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## Leuco-Anthocyanins

### 1. DETECTION AND IDENTIFICATION OF ANTHOCYANIDINS FORMED FROM LEUCO-ANTHOCYANINS IN PLANT TISSUES

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(Received 10 October 1953)

A method for the detection of leuco-anthocyanins, and the chromatographic identification of the anthocyanidins formed from them by boiling with mineral acid, has been described in an earlier paper (Bate-Smith, 1953). This method has been improved and applied to the leaves and other tissues of numerous species of plants. In the present paper the results of identification of anthocyanidins produced from leuco-anthocyanins in some of these species are reported. In the paper which follows the systematic distribution of the leuco-anthocyanin reaction in leaves is discussed.

### EXPERIMENTAL AND RESULTS

#### *Paper chromatographic separation of anthocyanidins*

The main problem is to obtain a solution of the anthocyanidins in sufficient concentration for paper chromatography, without at the same time concentrating irrelevant substances. This is accomplished by converting the leuco-anthocyanins into anthocyanidins in aqueous solution (rather than in methanol as described previously) and extracting the anthocyanidins with *isoamyl* alcohol.

Conveniently, 0.2–1.0 g. of tissue is covered with 2N-HCl (about 3 ml.) in a test tube, and heated in boiling water for 20 min. The aqueous solution is decanted (filtered if necessary) into a small narrow test tube and shaken with sufficient *isoamyl*

alcohol (3-methylbutan-1-ol) to give a supernatant layer just deep enough to be drawn cleanly into a capillary tube, from which the solution is spotted on the starting line of the chromatogram. The applications are repeated, employing a current of hot air to accelerate drying, until the colour is deep enough to ensure visibility of the anthocyanidins on the developed chromatogram. A marker of known identity is applied on each paper.

In order to prevent the anthocyanidins from fading, it is necessary to maintain a low pH during chromatography. This was originally achieved (Bate-Smith & Westall, 1950) by using the upper phase of the mixture *n*-butanol-2N-HCl (1:1, v/v). A solvent brought to our notice by Forestal Laboratories has given better-defined spots and more consistent results. This 'Forestal solvent' consists of water-acetic acid-conc. HCl (10:30:3, v/v). Solvents containing *m*-cresol and HCl have also been tested, and one of these consisting of *m*-cresol-5.5N-HCl-acetic acid (1:1:1, v/v) (acetic acid is added in order to bring the aqueous and phenolic constituents into a single phase) has given promising results. As was found with the phenolic solvents employed in earlier work on the chromatography of flavonoid compounds (Bate-Smith, 1949), this solvent has the effect of suppressing the lyophilic properties of methoxyl groups to a greater extent than the aliphatic solvents, so that the order

Table 1.  $E_{\max}$  and  $R_f$  values of some anthocyanidins

	$E_{\max}$ (m $\mu$ .)	Solvent system		
		<i>n</i> -Butanol-HCl	Acetic acid-HCl	<i>m</i> -Cresol-HCl-acetic acid
		$R_f$ value		
Pelargonidin	530	0.80	0.68	0.82
Cyanidin	545	0.69	0.50	0.69
Paeonidin		0.72	0.63	0.87
Delphinidin	555	0.35	0.30	0.52
Petunidin		0.45	0.45	0.75
Malvidin		0.53	0.60	0.90

Table 2. Spectrographic examination of anthocyanidins from leaves

Species	$E_{\max}$ (m $\mu$ .)	Chromatographic identification
<i>Cinnamomum sericeum</i>	544	Cyanidin
<i>Eriobotrya japonica</i>	543	Cyanidin
<i>Persea gratissima</i>	545	Cyanidin
<i>Raphiolepis indica</i>	543	Cyanidin
<i>Rhoicissus rhomboidea</i>	547, 553	Cyanidin, delphinidin
<i>Rumex lunaria</i>	545	Cyanidin
<i>Thea sinensis</i> (dried green leaf, China type clone)	543, 557	Cyanidin, delphinidin

## Application to various plant tissues

The earlier work (Bate-Smith, 1953) was confined to a study of white flowers, in which interference from other substances present is reduced to a minimum. No difficulty has been experienced in extending the study to leaves, in spite of the presence of chlorophyll, since this is largely destroyed in the process of heating with HCl. Any traces of chlorophyll which may remain are extracted by the *isoamyl* alcohol, and appear on the chromatogram near the solvent front. Leaves and other tissues pigmented with anthocyanin are, however, not easy to work with and have so far been avoided. The leaf tissues examined were from mature leaves not evidently pigmented with anthocyanins, freed from petioles and larger veins. Both immature and senescent leaves may contain anthocyanin pigment which may not be visible on mere inspection, and were therefore avoided in a survey confined to the incidence of leuco-anthocyanins. If anthocyanin pigmentation is not excessive, leuco-anthocyanins might still be detected in such instances by comparison with a control prepared from an aqueous extract of the tissue hydrolysed with dilute acid. Robinson & Robinson (1933) suggested such a procedure, selectively extracting the hydrolysed anthocyanidin with *isoamyl* alcohol and developing the leuco-anthocyanin in the underlying aqueous layer. This would of course only apply if the leuco-anthocyanin were itself insoluble in *isoamyl* alcohol. Rarely, tissues may contain pre-formed anthocyanin in colourless form which is converted into the normal coloured form on acidification; these also have been excluded from the present study.

Catechins, when heated with hydrochloric acid, are converted into 'phlobaphenes' which dissolve in *isoamyl* alcohol to give deep golden (catechins proper) or brown (gallo catechins) solutions.

The tissues of species belonging to certain families give strong colour reactions which might interfere with the detection and identification of anthocyanidins. Such species are *Caryopteris tangutica* and *Tectona grandis* in Verbenaceae,

of the  $R_f$  values of the anthocyanidins is different in this solvent from those in acetic acid-HCl and *n*-butanol-HCl (Table 1).

Most of the results recorded below have been obtained with the Forestal solvent. In every instance so far tested, however, the chromatographic results with the other solvents have agreed with those obtained with the Forestal solvent. Valuable further confirmation that the substances produced are, in fact, anthocyanidins lies in the observation that in all solvents other than those containing strong acid the substances are decolorized.

## Spectrographic examination

Anthocyanidins have well-defined peaks in the visible region, either in ethanolic HCl solution or when examined directly as a spot on a paper chromatogram by the method of Bradfield & Flood (1953). By the latter method the values are accurate to no more than  $\pm 2$  m $\mu$ . Values for six anthocyanidins are given in Table 1.

The anthocyanidin spots from digests of leaves were examined spectrographically on paper chromatograms and gave values of  $E_{\max}$  shown in Table 2. It will be seen that the spectrographic data in every case confirmed the chromatographic identification. In the case of leuco-anthocyanin prepared from wood of *Pinus maritima*, and supplied by Professor J. Masquelier, the anthocyanidin having the  $R_f$  value of cyanidin gave a maximum of 545 m $\mu$ .

Table 3. *Anthocyanidins formed from leuco-anthocyanins in leaves*

The following results record the detection, by paper chromatography, of cyanidin (C) and delphinidin (D) formed from leuco-anthocyanins by treatment with hydrochloric acid (see text). Where the concentration of anthocyanidin was appreciably different from the average this is indicated by the letters *s* (strong) and *w* (weak).

**Dicotyledons**

Actinidiaceae, *Actinidia chinensis*, C; Aizoaceae, *Carpobrotus acinaciformis*, C; Anacardiaceae, *Cotinus coggygria*, C, D; Anonaceae, *Asimina triloba*, C; Asclepiadaceae, *Periploca graeca*, C; Betulaceae, *Corylus avellana*, D; Caprifoliaceae, *Viburnum lantana*, Cw; Celastraceae, *Euonymus fortunei* 'Silver Queen', C, Ds; *E. japonicus*, C, D; *E. radicans* 'minimus', C, D; Cunoniaceae, *Callicoma serratifolia*, C; Ebenaceae, *Diospyros lotus*, Cw, Dw; Ericaceae, *Arbutus unedo*, C, Dw; *Macleanea punctata*, C; Rhododendron *smirnowii*, C; Fagaceae, *Fagus sylvatica*, Cs, D; *F. sylvatica*†, C; Juglandaceae, *Juglans regia*, Cs; Lauraceae, *Cinnamomum sericeum*, C; *Persea gratissima*, C; Leguminosae, *Bauhinia yunnanensis*, C; *Caesalpinia echinata*, D; *C. japonica*, C; *Cercis siliquastrum*, C, D; *Coronilla varia*, C, D; *Derris elliptica*, C; *Desmodium canadense*, Cs, Ds; *Hedysarum multiflorum*, Cw, Ds; *Lespedeza thunbergii*, C, D; *Onobrychis sativa*, C, Ds; *Pithecolobium dulce*\*, C; *Poinciana regia*\*, C; *Pterocarpus fraxinifolius*, C; *Rhynchosia phaseoloides*, C, Ds; *Scorpiurus muricatus*, C, D; Limnathaceae, *Limnanthes douglasii*, C, Ds; Magnoliaceae, *Magnolia denudata* (= *M. conspicua*), Cw; Melastomaceae, *Tibouchina semidecandra*, C, Ds; Moraceae, *Ficus sycomorus*, C; *Humulus lupulus*, C, D; Myricaceae, *Myrica gale*, C, Ds; Oxalidaceae, *Oxalis bupleurifolia*, D; Platanaceae, *Platanus orientalis*, C, Ds; Plumbaginaceae, *Limonium latifolium*, D; *L. sinuatum*, D; Polygonaceae, *Fagopyrum tataricum*, C; *Polygonum aviculare*, D; *Rheum raphaniticum*†, C; *Rumex lunaria*, C; Proteaceae, *Grevillea robusta*, D; Rhamnaceae, *Rhamnus cathartica*, C, D; *R. purshiana*, C, Dw; Rosaceae, *Agrimonia eupatoria*\*, C; *Chaenomeles speciosa* (= *Cydonia japonica* auct.), C; *Eriobotrya japonica*\*, C; *Fragaria* (strawberry) 'Auchincruive Climax', C; *Kerria japonica*\*, C; *Malus sargentii*\*, C; *Mespilus germanica*\*, Cs; *Neillia longiracemosa*\*, C; *Osteomeles schuerinae*\*, C; *Potentilla fruticosa*, C; *P. montana*, C; *Prunus pissardii*†, C; *Raphiolepis indica*\*, C; *Sorbaria aitchisonii*, C; *Sorbus aucuparia*, C; *Spiraea salicifolia*\*, C; Salicaceae, *Populus canescens*, C; *Salix elaeagnos* (= *S. incana*), C; Sapindaceae, *Koeleruteria paniculata*, C, D; *Xanthoceras sorbifolia*, C, Ds; Saxifragaceae, *Astilbe rosea* 'Queen Alexandra', C; *Boykinia aconitifolia*, C; *Hydrangea hortensis*†, C; *Ribes grossularia*, D; *R. sanguineum* 'splendens', D; *Tellima grandiflora*, C, D; Theaceae, *Camellia japonica*, C; *Thea sinensis*, Cw, Dw; Tiliaceae, *Tilia europaea*, C; Ulmaceae, *Ulmus procera*, C; Vitaceae, *Parthenocissus quinquefolia*, C; *Rhoicissus rhomboidea*, C, D; *Vitis vinifera*†, C.

**Monocotyledons**

Iridaceae, *Iris pseudacorus*, C, Ds; Liliaceae, *Smilax rotundifolia*, C.

**Gymnosperms**

Ginkgoaceae, *Ginkgo biloba*, D; Gnetaceae, *Ephedra americana*, Ds; *E. nebrodensis*, Ds; Pinaceae, *Araucaria bidwillii*, Cw, D; *Metasequoia glyptostroboides*, C; *Sequoiadendron giganteum*, D; Taxaceae, *Taxus baccata*, C, Dw.

**Pteridophyta**

Equisetaceae, *Equisetum telmateia*, C; Polypodiaceae, *Blechnum occidentale*, C, D; *Dryopteris elongata*, C, D; *Pellea rotundifolia*, Cw, D; *Pteridium aquilinum*, C; *Pteris childsii*, C.

\* Paeonidin also present.

† Results recorded by Robinson & Robinson (1933, 1934).

Table 4. *Anthocyanidins formed from leuco-anthocyanins in fruits, etc.*

Fruits and nuts	Plant part	Anthocyanidin
Apple 'Bramley Seedling'	Flesh	Cyanidin
Betel nut	—	Cyanidin
Cacao, Criollo	Bean, cotyledons	Cyanidin
	Skin	Cyanidin
Grape, purple		(Malvidin and delphinidin preformed)
	Seed	Cyanidin
Groundnut	Testa	Cyanidin
Hazel nut	—	Cyanidin
Pear 'Conference'	Flesh	Cyanidin
Plum 'Victoria'	Skin	Cyanidin
Walnut	Seedcoat	Cyanidin
Roots, etc.		
<i>Bergenia crassifolia</i>	Rhizome	Cyanidin
<i>Fagopyrum esculentum</i>	Root	Cyanidin
<i>Pinus maritima</i> *	Wood	Cyanidin
Flowers (Bate-Smith, 1953)		
<i>Dombeya spectabilis</i>	White petals	Cyanidin
<i>Erica carnea</i> var. <i>alba</i>		Cyanidin
<i>Raphiolepis ovata</i>		Cyanidin

\* Material as named and supplied by Professor J. Masquelier, Faculté de Médecine et de Pharmacie, Bordeaux, France.

*Galium* spp. in Rubiaceae, *Escallonia* spp. in Saxifragaceae, *Garrya elliptica* in Garryaceae, *Incarvillea olgae* in Bignoniaceae, and *Aucuba japonica* in Cornaceae. In some instances the substances responsible for these interfering reactions are known (cf. Trim & Hill, 1952).

Sugars in exceptionally high concentration, as in some ripe fruits, when heated with hydrochloric acid give a golden-brown colour, soluble in *iso*amyl alcohol to give a red-brown solution. On the chromatogram a brown zone due to this pigment, which appears cream-coloured when viewed in ultraviolet light, is visible near the solvent front.

Tentative identifications, based on measurements of  $R_f$  values, of the anthocyanidins formed from leuco-anthocyanins present in leaves, seeds and fruits, are recorded in Tables 3 and 4.

### DISCUSSION

A remarkable feature of the results obtained is that, with only a few exceptions, the leuco-anthocyanins present in the tissues examined yielded only cyanidin and delphinidin when digested with hydrochloric acid. The exceptions were confined to Rosaceae and Leguminosae, where, occasionally,

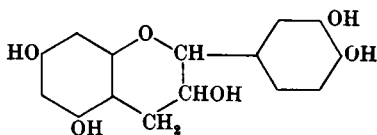
lation is the same as that found in cyanidin (III) and delphinidin (IV), respectively, and it is therefore a matter of some importance, in connexion with the origin and function in the plant of the leuco-anthocyanins and catechins, that the leuco-anthocyanins should be restricted to the patterns of hydroxylation found in the catechins. The systematic significance of these results is discussed in the following paper.

### SUMMARY

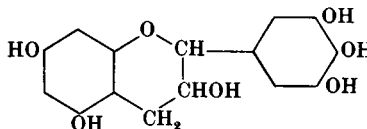
1. The leuco-anthocyanins present in leaves, fruits and other tissues are converted into anthocyanidins by heating the tissues with 2N aqueous hydrochloric acid. The anthocyanidins are identified by paper chromatography using the three solvent systems, *n*-butanol-2N hydrochloric acid (1:1, v/v), water-acetic acid-conc. hydrochloric acid (10:30:3, v/v), and *m*-cresol-acetic acid-5.5N hydrochloric acid (1:1:1, v/v).

2. Except in Rosaceae and a few Leguminosae (which appear to contain leuco-paeonidin) the leuco-anthocyanins appear to be restricted to leuco-cyanidin and leuco-delphinidin.

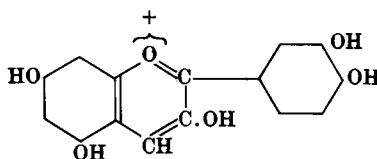
The author wishes to thank Dr T. White of the Forestal Land, Timber and Railways Company Ltd., Harpenden,



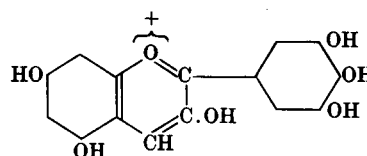
(I)



(II)



(III)



(IV)

an anthocyanidin corresponding in  $R_f$  value with paeonidin was present on the chromatogram. These areas on the chromatogram were usually confused with a pinkish brown trail, which occurred in most species of Rosaceae, and also in those of *Fagopyrum*, *Vitis*, *Fagus*, *Populus*, *Boykinia*, *Lespedeza* and *Desmodium*.

The near relationship, from the chemical point of view, of the leuco-anthocyanins and the catechins has been discussed previously (Robinson & Robinson, 1933; Bate-Smith, 1953). Up to the present no hydroxyflavans other than catechin (I) and gallo-catechin (II) have been found in nature (cyanomaclurin, occurring uniquely in *Artocarpus integrifolia*, is excepted). Their pattern of hydroxy-

lation is the same as that found in cyanidin (III) and delphinidin (IV), respectively, and it is therefore a matter of some importance, in connexion with the origin and function in the plant of the leuco-anthocyanins and catechins, that the leuco-anthocyanins should be restricted to the patterns of hydroxylation found in the catechins. The systematic significance of these results is discussed in the following paper.

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