THE CORRELATION BETWEEN STRUCTURE AND PAPER CHROMATOGRAPHIC BEHAVIOUR OF SOME FLAVONOID* COMPOUNDS AND TANNINS

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INTRODUCTION

The chromatographic behaviour of compounds belonging to the C_6-C_3-C_6 group of naturally-occurring "flavonoids" has been examined by BATE-SMITH[1,2], BATE-SMITH AND WESTALL[3], BRADFIELD AND BATE-SMITH[4], GAGE, DOUGLASS AND WENDER[5], and also by ROBERTS AND WOOD[6] and ROBERTS, CARTWRIGHT AND WOOD[7]. The precise mechanism of the migration of these compounds relative to the cellulose substrate is still largely unknown under those conditions where either partitioning or adsorption effects are considered to predominate[8,9,10].

BATE-SMITH and co-workers[3,4] observed regularities in the behaviour of certain phenolic compounds during partition paper chromatography, and found that the R_F values were related to the number of hydroxy groups on the C_{15} skeleton, and were also influenced by the spatial orientation of the "flavonoid" unit as a whole. In many instances a straight line relationship was obtained when the number of substituent hydroxyl groups was plotted against log $(1/R_F - 1)$.

ROBERTS, CARTWRIGHT AND WOOD[7] examined the chromatographic behaviour of phenolic compounds in water or in 2% acetic acid where adsorptive rather than partitioning effects appear to play the main role. They showed that for catechins and their gallates, and also other C_6 polyhydroxy-phenols and their derivatives the R_F values are slightly decreased by increases in the degree of hydroxylation. ROBERTS AND WOOD[6] had previously shown that water caused the separation of the optical antipodes of catechins, epicatechins, gallocatechins and epigallocatechins on paper chromatograms.

In the present work the R_F values of certain catechins, flavonols, 2,3-dihydroflavonols and flavan-3,4-diols are studied in water-saturated *sec.*-butanol as a "partitioning" mixture, and in water only as an "adsorptive" solvent. Reference is also made to their behaviour in the conventional *n*-butanol–acetic acid–water (4:1:5) "partitioning" mixture. The behaviour of condensed tannins in relation to molecular weight is studied in the same solvent systems.

EXPERIMENTAL

Compounds

The catechins, flavonols, 2,3-dihydroflavonols were obtained from natural sources.

* The term "flavonoid" is used in this paper to cover the broad group of C_6-C_3-C_6 compounds.

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DL-*epi*-7,3′,4′-Trihydroxyflavan-3-ol was kindly presented by Dr. P. MAITLAND, Cambridge University. Of the flavan-3,4-diols, melacacidin, was obtained from *Acacia melanoxylon*[11]. 7,3′,4′,5′-Tetrahydroxyflavan-3,4-diol and 7,3′,4′-trihydroxyflavan-3,4-diol were obtained by the hydrogenation of dihydrorobinetin and dihydrofisetin (fustin), using platinum oxide as catalyst[12]. 5,7,3′,4′,5′-Pentahydroxyflavan-3,4-diol and 5,7,3′,4′-tetrahydroxyflavan-3,4-diol were obtained by reduction of ampeloptin and taxifolin (dihydromyricetin and dihydroquercetin resp.) with sodium boron hydride using the method of SWAIN[13].

Due to the complexity of the wattle tannin mixture, the mature fresh bark of *Acacia mollissima* was initially extracted with ethyl acetate and finally with methanol[14]. The ethyl acetate fraction C_1 (see ref.[14]) and the methanol extracted fraction C_3 of average molecular weights 1290 and 1507, were used in this work.

Chromatographic conditions

Paper chromatograms of the "flavonoid" compounds were run on Whatman No. 1. paper in a constant temperature room at 21.1°. Downward migration was used for the water-saturated *sec.*-butanol and *n*-butanol–acetic acid–water mixtures, and upward migration for water only.

Although the *n*-butanol–acetic acid–water mixture has been widely used for the chromatographic study of "flavonoid" and related compounds, the water-saturated *sec.*-butanol (2-butanol) mixture permits the migration of C_{15} compounds as discrete almost flat spots. By comparison this group of compounds migrates as characteristically oval spots with slight trailing in the upper phase of the *n*-butanol–acetic acid–water (4:1:5) mixture.

Spraying reagents

Ammoniacal silver nitrate was generally used to locate flavonoid compounds. The authors' toluene-*p*-sulphonic acid reagent which gives pink colorations on paper sheets with leuco-anthocyanins (flavan-3,4-diols), and which has been used for locating such compounds[15], was found to give orange-yellow colours with the flavan-3,4-diols in which the 5-position is hydroxylated.

R_F values were obtained by measurement from the starting line to the centre of the spot.

Fractionation of tannins by the "partitioning" principle

The chromatographic behaviour of the tannins was studied on thick (Whatman No. 3) paper sheets using the "preparative" method[16] with *n*-butanol–acetic acid–water (6:1:2) as irrigant. The acid medium was preferred in order to minimise possible oxidation of the tannins. The tannin mixture was streaked on to the sheet and after development of the chromatogram, and drying, bands were cut to correspond with each of the R_F values 0.0–0.6 and also 0.7 + 0.8, so that the centre of each band corresponds to these R_F values. The band strips were eluted with ethanol–water mixtures[16] and the eluants from each band concentrated under vacuum.

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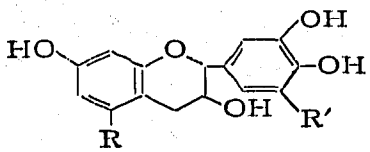
Fractionation of tannins by the "adsorption" principle

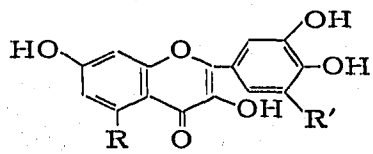
Fractionation was performed as above by the "preparative" method using 2% acetic acid as irrigant. For details see ref.[14].

Molecular weight estimation

The fractions obtained were methylated with excess diazomethane[17] and analysed for methoxyl content by using the absorption method of VIEBOCH AND BRECHER[18]. Molecular weights of the methylated tannin fractions were determined using the RAY[19] ebulliometer as modified by EVELYN[17,20] and incorporating improvements detailed by SMITH[21]. The molecular weights of the unsubstituted tannins were calculated from the determined molecular weights of the methylated derivatives and their methoxyl values.



(I)



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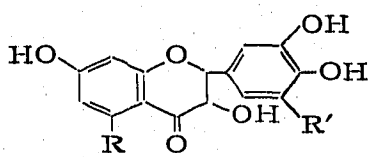

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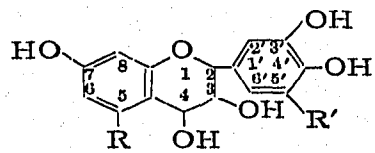

(IV)

RESULTS

Partition and adsorption paper chromatography of flavonoid compounds

Table I gives the R_F values of the following classes of compounds in the water-saturated *sec.*-butanol and in water: *catechins or flavan-3-ols*, *d*-gallocatechin (I, R = OH, R′ = OH), *d*-catechin (I, R = OH, R′ = H) and *dl-epi*-7,3′,4′-trihydroxyflavan-3-ol; *flavonols*, myricetin (II, R = OH, R′ = OH), quercetin (II, R = OH, R′ = H), robinetin (II, R = H, R′ = OH), fisetin (II, R = H, R′ = H); *dihydroflavonols*, ampeloptin (III, R = OH, R′ = OH), taxifolin (III, R = OH, R′ = H), dihydrorobinetin (III, R = H, R′ = OH) and fustin (III, R = H, R′ = H); *flavan-3,4-diols* (IV), the nomenclature of these compounds is obvious and follows the same sequence of hydroxylation as above. The number of phenolic hydroxyl groups is indicated for each compound in Table I.

Partition and adsorption chromatography of condensed tannins from black wattle barks

Table II gives the average molecular weights of the various tannin fractions in the partitioning mixture in relation to their mean R_F values. Fig. 1 illustrates the relationship between log $(1/R_F - 1)$ or R_M and average molecular weight.

Wattle and quebracho tannins show similar average molecular weight/R_F

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TABLE I

R_F VALUES OF "FLAVONOID" COMPOUNDS IN WATER-SATURATED *sec.*-BUTANOL AND IN WATER

Compound	*Number of OH groups*	R_F *in sec.-butanol*		R_F *in water*	
Catechins or flavan-3-ols					
d-Gallocatechin	6	0.54		0.32	
d-Catechin	5	0.67		0.35	
dl-epi-7,3′,4′-Trihydroxyflavan-3-ol	4	0.79		0.42	0.47
Flavonols					
Myricetin	6	0.65		0.0	
Quercetin	5	0.74		0.0	
Robinetin	5	0.59		0.0	
Fisetin	4	0.71		0.0	
7,8,3′,4′-Tetrahydroxyflavonol	5	0.41		0.0	
2,3-Dihydroflavonols					
Ampeloptin	6	0.73		0.24	
Taxifolin	5	0.80		0.28	
Dihydrorobinetin	5	0.68		0.35	
Fustin	4	0.81		0.36	
Flavan-3,4-diols					
5,7,3′,4′,5′-Pentahydroxyflavan-3,4-diol	7	0.48		0.45	
5,7,3′,4′-Tetrahydroxyflavan-3,4-diol	6	0.64		0.49	
7,3′,4′,5′-Tetrahydroxyflavan-3,4-diol	6	0.54		0.47	
7,3′,4′-Trihydroxyflavan-3,4-diol	5	0.68		0.48	
7,8,3′,4′-Tetrahydroxyflavan-3,4-diol	6	0.41	0.43	0.43	0.48

TABLE II

THE VARIATION OF THE AVERAGE MOLECULAR WEIGHT OF WATTLE TANNINS WITH R_F IN *n*-BUTANOL–ACETIC ACID–WATER (UPPER PHASE)

Mean R_F	*Wattle fraction* C_1		*Wattle fraction* C_2	
	*% extract eluted**	*molecular** weight of tannins*	*% extract eluted*	*molecular** weight of tannins*
0.0	1.1	—	12.5	2471
0.1	5.7	2045 *a*	27.0	1952 *b*
0.2	14.2	1457	33.3	1661
0.3	24.6	1271	20.6	1354
0.4	28.9	1018	4.1	1210 *b*
0.5	15.9	779	0.6	—
0.6	6.3	821 *a*	—	—
0.7 + 0.8	3.4	—	—	—
% recovery	80.6		68.6	
Determined av. mol. wt.		1284		1507

* Sugars run to R_F = 1–2 in the solvent system and the weight distribution therefore gives no indication of the tannin content of each fraction.

** The number-average molecular weights were obtained from the mean of readings at four concentrations, as readings are independent of concentration. Due to insufficient material resulting from fractionation only two readings were taken for samples designated *a*, and only one reading for samples marked *b*.

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relationships during "adsorption" separation in 2% acetic acid on thick sheets[13]. The average molecular weight of the "condensed" tannins increases progressively with decrease of R_F over the ranges 554–3240 for wattle and 790–2350 for quebracho.

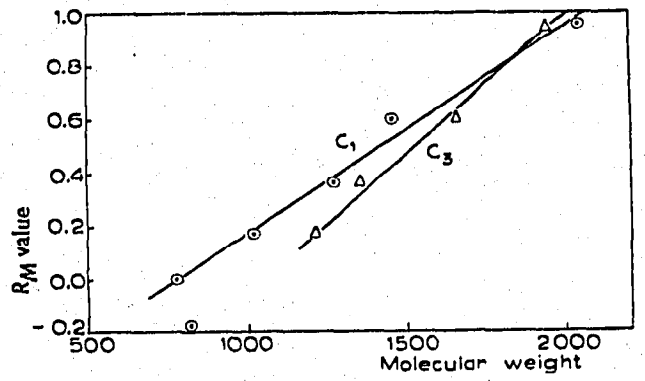

Fig. 1

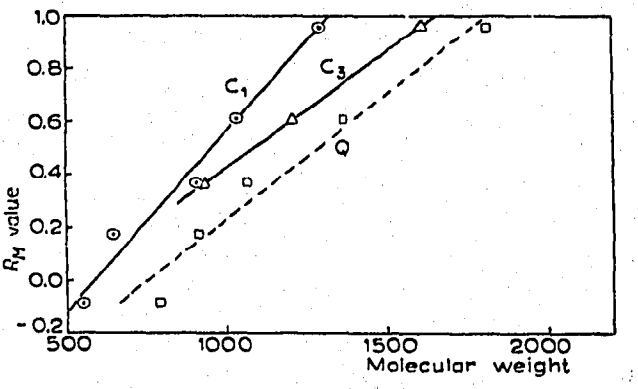

Fig. 2

Fig. 1. The relationships between molecular weight and R_M (= log ($^1/R_F$ — 1)) for wattle tannin fractions C_1 (ethyl acetate soluble) and C_3 (methanol soluble) in *n*-butanol–acetic acid–water (6:1:2).

Fig. 2. The relationship between molecular weight and R_M for wattle tannin fractions C_1 and C_3, and for commercial quebracho tannins (Q) in 2% acetic acid.

Fig. 2 shows the relationship between R_M and average molecular weight in 2% acetic acid for the same wattle tannin fractions as used for partitioning separation, and also for quebracho tannins.

DISCUSSION

One of the most notable phenomena of the paper chromatography of flavonoid compounds in aqueous medium is the immobility of the aglycones of flavonols, flavones and anthocyanidins, aurones and chalcones[5,22,23]. ROUX[10] considered that the zero R_F in water was associated with the planar and also resonating nature of the C_{15} compound as a whole, and this view was later supported by ROBERTS and co-workers[7]. Further support comes from observation that in order that a dye may have affinity for cellulose (cotton), the molecule should have the ability of taking up a linear or planar configuration, and should contain hydrogen-bonding groups, preferably spaced some multiple of 10.3 Å apart[24–26]. Evidence is strongly in favour of a lock and key process of adsorption[27]. As soon as the planar nature of the C_{15} unit is modified through the attachment of sugar residues, *e.g.* glycosides of flavonols and anthocyanins, or through the removal of a double bond in the heterocyclic ring, *e.g.* 2,3-dihydroflavonols, mobility is conferred on the unit in aqueous medium. This behaviour of the above classes of compounds is of great diagnostic value in two-dimensional paper chromatography.

The principle of slight reduction of R_F with increase of hydroxylation in water as solvent, first shown by ROBERTS, CARTWRIGHT AND WOOD[7], is clearly demonstrated for the catechin series in Table I, and for the catechins, gallocatechins and their gallates[7]. The 2,3-dihydroflavonols also show a decrease of R_F with increase of hydroxylation (Table I) although their relative configurations are not known. This

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rule by ROBERTS and co-workers does not apply in all instances, however, and appears to be valid only for increases of *phenolic hydroxylation*. For example, on hydrogenation of the 2,3-dihydroflavonols the R_F of the reduction product is increased from 0.24–0.36 to 0.45–0.49. This large increase is due to the introduction of an aliphatic hydroxyl group in the 4 position in the place of a carbonyl group. The flavan-3,4-diols (two aliphatic hydroxyls) also have higher R_F values than the corresponding catechins (one aliphatic hydroxyl, Table I) in aqueous irrigants. The general rule thus appears to be that increase in *phenolic hydroxylation* decreases the R_F of C_{15} compounds in water, but that increase of *aliphatic hydroxylation* (in the heterocyclic ring), increases the R_F.

From data by GAGE, DOUGLASS AND WENDER[5] it may be deduced that for flavonols and flavones, increase of phenolic hydroxylation results in a decrease in R_F also in 15% and 60% aqueous acetic acid.

In the partitioning mixture, *sec.*-butanol, the same rule demonstrated by BATE-SMITH and co-workers[3,4] for *n*-butanol–acetic acid–water solvent mixture applies; namely, that the R_F is reduced with increase of hydroxylation (see catechin series in Table I and also note the following R_F's in butanol–acetic acid–water: *l*-epiafzelechin (0.73)[28], *l*-epicatechin (0.65)[4] and *l*-epigallocatechin (0.47)[4]). This phenomenon is valid also for the introduction of aliphatic hydroxyl groups, *e.g.* the hydrogenation of the 2,3-dihydroflavonols (one aliphatic hydroxyl) to the corresponding flavan-3,4-diols (two aliphatic hydroxyl groups), and is accompanied by a decrease in R_F in each instance in partitioning solvents. Also the presence of two *ortho*-hydroxy groups on different benzene nuclei, *e.g.* 7,8,3′,4′-tetrahydroxyflavonol and 7,8,3′,4′-tetrahydroxyflavan-3,4-diol, appears to have an abnormally large effect in reducing the R_F compared with compounds of the same type, and containing the same number of substituent hydroxyl groups (see Table I). Similarly data by GAGE, DOUGLASS AND WENDER[5] show that quercetagetin (5,6,7,3′,4′-pentahydroxyflavonol) and gossypetin (5,7,8,3′,4′-pentahydroxyflavonol) both possessing two *ortho*-hydroxy groups on different benzene nuclei, have lower R_F values (0.22 and 0.21 respectively) in *n*-butanol–acetic acid–water (4:1:5) than myricetin (5,7,3′,4′,5′-pentahydroxyflavonol, $R_F = 0.45$) with the *ortho*-hydroxy group present on only one phenyl nucleus.

Amongst the dihydroflavonols (Table I) the similar R_F values of the pairs ampeloptin (six OH groups) and dihydrorobinetin (five OH groups), and of taxifolin (five OH groups) and fustin (four OH groups) is of interest. The abnormally high R_F values of ampeloptin and taxifolin, both of which have hydroxyl groups in 5-position, is due to the strong intra-molecular hydrogen bond formed between this hydroxyl and the carbonyl group at the 4-position. Ampeloptin and taxifolin, therefore, have similar R_F's as dihydrorobinetin and fustin respectively where the hydroxy group in the 5-position is absent. The reduction of hydroxyl character with the formation of chelate rings was previously observed by BATE-SMITH AND WESTALL[3] for phenolic carboxylic acids.

Finally, it should be noticed that, by comparison, the 2,3-dihydroflavonols always have higher R_F values than the corresponding flavonols themselves, *e.g.*

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myricetin → ampeloptin ΔR_F = 0.08, quercetin → taxifolin ΔR_F = 0.07, robinetin → dihydrorobinetin ΔR_F = 0.09 and fisetin → fustin ΔR_F = 0.10. This increase in R_F is probably associated with the conversion from a planar flavonol structure to non-planar (two asymmetrical carbon atoms) dihydroflavonol structure. The planar flavonol structures would show slightly higher affinity for cellulose than the corresponding dihydroflavonols. This theory was first advanced by ROBERTS[29] to explain differences in R_F between epicatechins (lower R_F) and catechins. Similar behaviour in the butanol–acetic acid–water partitioning mixture of the above series of flavonoid compounds may also be deduced from tables previously presented in ref.[4].

The chromatographic behaviour of condensed tannins some of which may be regarded as polymers of flavan-3-ols (catechins)[30–32] and of flavan-3,4-diols (leucoanthocyanins)[33–36], is of interest. The condensed tannins of black wattle bark (*Acacia mollissima*) show an increase of R_F with decrease of average molecular weight in both "partitioning" (Table II) and "adsorption"[14] solvents on paper chromatograms. The straight-line relationship between R_M and molecular weight in both instances emphasises the regularity of this relationship (Figs. 1 and 2). BATE-SMITH AND WESTALL[3] had previously found a straight-line relationship between R_M and the *degree of hydroxylation* for C_{15} "flavonoid" compounds of related structure.

PETERS AND SUMNER[37] (see also ref.[7]) have shown the correlation between affinity for cellulose and molecular weight of a large number of anthraquinone derivatives. This observation apparently agrees with the present findings of the linear R_M/molecular weight relationship of the tannins, if the mobility of the tannins on the cellulose substrate may be regarded as a measure of their "affinity" for cellulose. This represents the first occasion on which a linear relationship between R_M and average molecular weight of naturally-occurring compounds has been established.

The studies of chromatographic behaviour of the "flavonoid" compounds and of the tannins are of diagnostic value in interpreting the nature of the complex mixture of compounds usually present in natural plant extracts by means of paper chromatography.

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SUMMARY

Various factors influencing the paper chromatographic behaviour of "flavonoid" compounds in "partitioning" and "adsorption" solvent systems are examined Naturally-occurring "condensed" tannins are also examined in these solvent systems, and it is shown that the average molecular weight of the tannins increases with decrease of R_F, and that a linear relationship exists between R_M and their average molecular weight.

This study is of value in interpreting chromatograms of naturally-occurring tannin–"flavonoid" mixtures.

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