Phytochemistry, 1964, Vol. 3, pp. 151 to 160. Pergamon Press Ltd. Printed in England

PLANT POLYPHENOLS—XI.
THE STRUCTURE OF ACYLATED ANTHOCYANINS*

J. B. HARBORNE
John Innes Institute, Bayfordbury, Hertford, Herts.

(*Received* 14 *November* 1963)

Abstract—Fifteen acylated anthocyanins have been oxidized with H_2O_2 to give, in each case, an acylated sugar. These experiments prove that the acyl groups (*p*-coumaroyl, feruloyl or caffeoyl residues) are attached to the sugar groups in the 3-position in all these anthocyanins. Using the same procedure, the acylated flavonol tiliroside, described previously as a 7-*p*-coumaroyl derivative, has been shown to be kaempferol 3-(*p*-coumaroyl-glucoside). No evidence could be found for the presence of sinapic, *p*-hydroxybenzoic or malonic acids in pigments reported earlier to contain these as acyl residues.

INTRODUCTION

ACYLATED or "complex" anthocyanins, i.e. pigments containing covalently linked† acyl (e.g. *p*-coumaroyl) residues, were first described by Willstäter and his co-workers in 1915.[1] For example, delphinin, isolated from *Delphinium consolida*, was found to give on hydrolysis delphinidin, glucose and *p*-hydroxybenzoic acid in the ratio of 1:2:2.[1] Later Karrer and Widmer[2] examined a number of similar pigments, particularly from the Labiatae; one such compound, monardein, was reported to be a pelargonidin 3,5-diglucoside acylated with *p*-coumaric and malonic acids. In recent years, several series of acylated anthocyanins have been isolated from a range of plants, notably from the Vitaceae,[3] Solanaceae[4] and Cruciferae;[5] over fifty pigments of this type are now known. For a long time acylated anthocyanins were the only class of flavonoid known to have such substituents but in 1959 an acylated flavonol, tiliroside, was reported[6] to occur in flowers of *Tilea argentea* and several other acylated flavonols have been isolated since then.[7,8]

Acylated anthocyanins are primarily of interest because of their restricted taxonomic distribution[9] and their biogenetic relationship to other classes of hydroxycinnamic acid ester, particularly to the sugar esters.[10,11] Furthermore, they are of genetic interest, since

* Part X, J. B. HARBORNE, *Phytochemistry*, **2**, 327 (1963).

† All anthocyanins are thought to be ionically bound in the cell vacuole to aliphatic organic acids such as malonic, malic or citric acid.

[1] R. WILLSTÄTER and W. MIEG, *Liebig's Ann. Chem.* **408**, 61 (1915).
[2] P. KARRER and R. WIDMER, *Helv. Chim. Acta* **11**, 837 (1928).
[3] P. RIBEREAU-GAYON, *Ph.D. Thesis*, Paris (1959).
[4] J. B. HARBORNE, *Biochem. J.* **74**, 262 (1960).
[5] W. SEYFFERT, *Z. Pflanzenzücht.* **44**, 4 (1960).
[6] L. HÖRHAMMER, L. STICH and H. WAGNER, *Naturwissenschaften* **46**, 358 (1959); *Arch. Pharm.* **294**, 687 (1961).
[7] L. BIRKOFER and C. KAISER, *Z. Naturforsch.* **17b**, 359 (1962).
[8] J. B. HARBORNE, *Experientia* **19**, 7 (1963).
[9] J. B. HARBORNE in *Chemical Plant Taxonomy* Ed. T. SWAIN, p. 359, Academic Press (1963).
[10] J. B. HARBORNE and J. J. CORNER, *Biochem. J.* **81**, 242 (1961).
[11] L. BIRKOFER, C. M. KAISER, W. NOUVERTNÉ and U. THOMAS, *Z. Naturforsch.* **16b**, 249 (1961).

Mendelian factors controlling acylation have been demonstrated in the cultivated diploid potato,[4] in the aubergine, *Solanum melongena*,[12] and in the garden stock, *Matthiola incana*.[5]

Work on the structure of acylated anthocyanins has been hindered by the difficulty of purifying them and because of the relative lability of the acyl-anthocyanin linkage. However, for most acylated anthocyanins, the nature and position of attachment of the sugar residues and the nature and number of acyl substituents have been determined.[13,14] The last remaining structural feature requiring confirmation is the position in the molecule of the acyl group or groups.

A structure involving linkage of *p*-coumaric acid to the phenolic hydroxyl group in the 7-position of pelargonidin was favoured by Karrer[2] for monardein and a similar linkage has been proposed recently for the attachment of *p*-coumaric acid to kaempferol 3-glucoside in tiliroside.[6] By contrast, other pigments, e.g. ensatin from *Iris ensata*,[15] were considered to have their acyl groups linked through the sugar moieties and all acylated flavonols, except tiliroside, have been shown to have such a linkage. The spectral properties of a range of acylated anthocyanins[14] also suggested that the acyl groups were linked to sugar.

The present paper provides experimental evidence in favour of the view that acyl groups are indeed always attached through the sugar unit present in the 3-position of both anthocyanidins and flavonols. Other work reported here indicates that only three acids (*p*-coumaric, caffeic and ferulic) occur as acyl substituents in these complex pigments.

RESULTS

Purification and Characterization

General procedures for the purification of acylated pigments by paper chromatography and characterization by their chromatographic behaviour before and after alkaline treatment and by spectral measurements have already been described (e.g.[4,14]); these were used throughout this work. In all cases, special care was taken during purification to remove other cinnamic acid derivatives, especially sugar esters, which have very similar R_f's to acylated anthocyanins in some solvents; repeated paper chromatography (6–8 separations was frequently necessary. Some anthocyanins, e.g. negretein, were isolated on a larger scale by chromatography on columns of polyamide.[16]

The spectral, chromatographic and colour properties of most anthocyanins studied in this paper have already been reported in the literature; data for the remainder are given in Table 1. This table includes some pigments which were not further characterized by H_2O_2 oxidation (see below); detailed comments on these pigments are given in a later section.

H_2O_2 *Oxidation: Isolation of Acylated Sugars*

Fifteen purified pigments were oxidized with H_2O_2 in methanolic solution, using the procedure developed by Chandler and Harper,[17] and the acylated sugars formed (see Table 2) were isolated by paper chromatography and characterized by the usual methods.[10,18] The properties and hydrolysis products of the seven sugar esters obtained variously from these

[12] Y. ABE and K. GOTOH, *Botan. Mag. Tokyo* **72**, 432 (1959).
[13] J. B. HARBORNE, *Phytochemistry* **2**, 85 (1963).
[14] J. B. HARBORNE, *Biochem. J.* **70**, 22 (1958).
[15] K. HAYASHI, *Acta Phytochim. Japan* **12**, 65 (1941).
[16] B. V. CHANDLER and T. SWAIN, *Nature* **183**, 989 (1959).
[17] B. V. CHANDLER and K. A. HARPER, *Austral. J. Chem.* **14**, 586 (1961).
[18] J. J. CORNER, J. B. HARBORNE, S. G. HUMPHRIES and W. D. OLLIS, *Phytochemistry* **1**, 73 (1962).

anthocyanins are shown in Table 3. In none of these esters is the reducing end of the sugar molecule bound to the acyl substituent, as is the case with the naturally occurring hydroxycinnamic acid sugar esters. Thus, except for those containing sophorose, none is hydrolysed

TABLE 1. PROPERTIES OF ACYLATED ANTHOCYANINS

Pigment	Source	$\lambda_{max.}$ in MeOH–HCl (mμ)			Ratios (%)	
		Band I	Band II	Band III	E_{440} / E Band III	E Band II / E Band III
Matthiolanin	Crimson petals *Matthiola incana*	288	328	509	20	92
Monardein	Red petals *Gilia coronopifolia*	285	314	507	21	67
Raphanusin C	Purple roots *Raphanus sativus*	278	310	523	17	60
Rubrobrassicin A	Red leaves *Brassica oleracea*					
Raphanusin D	Purple *Raphanus*	278	328	523	17	55
Rubrobrassicin B	Red *Brassica*					
Rubrobrassicin C	Red *Brassica*	—	333	530	14	88
Hyacinthin	Mauve bulb scales Garden *Hyacinthus*	284	310	527	21	64
Delphanin	Mauve petals Garden *Iris*	282	310	538	11	71
Tibouchinin	Mauve petals *Tibouchina semidecandra*	280	305	536	10	64

	R_f values in*			Products of alkaline† Hydrolysis and ratios‡
	BAW	BuHCl	1% HCl	
Matthiolanin	0·34	0·29	0·37	Pg 5-glucoside, 3-sambubioside, PC and FE (1:1:1)
Monardein	0·43	0·61	0·16	Pg 3,5-diglucoside and PC (1:1)
Raphanusin C / Rubrobrassicin A	0·34	0·21	0·40	Cy 5-glucoside, 3-sophoroside and PC (1:1)
Raphanusin D / Rubrobrassicin B	0·33	0·15	0·39	Cy 5-glucoside, 3-sophoroside and FE (1:1)
Rubrobrassicin C	0·21	0·13	0·39	Cy 5-glucoside, 3-sophoroside and FE (1:2)
Hyacinthin	0·33	0·63	0·04	Cy 3-glucoside and PC (1:1)
Delphanin	0·29	0·25	0·24	Dp 5-glucoside, 3-rutinoside and PC (1:1)
Tibouchinin	0·40	0·42	0·10	Mv 3,5-diglucoside and PC (1:1)

* On No. 1 paper; abbreviations: BAW, butan-1-ol–acetic acid–water (4:1:5); BuHCl, butan-1-ol–2 N HCl (1:1); 1% HCl, water–conc. HCl (97:3).

† Abbreviations: Pg, pelargonidin; Cy, cyanidin; Dp, Delphinidin; Mv, malvidin; PC, *p*-coumaric acid; FE, ferulic acid.

‡ Ratio of aglycone in glycoside(s) to cinnamic acid(s).

by β-glucosidase; and the sophorose esters are only hydrolysed to the corresponding glucose esters on β-glucosidase treatment. Furthermore, three of the esters, *p*-coumaroylglucose, *p*-coumaroylrutinose and caffeoylglucose have different R_f values in aqueous solvents from the isomeric 1-substituted sugars, which were available for comparison. Similarly linked glucose esters of sinapic and 3,4,5-trimethoxycinnamic acid were isolated recently in combined form from *Polygala senega*.[18] It has not yet been possible to determine which of the remaining

four positions (2, 3, 4 and 6) in the glucose molecule contain the acyl substituent in these esters; this determination awaits the isolation of larger amounts of the appropriate acylated sugars.

On acid hydrolysis of the *p*-coumaroylrutinose obtained from the *Solanum* pigments, *p*-coumaroylrhamnose is formed; the same is also obtained during acid hydrolysis of the original anthocyanins. This proves that the acyl group in this instance is linked to the terminal sugar unit in negretein and related pigments. A similar linkage is probable in other pigments,

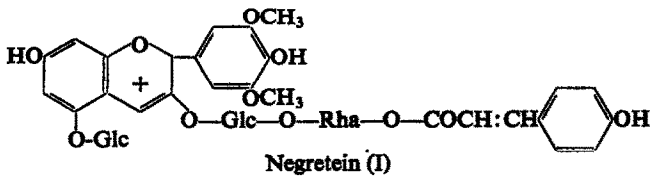
TABLE 2. OXIDATION PRODUCTS OF ACYLATED ANTHOCYANINS

Pigment	Source	Aglycone*	Sugar pattern	H_2O_2 oxidation product
Monardein	*Monarda didyma*	Pg	3,5-Diglucoside	*p*-Coumaroylglucose
Hyacinthin	*Hyacinthus*	Cy	3-Glucoside	
Tiliroside	*Tilea argentea*	Km	3,5-Diglucoside	
Tibouchinin	*Tibouchina*	Mv	3,5-Diglucoside	
Negretein	*Solanum tuberosum*	Mv	3-Rutinoside 5-glucoside	*p*-Coumaroylrutinose
Petanin		Pt		
Delphanin†		Dp		
Peonanin		Pn		
Pelanin		Pg		
Raphanusin A	*Raphanus sativus*	Pg	3-Sophoroside, 5-glucoside	*p*-Coumaroylsophorose
Rubrobrassicin A	*Brassica oleracea*	Cy		
Salvianin	*Salvia splendens*	Pg	3,5-Diglucoside	Caffeoylglucose
Raphanusin B	*Raphanus*	Pg	3-Sophoroside, 5-glucoside	Feruloylsophorose
Rubrobrassicin C	*Brassica*	Cy		
Rubrobrassicin B	*Brassica*	Cy	3-Sophoroside, 5-glucoside	Diferuloylsophorose
Matthiolanin	*Matthiola incana*	Pg	3-Sambubioside, 5-glucoside	Feruloyl-*p*-coumaroyl-sambubiose

* Abbreviations: Pg, pelargonidin; Cy, cyanidin; Pn, peonidin; Dp, delphinidin; Pt, petunidin; Mv, malvidin; Km, kaempferol.

† Delphanin, isolated from *Iris*, also gave, on oxidation, *p*-coumaroylrutinose.

but it has not been possible to prove this; thus the fact that acid hydrolysis of the acylated sophoroses yields acylated glucoses does not indicate which glucose unit in the sophorose carries the acyl substituent. Similarly, in the two disaccharides carrying more than one acyl substituent, it has not been possible to determine whether these acyl substituents are both present on the terminal sugar unit or not.



Negretein (I)

Chandler and Harper[17] have shown that H_2O_2 oxidation specifically removes the 3-substituent from anthocyanins and flavonol glycosides, so that the isolation of these acylated sugars proves that all the fifteen acylated anthocyanins examined have their acyl groups attached to the 3-*O*-sugar, and not to the 5-*O*-sugar or to one of the phenolic hydroxyl groups of the anthocyanidin molecule. The structure of negretein is thus (I) and similar structures

may be written for all the other anthocyanins studied. Birkofer *et al.* have recently derived formulae similar to I for six acylated anthocyanins present in the petals of *Petunia*.[19]

Structure of Tiliroside

Hörhammer *et al.*[6] proposed structure II (7-*p*-coumaroylkaempferol 3-glucoside) for tiliroside, an acylated flavonol present in flowers of the lime, *Tilea argentea*. This structure was partly based on the fact that the long waveband of the u.v. spectrum of tiliroside did not

TABLE 3. PROPERTIES OF ACYLATED SUGARS

Acylated sugar	R_f values in,* BAW	BN	H_2O	PhOH	$\lambda_{max.}$ in 95% EtOH: Alone	With alkali
p-Coumaroylglucose	0·61 (0·66)	0·20 (0·24)	0·64, 0·81 (0·76, 0·81)	0·75 (0·82)	229, 312	365
p-Coumaroylrutinose	0·50 (0·52)	0·12 (0·10)	— 0·83 (0·71, 0·77)	0·67 (0·67)	— 313	367
p-Coumaroylsophorose	0·43	0·04	0·62, 0·78	0·52	224, 313	367
Caffeoylglucose	0·42 (0·46)	0·02 (0·06)	0·47, 0·73 (0·62, 0·75)	0·47 (0·57)	223, 331	385
Feruloylsophorose	0·37	0·00	0·51, 0·74	0·62	235, 327	380
Diferuloylsophorose	0·12	0·00	0·79	0·21	240, 324	378
Feruloyl-*p*-coumaroyl-sambubiose	0·40	0·11	0·50, 0·75	0·69	237, 327	379
p-Coumaroylrhamnose†	0·74	0·70	0·63	—	229, 312	367

Acylated sugar	Colour in u.v.: Alone	+NH_3	Reaction with β-glucosidase	Products of alkaline hydrolysis §
p-Coumaroylglucose	Colourless	Blue	−ve	Glucose and PC
p-Coumaroylrutinose			−ve	Rutinose and PC
p-Coumaroylsophorose			*p*-Coumaroylglucose	Sophorose and PC
Caffeoylglucose			−ve	Glucose and CA
Feruloylsophorose	Blue	Green	Feruloylglucose‡	Sophorose and FE
Diferuloylsophorose			−ve	Sophorose and FE
Feruloyl-*p*-coumaroyl-sambubiose			*p*-Coumaroylglucose	Sambubiose, PC and FE
p-Coumaroylrhamnose†	Colourless	Blue	−ve	Rhamnose and PC

* On Whatman No. 1 paper; solvents are: BN, butan-1-ol–2 N ammonia (1:1); PhOH, phenol satd. with H_2O. Values in parentheses refer to figures for corresponding 1-glucose esters.

† Produced by acid hydrolysis of *p*-coumaroylrutinose.

‡ Differs in its R_f values from 1-feruloylglucose.

§ Abbreviations: PC, *p*-coumaric acid; CA, caffeic acid; FE, ferulic acid.

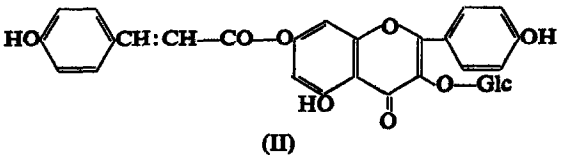
show a bathochromic shift in the presence of sodium acetate. However, it is well known (cf. Jurd[20]) that it is the lack of a shift of the short waveband in the presence of sodium acetate that indicates that the 7-position in flavonols is blocked. Both our own spectral measurements (see Experimental) and those of Swain[21] show that the 7-hydroxyl group in tiliroside

[19] L. BIRKOFER, C. KAISER, W. KOCH, M. DONIKE and D. WOLF, *Z. Naturforsch.* **18b**, 631 (1963).

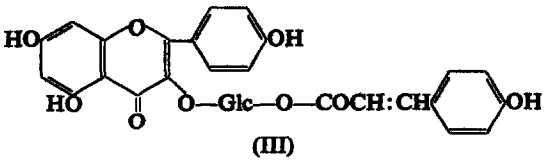
[20] L. JURD, *Arch. Biochem. Biophys.* **66**, 284 (1957).

[21] T. SWAIN, unpublished results.

is free. Since the spectral data also show that the 5- and 4'-hydroxyls are free, the only possible position for attachment of the *p*-coumaroyl residue is the sugar in the 3-position. That such a linkage is present in tiliroside has been confirmed by isolating a *p*-coumaroylglucose after either acid hydrolysis or H_2O_2 oxidation. The acylated sugar obtained was identical in R_f values with that produced from monardein and hyacinthin. Tiliroside must therefore have structure III and it thus does not differ in this respect from any of the other acylated flavonols (or anthocyanins) so far described.



(II)



(III)

The Acyl Groups of Complex Anthocyanins

Although a range of different acids has been recorded in the earlier literature as being associated with anthocyanins, more recent work has indicated that only a few common hydroxycinnamic acids are, in fact, present in these acylated pigments. Several of the pigments described earlier have therefore been re-examined and the results are recorded below. A number of new acylated pigments and some new occurrences of known pigments are also described.

Both monardein and salvianin are reported to contain, besides *p*-coumaric acid, two molecular equivalents of malonic acid.[2] On re-examination of the pigments purified by paper chromatography, no malonic acid could be detected in the alkaline hydrolysates and, furthermore, there was no indication from chromatographic behaviour or from controlled acid hydrolysis of the presence of any acyl group other than *p*-coumaric acid in these pigments. Monardein is thus pelargonidin 3-(*p*-coumaroylglucoside)-5-glucoside and salvianin the corresponding caffeic derivative. Monardein, originally isolated from *Monarda didyma* (Labiatae), has also now been found in the petals of *Gilia coronopifolia* (Polemoniaceae) and in the petals of red colour forms (e.g. "Scarlet O'Hara") of the garden *Hyacinthus* (Liliaceae). Mauve and blue hyacinth flowers (e.g. "Mauve Queen" and "Delft Blue") have the related cyanidin and delphinidin derivatives respectively. Surprisingly, the pigment in the bulb scales of "Delft Blue" is cyanidin 3-*O*-(*p*-coumaroylglucoside) (hyacinthin in Tables 1 and 2). The malvidin analogue of monardein has not been described before but has now been isolated from *Tibouchina semidecandra* (Melastomaceae) and called tibouchinin.

Delphanin, previously reported in *Solanum*,[4] *Viola*[22] and *Petunia*,[19] has now been found in flowers of the garden *Iris*. It is present as the only anthocyanin in all of 19 cultivars examined and also occurs in seven wild species. In two of these, *I. chrysographes* and

[22] T. ENDO, *Botan. Mag. Tokyo* **72**, 10 (1959).

I. delavayi, it is accompanied by a malvidin derivative, but this is not surprising, because ensatin [a malvidin 3-(*p*-coumaroylglucosylglucoside)] has already been noted in the genus.[15]

Studies of the pigments, raphanusin A and B, of red forms of the radish, *Raphanus sativus*, have already been reported.[13,14,23] With the results of H_2O_2 oxidation of raphanusin B (see p. 154), they may be formulated as the 3-(feruloylsophoroside)-5-glucoside and the 3-(*p*-coumaroylsophoroside)-5-glucoside of pelargonidin. Two cyanidin derivatives, raphanusins C and D, have now been found in purple radishes (the F_1 plants of a cross between red and white forms). The properties of the two pigments are shown in Table 1 and it is clear that they are structurally analogous to the pelargonidin derivatives.

Although only one acylated pigment, "rubrobrassicin", was isolated from red cabbage, *Brassica oleracea*, by Chmielewska,[24] pigments, rubrobrassicins A, B and C, have been obtained during the present work. None of these three pigments, once purified, contained sinapic acid, although this acid was reported to be present in rubrobrassicin by Chmielewska.[24] Rubrobrassicin A contained one *p*-coumaroyl residue, rubrobrassicin C had two residues of *p*-coumaric acid and the B isomer one of ferulic acid (see Table 1). All gave cyanidin 5-glucoside, 3-sophoroside on alkaline hydrolysis;[13] and H_2O_2 oxidation of rubrobrassicin C, the main component, gave a diferuloylsophorose (Table 2). There is no evidence that a sinapoyl-containing anthocyanin is present in red cabbage, although only one commercial strain of this plant has yet been examined.

The only other report of acylated anthocyanins containing sinapoyl residues is that of Seyffert,[5] who reported their presence in the flowers of pink and mauve strains of *Matthiola incana*. Since Seyffert did not take any special precautions to purify his pigments and did not make spectral measurements to determine the anthocyanin–acyl ratios in these compounds, his reported sinapoyl-containing anthocyanins must be viewed with some reserve. We have noted that large amounts of 1-sinapoylglucose are present in the petals of most forms of the garden stock (as they are present in red cabbage leaves[10]) and contamination of anthocyanin with this substance and with any sinapoyl disaccharides that may be present could easily lead to erroneous results. In order to obtain further information on this point, the acylated pigments of a crimson–red commercial form of *Matthiola incana* were examined. In fact, only one glycoside was present, matthiolanin, and this was identified (see Tables 1 and 2) as pelargonidin-(3-*p*-coumaroylferuloylsambubioside)-5-glucoside. Examination of blue forms again showed the presence of only two acylated cyanidin pigments, although Seyffert describes the presence of some ten or twelve such compounds in his various phenotypes. It is concluded that it is unlikely that sinapic acid is present as the acylating group in any anthocyanins so far described.

Finally, the first known acylated anthocyanin, delphinin, has been re-examined, in order to see if it did, in fact, have *p*-hydroxybenzoic acid present in its structure. Great difficulty was experienced in purifying delphinin from *Delphinium* flowers and, even after repeated paper chromatography, it could not be obtained completely pure. However, the best sample that could be obtained only gave delphinidin, delphinidin 3- and 5-glucoside and glucose on acid hydrolysis. No *p*-hydroxybenzoic acid was detected in the alkaline and acid hydrolysates and H_2O_2 oxidation also failed to yield a *p*-hydroxybenzoic glucose ester. It is concluded that the major pigment of *Delphinium* is probably delphinidin 3,5-diglucoside; it certainly does not appear to have *p*-hydroxybenzoic acid attached to it by an ester linkage.

[23] N. Ishikura and K. Hayashi, *Botan. Mag. Tokyo* **75**, 28 (1962).
[24] I. Chmielewska, I. Kakowska and B. Lipinski, *Bull. acad. polon. Sci.* cl. III, 3, 527 (1955).

DISCUSSION

The results described in this paper, together with those of other workers (e.g. Ref. 19), show that all known acylated flavonoids are of the same general type, in that the acyl group is always attached to the sugar substituted in the 3-position. The precise positions in the 3-sugars to which the acyl group are attached has yet to be determined, and it is suggested, as a matter of convenience, that trivial names be retained for these pigments for the time being. Furthermore, it has been shown in the present work that only three hydroxycinnamic acids, *p*-coumaric, caffeic and ferulic, are commonly present in these pigments. Previous reports of pigments containing sinapic, *p*-hydroxybenzoic and malonic acids have been found to be incorrect. While it has not been possible to examine all cases in which other unusual acids have been found, it seems likely that acyl groups of complex anthocyanins are, in fact, normally limited to the three types mentioned above.

These results have implications from both the biosynthetic and genetic points of view. Biosynthetically, it seems unlikely that acylated pigments are directly related to any other type of hydroxycinnamic acid ester and the co-occurrence of acylated flavonoids and hydroxycinnamic acid sugar esters in a number of plants, e.g. in *Petunia*, is perhaps fortuitous. It seems likely that the enzyme system controlling acylation is normally specific for anthocyanin or for flavonol, since such derivatives rarely occur together. *Petunia* is the only known exception; even so, the acyl groups in the case of the anthocyanins are *p*-coumaric acid and caffeic acid[4,19] and in the case of the flavonols, ferulic acid.[7]

Genetically, the discovery that the anthocyanins of the Solanaceae are of the general type 3-(*p*-coumaroylrutinoside)-5-glucoside (e.g. as in (I)) has an immediate bearing on the structure of the *Ac–ac* locus in the cultivated potato. This Mendelian factor controls the following three biochemical steps in anthocyanin synthesis: (*a*) addition of glucose to the 5-hydroxyl; (*b*) acylation with *p*-coumaric acid, and (*c*) methylation of the 3′-hydroxyl.[4] Now that it is clear that acylation occurs on the rhamnose of the rutinose group in the 3-position and not on the glucose in the 5-position, it is no longer possible to consider that the biochemical effects of *Ac* are related. Indeed, the simplest explanation is that *Ac* is a complex locus of three closely linked genes. It is noteworthy that a similar association of biochemically distinct functions is known also in *Solanum melongena* and *Matthiola*, in which the genetic control of acylation is again associated with glycosylation; methylation is not, however, involved in these examples.

EXPERIMENTAL

Plant Material

Sources of most of the acylated pigments were given in earlier papers.[4,13,14] The rubrobrassicins were isolated from an unidentified commercial variety of *Brassica oleracea*; the radish pigments from roots of *Raphanus sativus*, F_1 hybrid cv. "Red Turnip" × cv. "Icicle"; hyacinthin from bulbs of *Hycinthus* cv. "Delft Blue"; and delphanin from flowers of *Iris* cv. "Red Rover".

Isolation and Purification

All small scale isolations were carried out by paper chromatography on Whatman No. 3 filter paper in BAW or BuHCl. The pigments were then purified by repeated chromatography in these and in aqueous solvents. Larger scale isolation of the pigments of the "Congo" potato and of red cabbage leaves was carried out as follows. Plant material (2 kg) was

macerated in a blender with 500–800 ml MeOH–HCl (97:3, v/v) and the macerate was centrifuged. The supernatant liquid, after concentration to 100 ml, was adsorbed on to powdered nylon (3 × 10 g). The powder was dried in a desiccator and then eluted with MeOH–HCl (3 × 50 ml). The eluate was concentrated and then dried in a desiccator over KOH. The residue was purified by extraction with MeOH and precipitation with dry ether; this process was repeated three times. The product was a non-hygroscopic intensely dark-red powder, which was contaminated with cinnamic acid derivatives (fluorescing blue and green in u.v. light). The pure pigments were readily obtained from this powder by successive chromatography in BAW and 2% aqueous acetic acid.

Oxidation of Acylated Anthocyanins: Isolation and Characterization of Acylated Sugars

Pigments were dissolved in MeOH and oxidized with H_2O_2 by the procedure of Chandler and Harper.[17] The resulting solutions were chromatographed in BAW and the bands containing the acylated sugars were cut out, eluted and purified by chromatography. Oxidation of salvianin failed to give any acylated sugar, because the caffeic ester was further broken down by the reagent. Caffeoylglucose was therefore obtained from salvianin by acid hydrolysis (0·2 N HCl at 100° for 30 min or 10% HOAc at 100° for 2 hr). *p*-Coumaroylrutinose was obtained from negretein by both H_2O_2 oxidation and acid hydrolysis (0·2 N HCl at 100° for 20 min) in comparable yields. *p*-Coumaroylrhamnose was obtained from *p*-coumaroylrutinose by acid hydrolysis (N HCl at 100° for 20 min).

The acylated sugars were characterized by R_f values, colour reactions, spectra and by identification of the products of alkaline and β-glucosidase hydrolysis. Enzyme hydrolysis was carried out overnight at pH 5·0 and 37° with excess β-glucosidase; aesculin was used as a control. The resulting solutions were run on BAW and H_2O chromatograms to test whether hydrolysis had occurred. In the three cases in which hydrolysis had taken place (see Table 3), the products were co-chromatographed with the appropriate markers.

The sugar esters from the different sources (e.g. *p*-coumaroylglucose from monardein, hyacinthin and tiliroside) all had same R_f's in six solvent systems (the four shown in Table 3, and also BEW, Butan-1-ol–ethanol–water (4:1:2·2), and 15% aqueous acetic acid). They thus appear to be identical; they were also individually characterized from each source by spectral determination, by response to β-glucosidase and by identification of the products of alkaline hydrolysis.

Tiliroside

This pigment was isolated from petals of *Tilea argentea* by extraction with hot EtOH, followed by chromatography in BAW (R_f 0·88), H_2O (R_f 0·04) and BEW (R_f 0·87). Its spectral properties are as follows:

$\lambda_{max.}^{95\%\ EtOH}$ 267, 305 (infl.), 317, 357 (infl.);
$\lambda_{max.}^{EtOH/NaOAc}$ 273, 314, 360 (infl.);
$\lambda_{max.}^{EtOH/H_3BO_3}$ 270, 315, 357 (infl.);
$\lambda_{max.}^{EtOH/AlCl_3}$ 225 (infl.), 277, 304, 320, 392;
$\lambda_{max.}^{EtONa}$ 377 mμ.

(Hörhammer *et al.* report $\lambda_{max.}^{EtOH}$ 312; $\lambda_{max.}^{EtOH/NaOAc}$ 310 mμ.) Tiliroside was not hydrolysed by overnight treatment with β-glucosidase. On controlled acid hydrolysis (20 min at 100° with N HCl), it gave *p*-coumaroylglucose (see Table 3), *p*-coumaric acid, kaempferol, kaempferol 3-glucoside and some unchanged material.

"*Delphinin*"

The pigment of a dark-blue form of *Delphinium consolida* was isolated and purified by chromatography in BAW, 1% HCl, BuHCl, 15% HOAc and then BAW. It has $\lambda_{max.}$ 272, 543 mμ and E_{440}/E_{543} 13%, E_{310}/E_{543} 17%, E_{271}/E_{543} 63%. Its spectral characteristics were similar to delphin (delphinidin 3,5-diglucoside) except that the main peak was shifted 5–8 mμ towards the visible region, indicating contamination with some co-pigment or inorganic material. On acid hydrolysis (2N HCl for 35 min), "delphinin" gave delphinidin, glucose, delphinidin 3-glucoside and delphinidin 5-glucoside. On co-chromatography with delphin, it did not separate in BuHCl (R_f's 0·02, 0·02 resp.), 1% HCl (R_f's 0·10, 0·08) or in HOAc–HCl–H_2O (15:3:82) (R_f's 0·49, 0·51); in BAW it gave two spots, R_f 0·13 and 0·18 (delphin R_f 0·11). On acid or alkaline hydrolysis, it gave no detectable aromatic acids (*p*-hydroxybenzoic acid was used as a control). No *p*-hydroxybenzoic acid sugar derivative could be detected after the H_2O_2 oxidation of delphinin.

Acknowledgement—The author thanks Mr. E. Hall for his experimental assistance.