Biochem. J. (1969) **113**, 757
Printed in Great Britain

The Separation and Distribution of Simple and Condensed Leucoanthocyanins of the Tea Plant (*Camellia sinensis* L.)

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(*Received* 31 *December* 1968)

1. Leucoanthocyanin monomers of high mobilities in aqueous solvents on thin-layer chromatograms, assumed to be structurally simple, were characteristic of mature bulky tissues, whereas members of lower mobility were confined to young vegetative and floral tissues. 2. Flavylogens were separated by gel filtration on Sephadex columns into monomeric, oligomeric and polymeric fractions. 3. The polymeric fraction from young brown stems was heterogeneous, one-half having a molecular weight of about 3400, one-third a molecular weight between 3600 and 17000, and the remainder a molecular weight of over 17000. 4. Leaves had low flavylogen concentrations; only monomers were present. Stem tissues were rich in polymers, which increased with the age of the young stem and decreased inwards through the wood. The maximal flavylogen concentrations were in the phloem and cambium from mature stems, where all three fractions were richly present. The periderm tissue and, to a lesser extent, the seed coat were characterized by a very high polymer/monomer ratio, exhibiting a much higher degree of polymerization than the wood. Root tissues contained high concentrations of monomers. 5. In general, there was an inverse correlation between the extent of polymerization and the complexity of the monomers present. 6. The results are in favour of the thesis that the function of the flavanols is, after polymerization to condensed tannins, to impregnate dead structural tissues and thereby to protect them from infection and decay.

A survey of the polyphenolic distribution over the tea plant (Forrest & Bendall, 1969) showed the leucoanthocyanins to occupy a special place in flavonoid metabolism, in that they were present in all tissues and were particularly characteristic of the woody tissues of stem, root and seed. The present work was designed to shed further light on the importance and function of the leucoanthocyanins by separating the polymers into fractions of different molecular size, and by describing the distribution of these different categories, and also of the several components of the monomeric fraction, within the plant.

MATERIALS AND METHODS

Plant material. The sources of material were as described in the preceding paper (Forrest & Bendall, 1969). In addition, a sample of commercial black-wattle-bark extract (*Acacia mearnsii* De Wild.) was kindly given by Dr T. Swain.

Separation and determination techniques. Tissues were extracted as in the preceding paper (Forrest & Bendall, 1969), and t.l.c. and colour reactions were similarly used for the separation and identification of polyphenols. Flavylogens were separated into different fractions by gel filtration on Sephadex (Pharmacia, Uppsala, Sweden) columns. The most suitable gel was found to be G-25 (fine grade), which was allowed to swell overnight in the solvent, aq. 50% (v/v) methanol, before the column was packed. A column of length 12·7cm. and of volume 2·82ml./cm. was used for most of the separations, having total bed volume (V_t) 36ml., and void volume (V_0) 13·5ml. (determined by running a solution of blue dextran in aq. 50% methanol through the column); the flow rate was about 58ml./hr. Alcohol-resistant Teflon tubing was used for all connexions. Extracts of tissues in aq. 50% methanol were applied to the column, and fractions of 104 drops (approx. 2·4ml.) were collected from the column with a fraction collector.

Flavylogen determinations were carried out on the fractions by a modification of the method described by Swain & Hillis (1959). Samples (1ml.) of the fractions were pipetted into small bijou bottles (capacity approx. 5ml.) with screw tops, and 3ml. of the leucoanthocyanin reagent (25ml. of conc. HCl, sp.gr. 1·18, diluted with butan-1-ol to 500ml.) were added from a Jencons Zipette. The tops were firmly screwed on and the bottles were heated at 97° for 15min. in a water bath, by which time maximum colour had developed. They were then removed and cooled, and the extinctions of the solutions were measured at 550nm. against distilled water on the Unicam SP. 500 spectrophotometer.

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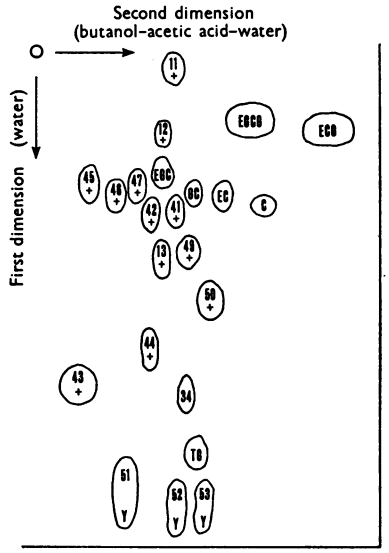

Fig. 1.* Thin-layer cellulose chromatogram (diagrammatic) showing the positions of the main leucoanthocyanin spots (numbered) relative to the catechins and theogallin. The solvent fronts are shown by solid lines.

RESULTS

Distribution of low-molecular-weight components

Thin-layer chromatograms showed a comparatively large number of discrete leucoanthocyanin spots, although in tissue extracts containing polymerized material some of these were obscured by superimposed streaking. Fig. 1, a generalized chromatogram, shows the positions of the main spots relative to the catechins and theogallin, and Table 1 summarizes results obtained by inspection of chromatograms of extracts of various tissues, which are arranged approximately in order of increasing 'bulk', the first being the young, actively synthesizing, apical tissues and the last being the massive tissues of stem and root, which are presumed to have a lower rate of metabolism and general synthetic ability. The leucoanthocyanins are arranged approximately in order of increasing mobility in the two solvent systems normally used, water and butan-1-ol–acetic acid–water (4:1:5, by vol., upper phase, aged). A positive correlation is evident, the most metabolically active tissues tending to contain components of lower mobility. The structural variations on the molecule responsible for differences in mobility are unknown, but it is reasonable to suppose that increasing hydroxylation is one important factor causing a decrease in mobility in both solvent systems (Harborne, 1959), in which case the leucoanthocyanin distribution would parallel that of the catechins (Forrest & Bendall, 1969) in having the more fully hydroxylated members restricted to apical tissues. However, there are also the possibilities of esterification (e.g. with gallic acid), of glycosylation (King, 1966; Kumari, Mukerji & Seshadri, 1966) and of conjugation with catechins to form flavanol dimers (Bate-Smith & Swain, 1965; Creasy & Swain, 1965); but in general it may be said that greater molecular complexity will lead to decreased mobility. Certainly the leucoanthocyanins staining yellow, and therefore assumed to lack 5-hydroxyl groups (Roux & Maihs, 1960), had the highest mobilities in aqueous solvents and were very characteristic of mature stem and root tissues, whereas those with low mobilities, particularly components LA-11, -12, -45 and -46 were typical of leaves and flowers, being practically absent from most other tissues. Even within the flower it was found that the ovary, the bulkiest tissue, had a higher proportion of leucoanthocyanins of high mobility, and the most fragile tissue, the corolla, was rich in components of lower mobility. The calyx, intermediate in bulk, was correspondingly intermediate in contents, the main leucoanthocyanins being those usually found in leaves, as might have been expected from its foliar nature.

Separation of flavylogen fractions by gel filtration

Aromatic compounds are adsorbed to different degrees on the polysaccharide network of Sephadex gels, and hence are retarded on a column to a greater degree than would otherwise be expected from their molecular weights (see, e.g., Somers, 1966). The use of aq. 50% (v/v) methanol as solvent allowed the polymers from tea extracts to pass through unretarded, whereas the monomers suffered from adsorptive retardation.

Stem tissues. Extracts of apical soft green stem, mature green stem and first-year brown stem from bushes of various clones growing in Cambridge were applied to the column; the results of flavylogen analyses of the eluates are shown in Fig. 2. The first of the three main peaks was completely excluded from the column and must therefore be of high molecular weight. When this fraction was concentrated and chromatographed on the thin-layer system, the only material staining with the benzidine reagent was two brown streaks extending from the origin for a short distance in the direction of each solvent, and most of the material did not move at

* Abbreviations used in figures and tables: B, blue; C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; FW, fresh weight; G, green; GC, (+)-gallocatechin; LA or +, leucoanthocyanin; O (at top left-hand corner), origin; O, orange; P-, pale; P, purple; TG, theogallin (galloylquinic acid); Y, yellow.

Table 1. *Distribution of the main leucoanthocyanins within the plant*

Only occurrences of significant concentration are tabulated. The relative amounts of substances, estimated by inspection of chromatograms, are shown by numbers of × signs.

Compound (LA-) ...	11	12	45	46	47	43	44	42	41	13	49	50	51	52	53
Leaf															
Young	×			×		××	×	×		××	×				
Mature	××	×	××			××	×	×		×					
Flower															
Bud	×	××	××	×	×			×	×	×	×	×			
Corolla	×	×	×												
Stamens		×							×	×	×				
Calyx		××	××	×	×			××	×		××				
Ovary					×	×		×	×		××	××			
Seed															
Coat								×	×	×	×	××			
Stem															
Young				×		××		××		×	××	×	×		××
Old															
Periderm												×	×	×	×
Phloem	×	×							××			×			×
Xylem									×	×	×	××			×
Root															
Young											×	×	×	×	××
Old															
Bark											××	××	×	×	×
Xylem										×	×	×	×	×	××

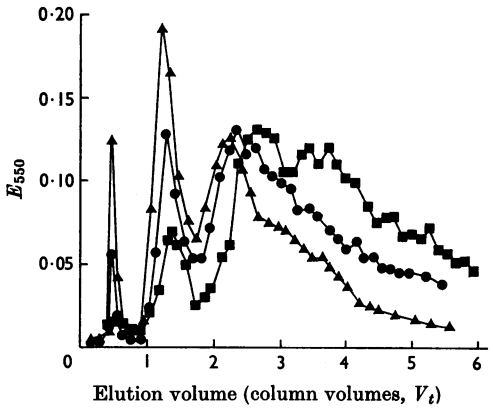

Fig. 2. Flavylogen concentrations of fractions of young stem extracts eluted from a Sephadex G-25 (fine grade) column in aq. 50% (v/v) methanol. ■, Apical soft green stem, load 410mg. fresh wt.; ●, mature green stem, load 780mg. fresh wt.; ▲, first-year brown stem, load 660mg. fresh wt.

all. The fraction as it moved down the column was greenish yellow. An estimate of its minimal molecular weight was made: since 1g. of dry Sephadex G-25 powder swells to 4ml. of gel in aq. 50% methanol, i.e. about 80% of the corresponding value in water, the minimal molecular weight for complete exclusion is about 3200 (the limit for complete exclusion of dextran homologues in water being a molecular weight of 4000). If a molecular weight of about 300 is assumed for the monomeric precursors, this polymeric fraction would be at least decameric. Roux & Paulus (1962) found that the mobilities of leucofisetinidin polymers in both alcoholic and aqueous solvents decreased progressively as the degree of polymerization increased; the pentamer moved in each solvent but the decamer remained at the origin, in agreement with the immobile fraction from tea being at least decameric.

Further indication of the molecular weight was obtained by running the young-brown-stem extract down columns of less highly cross-linked Sephadexes in aq. 50% methanol. The results given in Table 2 show that the polymeric fraction that is completely excluded from Sephadex G-25 is heterogeneous: 48% has a molecular weight of about 3400, 36% has a molecular weight of 3600–17000, and the remaining 16% has a molecular weight of over 17000. Sephadex G-50 and G-75 did not give good separations of the remaining fractions; in both cases the retarded fraction of the polymer produced a peak that merged with that of the oligomer, which in turn merged with that of the monomers. Adsorption of the monomers was practically nil, and no trailing

Table 2. *Fractionation of polymeric flavylogens from tea stem on Sephadex gels*

Sephadex	Degree of swelling in aq. 50% methanol (% of that in water)	Calculated minimum mol.wt. for complete exclusion	Proportion of polymeric fraction completely excluded (%)
G-25	80	3200	100
G-50	40	3600	52
G-75	38	17000	16

was observed. Thus the good separations of the monomeric and oligomeric fractions achieved on Sephadex G-25 were largely due to adsorptive retardation.

The second peak of the young-stem flavylogens, occurring in the eluate of Sephadex G-25 columns after about $1{\cdot}3\,V_t$, was orange–brown, and on t.l.c. gave an orange streak with the benzidine reagent at R_F 0·80 in water and streaking (R_F 0·0–0·4) in butanol–acetic acid–water. There were a small number of discrete orange spots distinguishable within this streak. The fraction probably consisted mainly of oligomeric material of high mobility in water (see below), but also contained gallic acid, the gallate G-36 (Forrest & Bendall, 1969), and the depsides.

The third peak was clearly composite, exhibiting maxima at different positions in the eluates of the three types of stem, and included a subsidiary peak centred at about $3{\cdot}5\,V_t$. T.l.c. of the various fractions showed that the leucoanthocyanins were those that run as discrete spots (see Fig. 1), and that partial separations were achieved on the column. It was found that, with increasing volume of eluate, the concentration of each leucoanthocyanin reached a maximal value at a definite point, and that these maxima occurred strictly in order of decreasing mobility of the spots on cellulose thin layers in aqueous solvents. At $2{\cdot}1\,V_t$ the eluate contained predominantly components LA-43 and -44 (Fig. 1) and also the simple catechins; at $2{\cdot}2\,V_t$, component LA-50 predominated; at $2{\cdot}6\,V_t$, components LA-49 and -13, and also the catechin gallates in the young-soft-stem extract; at $3{\cdot}1\,V_t$, component LA-41, with smaller amounts of component LA-49; at $3{\cdot}5\,V_t$, component LA-41 in mature stem and components LA-42 and -46 in young stem. In all later fractions there were only leucoanthocyanins of low mobility in water, chiefly components LA-11 and -12, which gradually decreased in definition and gave way to a small region of trailing of low mobility. Differences in degree of hydroxylation were probably important in this preferential retention on both cellulose and Sephadex, whether due to direct hydroxylation of the flavonoid skeleton or due to esterification with a hydroxyl-rich residue such as gallate.

The properties of the three main peaks are thus consistent with their identification as polymeric, oligomeric and monomeric species respectively. The polymeric fraction, absent from the young stem, increased in the older stem with age. The oligomeric fraction also increased with age until it was the predominant fraction in the mature brown stem. The monomers decreased in importance with age; within this fraction there were very marked differential decreases in the peak at $3{\cdot}5\,V_t$ and in the trailing fraction thereafter, and a gradual shift in the fraction maximum from about $2{\cdot}6\,V_t$ to $2{\cdot}2\,V_t$ as the stem matured. This indicated that during stem maturation the proportions of leucoanthocyanins of lower mobility (and therefore greater complexity) gradually decreased, the predominant components changing to those of the highest mobility. Since there was a decrease in the concentrations of the less mobile monomers, this may have been due to preferential polymerization of the most complex monomers. The polymeric fractions must be present in greater concentrations relative to the monomers than would appear from the graphs, since Roux & Paulus (1962) showed that the yield of anthocyanidin in the acid–butanol reaction decreased with increasing degree of polymerization, the monomers giving about four times the percentage yield of the polymers. However, the widths of the peaks were approximately proportional to the percentage yield of anthocyanidin of each polymeric fraction, so that it was considered that, rather than converting the areas under the peaks into the corresponding values for total flavylogen, it would be simpler merely to record the height of each peak per unit weight of material applied to the column. This would give an approximate estimate of the proportions of each fraction present, the underestimation on polymeric peaks due to low percentage yields of anthocyanidin being counterbalanced by the relatively narrow widths of these peaks. The results obtained in this way for various tissues are given in Table 3.

In the old mature stem, the secondary phloem (including the cortex and cambium) contained very high concentrations of all three fractions which exceeded those in any other part of the plant (see Table 3). As one proceeded outwards to the peri-

Table 3. *Relative concentrations of flavylogen fractions from extracts of different tissues after separation by gel filtration*

Concentrations are given as the peak extinctions/g. fresh wt. applied to the Sephadex G-25 column (see the text for justification of this). The elution positions are given in column volumes (V_t) of each fraction. The amounts of trail material are shown by numbers of × signs.

Tissue	Polymer	Oligomer	Monomers		Trail
	$0{\cdot}5\,V_t$	$1{\cdot}3\,V_t$	$2{\cdot}3\,V_t$	$3{\cdot}3\,V_t$	
Apical bud	—	0·04	0·13	0·02	—
Young leaf	—	—	0·14	0·01	—
Mature leaf	—	—	0·06	0·03	—
Old leaf	—	0·04	0·08	0·07	(×)
Flower bud	—	0·10	0·30	0·32	× ×
Ovary	—	0·14	0·29	0·14	×
Seed coat	0·44	0·28	0·24	0·17	× ×
Young stem					
Apical	—	0·15	0·32	0·27	× ×
Green	0·07	0·16	0·17	0·11	×
Brown	0·18	0·29	0·19	0·09	—
Old stem					
Periderm	0·19	0·15	0·04	0·03	—
Phloem	1·12	0·91	0·93	0·71	× ×
Xylem					
Outer	0·073	0·044	0·047	0·033	×
Middle	0·018	0·042	0·039	0·023	(×)
Inner	0·003	0·036	0·032	0·021	—
Root					
Apex	0·01	0·16	0·17	0·14	× ×
Young	0·07	0·50	0·55	0·43	× ×
Bark	—	0·13	0·42	0·35	× × ×
Xylem	0·01	0·13	0·14	0·08	(×)

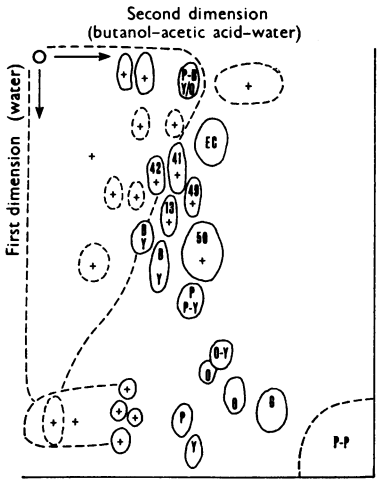

Fig. 3. Thin-layer cellulose chromatogram of extract of outer wood from a 6-year-old stem of clone Likanga 8/D4. The solvent fronts are shown by solid lines.

derm the concentrations fell off markedly, the monomers being finally nearly absent; and inwards through the wood the concentrations were even lower, the proportion of polymers falling rapidly towards the centre of the stem, although the monomers and oligomers were relatively well represented throughout. The appearance of a thin-layer chromatogram of the outer-wood extract is shown in Fig. 3. The polymeric fraction consisted solely of immobile or hardly mobile material; the oligomeric fraction contained the streak and spots situated near the aqueous solvent front, with low mobility in the alcoholic solvent; and the two monomeric peaks, at $2{\cdot}5\,V_t$ and $3{\cdot}5\,V_t$, consisted predominantly of the spots LA-50 and LA-49 respectively. The leucoanthocyanins of low mobility in water were confined to the trail beyond $3{\cdot}5\,V_t$.

Root tissues. The greatest flavylogen concentrations occurred in the young root (see Table 3) and monomers generally formed a much higher proportion of the total than in the stem tissues. Considerable trailing was evident, especially with the root-bark extract, when the column still retained some flavylogen even after more than $12\,V_t$ of solvent had passed through. A further characteristic of roots was the high proportion of 5-deoxyleucoanthocyanins staining yellow with the benzidine reagent. Fig. 4 shows a chromatogram of root wood with ten yellow-staining monomers; these seemed

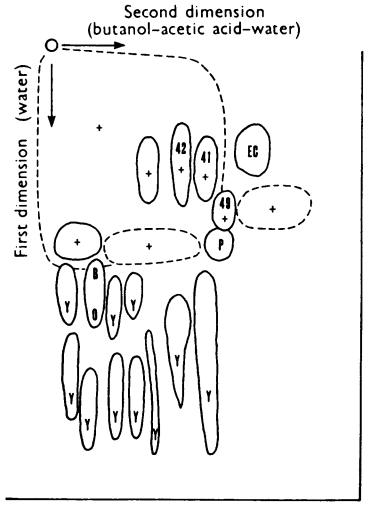

Fig. 4. Thin-layer cellulose chromatogram of extract of root wood of clone Likanga 8/D4. The solvent fronts are shown by solid lines.

to replace to some extent the 5,7-dihydroxyleucoanthocyanins of the stem wood.

Other tissues. Leaves were characterized by the absence of polymers and a much smaller oligomeric fraction (Table 3). The monomeric fraction of young leaves consisted primarily of a peak at 2·0 V_t, and with increasing age of the leaves a second peak at 3·7 V_t became important. The separation of these two monomeric peaks was very marked compared with the stem extracts, and the reason for this is clear on inspection of Table 1 and Fig. 1. The main monomers of mature leaves were the highly mobile compounds LA-43 and -44, and the much less mobile group of compounds LA-45, -11 and -12. All intervening monomers of intermediate mobility, mainly the group of compounds LA-13, -41, -49 and -50, so characteristic of stem tissues, were much less well represented in leaves, so that there was a definite minimum on the gel-filtration curve in the centre of the monomeric region. Leaves, then, exhibited an almost complete inability to polymerize leucoanthocyanins. The only change that occurred between the youngest leaves newly separated from the apical bud and leaves about 1 year old was a decrease in the simplest monomers and an increase in the most complex group of monomers.

Similarly, flower buds contained predominantly monomers (Table 3), as would be expected from the large number of discrete leucoanthocyanin spots on chromatograms (Forrest & Bendall, 1969), but a small oligomeric peak was also present. Ovary tissue gave a higher oligomeric peak, and a predominance of the simpler monomers (confirming the results in Table 1). The floral tissues showed a greater capacity for flavylogen synthesis than the leaves (see Table 3) but were almost equally incapable of effecting polymerization. The seed coat, on the other hand, was characterized by high concentrations of oligomers and polymers, monomers still being abundant as in young floral tissues.

Confirmation of fraction identifications. As an independent means of confirming the identities of the three main flavylogen fractions, tannins of different molecular size were separated by the technique of Goldstein & Swain (1963), after which each fraction was subjected to the standard gel-filtration procedure. For this purpose it was necessary to use a plant extract containing a suitably wide range of polymeric fractions, and commercial black-wattle-bark extract (*Acacia mearnsii* De Wild.), known to have a high tannin content (Roux, 1958; Roux, Maihs & Paulus, 1961; Drewes & Roux, 1963), was chosen. The powdered bark was extracted successively with absolute methanol to extract monomers and oligomers, aq. 50% (v/v) methanol to extract polymers and residual oligomers, and water to extract any remaining polymeric material. Two extractions were performed with each solvent, and each extract, after concentration, was passed down the Sephadex G-25 column. The monomeric extract had a peak at 3·3 V_t, being adsorbed similarly to the tea monomers; polymers and oligomers passed through at 0·6 V_t and 1·4 V_t, corresponding to the two fractions from tea thus identified. The final aqueous extract had a composition similar to the aqueous-methanol extract, but contained a smaller proportion of monomers. These results confirm the identification of the three flavylogen peaks as polymeric, oligomeric and monomeric.

DISCUSSION

These studies indicate that leucoanthocyanin synthesis was characteristic of axial rather than of foliar tissues. Leaves not only had a low synthetic ability for leucoanthocyanins, but also were unable to effect polymerization. Within the plant axis, moreover, monomeric leucoanthocyanins predominated in the root, whereas oligomers and polymers characterized the mature stem. A comparison of Tables 1 and 3 shows that those organs with the simplest monomers were also the most efficient at synthesizing polymers; it may therefore be that the more complex monomers, owing to structural hindrance or to enzyme specificity, can be polymerized to a lesser extent than can the simple monomers. Extensive polymerization was confined to the outer stem and seed coat, tissues marked both by comparative bulkiness and by the presence of a small group of highly mobile monomers.

Forrest & Bendall (1969) showed that the bulkier tissues had mainly simple rather than complex catechins, and the same seems to be true of the flavandiols. However, whereas catechin synthesis was much decreased in such tissues, flavandiol synthesis was not.

The highest flavylogen concentrations were in the region of the vascular cambium and secondary phloem. Hillis (1955) and Hillis & Carle (1960) also observed high leucoanthocyanin concentrations in the cambium and phloem of eucalypt stems, with much lower concentrations in the wood. The high efficiency of polymerization in the periderm compared with the wood of tea may be compared with similar observations on a number of trees by Drewes & Roux (1963). Suggestions have been made (e.g. Roux & Evelyn, 1958*b*, 1960) that the monomeric precursors of tannins are, in certain species, synthesized in the vascular cambium and then translocated outwards to the bark and inwards to the xylem, where they are polymerized to different degrees by separate enzyme systems. Nevertheless, the directions of flavylogen metabolism clearly differed in periderm and wood, only the former exhibiting a conversion into tannins so intensive as to lower dramatically the proportion of monomers.

As in all other cases studied, the polymeric flavylogens were found to be heterogeneous. However, the molecular-weight range found in tea stem, with a substantial proportion higher than 17000, extends considerably beyond the range reported in bark and wood of other trees, in which the molecular weights lie mainly in the region 600–3000 (Roux, 1958; Roux & Evelyn, 1958*a*,*b*, 1960; Roux & Paulus, 1962). However, the bulk of the condensed tannins of wine studied by Somers (1966), using gel filtration, were in the molecular-weight range 2000–5000, and some material reached values of up to 50000; this range and diversity of polymerization is similar to that encountered in the young-tea-stem flavylogens.

Polymerization was maximal in those dead tissues that were also external, i.e. the bark and seed coat; in the root and inner-stem wood the extent of polymerization was much less. This distribution offers support for the thesis that the basic function of the flavanols is polymerization to condensed tannins, which, by deposition in dead lignified or suberized tissues liable to infection, protect the plant from invasion by harmful organisms and from the initiation of decay.

G.I.F. thanks the Science Research Council for financial support.

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