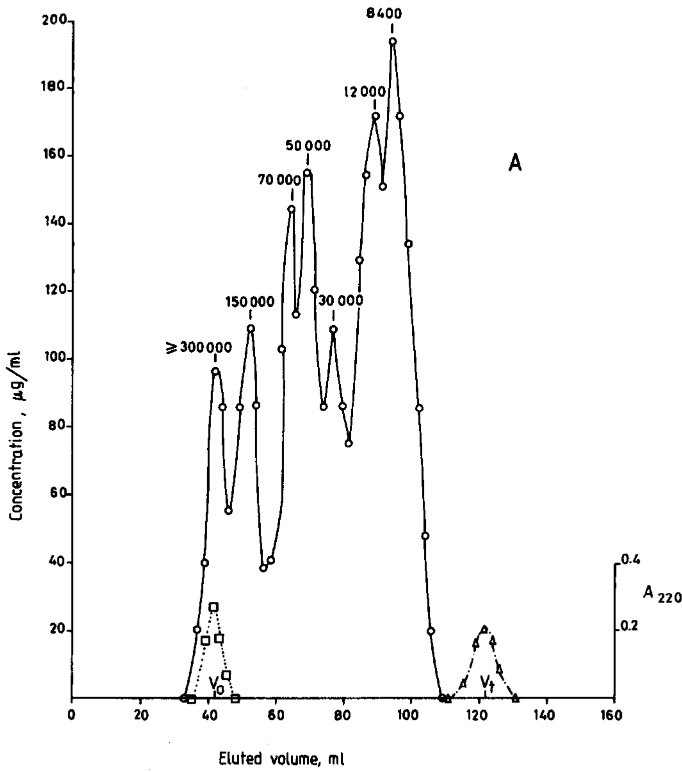
Fractionation with aqueous acetone

Fractionation of R2 with aqueous acetone resulted in precipitation of all carbohydrate components having molecular weight of 8000 or above, together with the protein. The small amount of tannin found in the acetone-insoluble fraction, R2 I, was probably coprecipitated by adsorption to the polysaccharide. SEC of R2 I on Sepharose 4B separated the polysaccharide–protein conjugate (corresponding to fraction Ia isolated from R1), which was eluted at V_0, from the bulk of the carbohydrate, which emerged as a single, broad peak ($\bar{M}_w$ 70 000; *cf.*, fractions Ib and Ic), overlapping with the phenolic component, eluted in the region of V_t. The carbohydrate in the acetone-soluble fraction, R2 S, was co-eluted with the tannin at V_t on this column. With the Bio-Gel P-10 column, all carbohydrate and protein in R2 I were eluted at V_0, well removed from the phenolic component at V_t. Optimum resolution of the polysaccharide components of R2 I was given by Sephacryl S-300, which also separated these from the phenolic material (Fig. 6A). SEC of R2 S on this column showed the presence of two carbohydrate components, the major one eluting at K_{av} 0.84 (corresponding to $\bar{M}_w$ 3300) and the minor one at K_{av} 0.90 ($\bar{M}_w$ 2400). The latter peak coincided with one due to phenolic material, which was resolved from the main tannin fraction, eluted (with low recovery) at V_t. Further fractionation of R2 S with aqueous acetone distinguished the two types of phenolic component, that eluting at K_{av} 0.90 being precipitated with most of the carbohydrate in a sub-fraction that did not react with lead acetate.

Paper chromatography (solvents A and B) of hydrolysates of R2 I and R2 S showed that all the uronic acid was recovered in R2 I, but the neutral sugar constituents of both fractions were the same. In TLC on polyamide, R2 I was not detectable by spray reagent a, but R2 S showed the characteristic blue streak given by free tannin. When the plate was subsequently sprayed with acid reagent b and heated, a further component of R2 S was revealed as a blue spot ($R_F \approx 0.9$), not seen with R2 I. This behaviour and the absence of reaction with lead suggests strong association between carbohydrate and phenolic moieties in the minor component (7% by weight) of R2 S, this component corresponding to fractions II and III from R1.

Hide-powder fractionation

The "non-tannins" recovered after fractionation of R2 with hide powder were



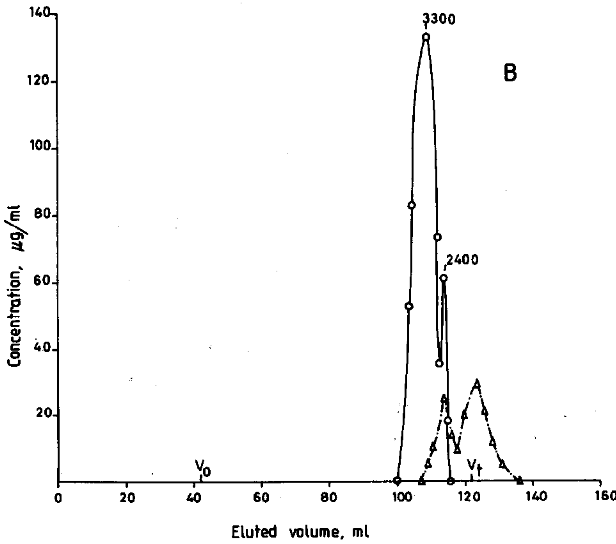


Fig. 6. SEC on Sephacryl S-300 of (A) acetone-insoluble fraction (R2 I) of R2 and (B) acetone-soluble fraction (R2 S). (A) ○ = Carbohydrate; △ = phenolics; □ = protein, (B) ○ = Carbohydrate; △ = tannin.

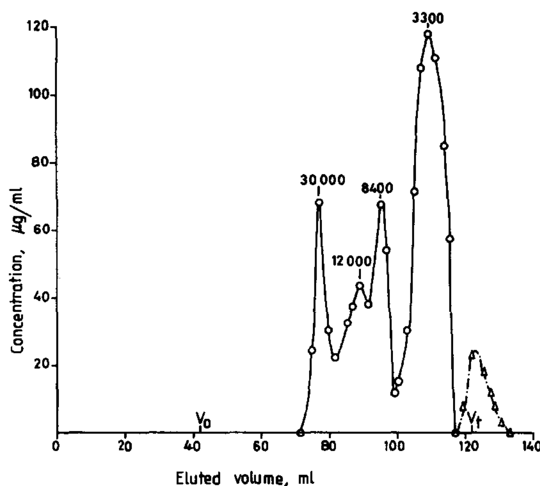


Fig. 7. SEC on Sephacryl S-300 of "non-tannins" from hide-powder fractionation of R2. ○ = Carbohydrate; △ = phenolics.

found, by SEC on Sephacryl S-300, to contain carbohydrate components having molecular weights in the range 3000–30 000 (Fig. 7), which were completely separated on the column from the residual phenolic material (probably not tannin). The corresponding fraction from A gave a similar elution profile, but with more carbohydrate of lower molecular weight and some overlap between this and UV-absorbing material at V_t. These results show that all carbohydrate having molecular weight >30 000 is adsorbed with tannin on hide powder. There is no evidence for carbohydrate–tannin complexes (such as fractions II, III and R2 S) in the non-tannins from R2; these conjugates are apparently also adsorbed by the hide powder.

Behaviour of fractions with gelatin and surfactant

The gelatin reagent, which gives a dense precipitate immediately when added to solutions containing free tannins, reacted less readily with fraction R2 P, in which the chemical nature of the tannin had been changed, and with II and III, in which the tannin appears to be strongly associated with carbohydrate. Addition of cetavlon was found to suppress the interaction of tannin and gelatin by strong competitive interaction (believed to be hydrophobic [41]) with the tannin. Nevertheless, the behaviour on polyamide TLC of a sample of II that had been exposed to Cetavlon was unchanged, there being no indication of free tannin unless the plate was sprayed with acid and heated after pre-spraying with a. This persistence of complexation between carbohydrate and tannin in II, despite the presence of the strongly competing surfactant, is further evidence for very close association, perhaps covalent linkage, between these two components in such fractions.

CONCLUSIONS

The problem of separating carbohydrate from phenolic components in wattle

bark extract is emphasized by this investigation, and the heterogeneity of the products obtained by accepted methods of fractionation has been clearly demonstrated by chromatographic methods, mainly SEC. Neither solvent fractionation nor dialysis is effective in separating tannin from carbohydrate, and strong interaction between the polyphenolic material and the acidic polysaccharides of intermediate molecular weight in the retentate from the non-tannin fraction causes these to coprecipitate on addition of lead (Fig. 5A) or ethanol (Fig. 1), and to be adsorbed together on hide powder (Fig. 7). The coprecipitation of polysaccharide in the molecular weight range 8000–70 000 with tannin on treatment with lead acetate has been clearly demonstrated and quantified by SEC, as has the adsorption by hide powder of polysaccharides of molecular weight above 30 000. The limitations of the hide-powder method have been described [8].

Polysaccharide of molecular weight above 8000 is precipitated by addition of acetone to an aqueous solution (Fig. 6A), but the presence of carbohydrate of lower molecular weight together with tannin in the acetone-soluble fraction has been shown by SEC (Fig. 6B). There is some evidence, mainly from TLC, of the presence of a carbohydrate–tannin complex in the acetone-soluble fraction, as in the material eluted with formamide and with aqueous urea on PVPP column chromatography of a similar retentate from the non-tannin fraction. These are, however, minor components and, if compounds of carbohydrate and tannin are present, they may be artefacts produced in the initial process of extraction of the bark with hot water. Methylation analysis of these fractions suggests that the carbohydrate moiety consists of single sugar units (mainly Ara*f*) and short chains (2 or 3 units).

More complex carbohydrate components, containing uronic acid, have been isolated from the aqueous eluate obtained on PVPP column chromatography of the retentate of the non-tannin fraction. The polysaccharide of highest molecular weight is associated with protein (covalent attachment has not been proved). This and the other polysaccharides thus isolated ($\bar{M}_w$ 150 000 and 50 000) were the only fractions completely freed of tannin, possibly because their molecular size was above the optimum range, as suggested by SEC, for interaction with the phenolic components of wattle bark extract.

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