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CHROMATOGRAPHIC INVESTIGATION OF ANTHOCYANIN PIGMENTS IN *VITIS CINEREA*

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Abstract—The anthocyanin skin pigments of the American grape, *Vitis cinerea* Engelmann, are shown to be qualitatively identical with the pigments of most *Vitis vinifera* L. varieties previously investigated. A unique chromatographic solvent system is described which permits greatly improved resolution on a preparative scale of the complex mixture of acylated anthocyanin-3-monoglucosides present in the cinerea skins. Only about 50 per cent of the acylated pigments are esterified with cinnamic acid derivatives (*p*-coumaric and caffeic acids).

INTRODUCTION

IT IS only relatively recently that the complexity of grape skin pigment mixtures has been fully appreciated. In general, *Vitis vinifera* L., the grape of the Middle East, Europe, South Africa, Australia, and California has acylated and non-acylated 3-monoglucosides of delphinidin, petunidin, cyanidin, peonidin and malvidin in the skin. Most of the American native species, in particular *V. riparia*, *V. rupestris*, and *V. rotundifolia*, have large proportions of 3,5-diglucoside pigments as well as the 3-monoglucoside pigments, both of which occur in acylated and non-acylated forms, in their skins.[1,2]

The pigments of the American species *V. cinerea* have never been thoroughly studied, although preliminary surveys indicated that cinerea resembled the viniferas in having only 3-monoglucosides. *V. cinerea* is native to the Mississippi Valley and middle-western parts of the United States. The vine is immune to attacks by phylloxera and is highly resistant to damage by many other insects and fungus infection.[3] The many highly desirable characteristics of *V. cinerea* suggest that it might be used in interspecific crosses with vinifera to produce hybrids having the desirable characteristics of both species.[4] A detailed investigation of the pigment composition of *V. cinerea* skins, a necessary adjunct to such a breeding program, is the subject of this report.

RESULTS AND DISCUSSION

Identification of Non-Acylated Anthocyanins

The skin pigments of *Vitis cinerea* are separable into six discrete fractions by unidirectional paper chromatography with butanol–acetic acid–water, v/v 4:1:5 (BAW). A solvent

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[1] P. RIBEREAU-GAYON, Thesis, Faculte des Sciences de Paris (1959).
[2] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, Academic Press, London (1967).
[3] T. V. MUNSON, *Foundations of American Grape Culture*, Orange Judd Co., New York (1909).
[4] H. P. OLMO, private communication (1968).

system[5] composed of *n*-pentanol–acetic acid–water, v/v 2:1:1 (PAW), was found to give better resolution and sharper bands (Fig. 1). Asen,[6] Hess and Meyer;[7] and Nybom[5] have also demonstrated the superior speed and resolution of TLC for analytical purposes in this type of investigation. These factors, in addition to the complexity of the pigment mixture of most grape skins, make the two-dimensional cellulose powder TLC technique described here especially valuable.

The separation of the complex pigment mixture of *V. cinerea* obtained by two-dimensional TLC is shown in Fig. 2. The two-dimensional thin-layer technique gives excellent resolution of the anthocyanin-3-monoglucosides (spots III to VII)* and partially resolves the complicated mixture of acylated pigments (spots I, II_A, $II_B{}^a$, $II_B{}^b$) in this grape variety. Relative

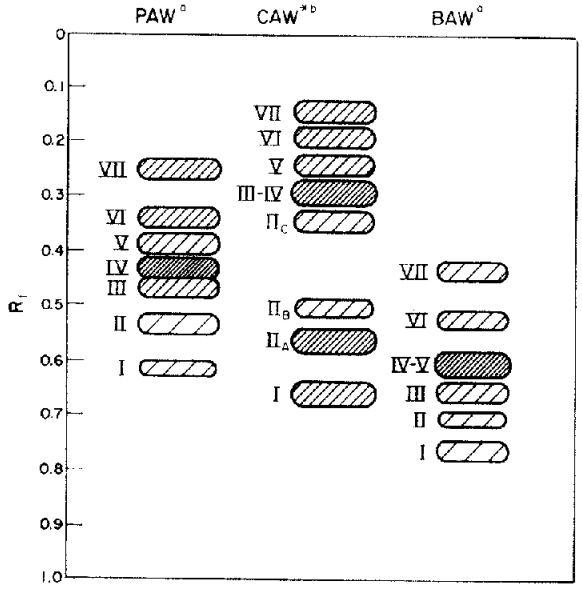

FIG. 1. SCHEMATIC REPRESENTATION OF THE SEPARATION OF *V. cinerea* PIGMENTS ON PAPER WITH VARIOUS SOLVENTS.

[a] Pigment extract chromatographed with 1% HCl prior to above development with PAW or BAW.
[b] Pentanol–acetic acid extract. No exposure to aq. HCl.

concentrations of the various components making up the pigment mixture can be estimated by densitometer measurements. The two-dimensional TLC plates (Fig. 2) contained a faint spot in the lower left region of the chromatogram where diglucoside pigments would be expected to appear. Employing the unidirectional paper chromatographic technique with 0·6%, w/v citric acid as the solvent,[1] however, no evidence for diglucoside pigmentation could be obtained. At most, trace amounts of diglucoside pigments are present in the *V. cinerea* skins.

* The same Roman numeral is used to designate comparable pigment fractions, regardless of method of isolation, throughout this discussion.

[5] N. NYBOM, *Physiol. Plantorum* **17**, 157 (1964).
[6] S. ASEN, *J. Chromatog.* **18**, 602 (1965).
[7] D. HESS and C. MEYER, *Z. Naturforsch.* **176**, 853 (1962).

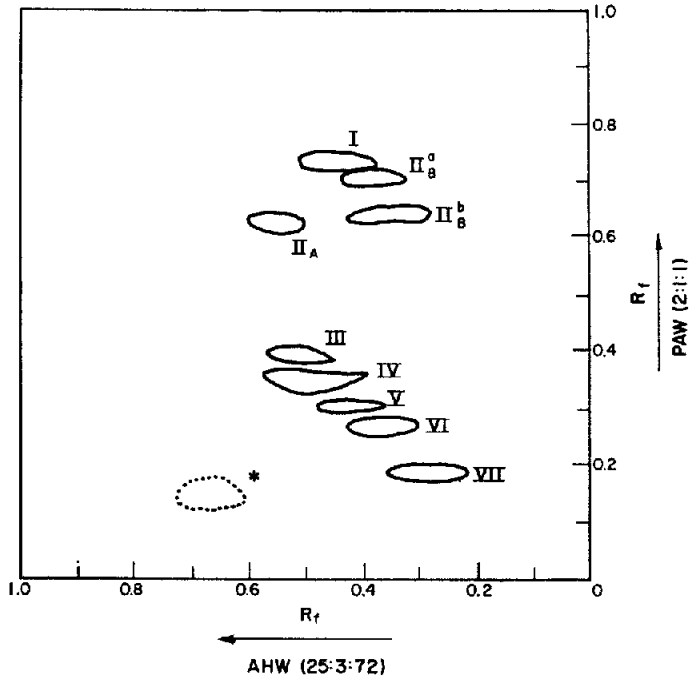

FIG. 2. TWO-DIMENSIONAL TLC OF *V. cinerea* PIGMENTS.

The recorded densities of the anthocyanin-3-glucosides, III, IV, V, VI, VII, are 0·80, 1·8, 0·74, 0·91, and 0·64, respectively. The densities of the acylated pigments, I, II$_A$, II$_B^a$, II$_B^b$, are 0·08, 0·12, 0·02, and 0·04, respectively. The possible diglucoside spot * was too faint for measurement. Order of development: 1, AHW; 2, PAW.

The structures of the five non-acylated anthocyanins were determined using standard techniques and both paper and TLC chromatography. Spectroscopic data and R_f values (Table 1), data from complete acid hydrolysis (Table 2) and from the alkaline degradation (Table 3) of the purified pigments confirmed the identities of the non-acylated pigments to be band III peonidin-, band IV malvidin-, band V cyanidin-, band VI petunidin- and band VII delphinidin-3-monoglucoside. Partial hydrolysis studies[8] further confirmed that each

TABLE 1. PROPERTIES OF ANTHOCYANINS ISOLATED FROM SKINS OF *V. cinerea*

Band	λ_{max} (nm) MeOH, HCl	$\Delta\lambda_{max}$ (nm) $AlCl_3$	E_{440}/E_{max} ×100	$R_f \times 100$ BAW*	PAW†	AHW‡
VII	277, 540	40	20	24	28	30
VI	279, 540	44	18	33	46	41
V	282, 526	44	25	39	50	46
IV	278, 538	0	20	41	57	53
III	280, 526	0	26	43	62	55

* Determined on Whatman No. 1 paper.
† Determined by TLC on cellulose (MN-300) plates.

[8] Y. ABE and K. HAYASHI, *Botan. Mag. Tokyo* **69**, 577 (1956).

TABLE 2. PRODUCTS* FROM ACID-HYDROLYSED ANTHOCYANINS OF *V. cinerea*

Material	$R_f \times 100$		
	Forestal†	FHW‡	AHW‡
V. cinerea			
Band VII	32	25	16
Band VI	46	41	22
Band V	50	35	25
Band IV	61	52	30
Band III	62	51	35
Known compounds			
Delphinidin	32	25	16
Petunidin	46	41	21
Cyanidin	49	35	26
Malvidin	60	51	30
Peonidin	63	—	36

* In each case glucose was the only sugar found.
† Determined on Whatman No. 1 paper.
‡ Determined by TLC on cellulose (MN-300) plates.

TABLE 3. ALKALINE DEGRADATION PRODUCTS OF *V. cinerea* PIGMENTS

Compound	$R_f \times 100$			Color, DSA reagent‡
	BAW*	PAW†	15HOAc†	
V. cinerea pigments				
Band VII	60, 70	56, 62	52, 63	Yellow, orange
Band VI	75, 70	76, 62	59, 63	Russet, orange
Band V	81, 70	81, 62	60, 63	Buff-yellow, orange
Band IV	85, 70	88, 62	70, 63	Orange, orange
Band III	87, 70	88, 62	69, 63	Yellow-brown, orange
Authentic compounds				
Phloroglucinol	70	62	63	Orange
Gallic acid	61	56	51	Yellow
3-Methoxy gallic acid	—	—	—	Russet
Protocatechuic acid	80	81	60	Buff-yellow
Syringic acid	83	90	68	Orange
Vanillic acid	87	91	70	Yellow-brown
p-Coumaric acid	88	93	56	Yellow
Caffeic acid	81	81	45	Buff
Ferulic acid	86	89	54	Brown

* Whatman No. 1 paper.
† Thin-layer cellulose (MN-300).
‡ All chromatograms visualized in color with DSA reagent spray.

of these pigments is a 3-monoside. The non-acylated anthocyanin pigments of *V. cinerea* were thus found to be the same as those found in the varieties of *V. vinifera* previously investigated in this laboratory[9-12] with malvidin-3-monoglucoside again being the major pigment present.

[9] R. F. ALBACH, R. E. KEPNER and A. D. WEBB, *Am. J. Enol. Viticult.* **10**, 164 (1959).
[10] R. F. ALBACH, R. E. KEPNER and A. D. WEBB, *J. Food Sci.* **28**, 55 (1963).
[11] M. AKIYOSHI, R. E. KEPNER and A. D. WEBB, *J. Food Sci.* **28**, 177 (1963).
[12] R. F. ALBACH, R. E. KEPNER and A. D. WEBB, *J. Food Sci.* **30**, 69 (1965).

Investigation of Acylated Anthocyanins

Separation of the anthocyan pigments in *V. cinerea* by unidirectional preparative paper chromatography using either the BAW or PAW solvent systems gives only two acylated pigment bands (I and II; Fig. 1), in marked contrast to the better resolution of the acylated pigments obtained by two-dimensional thin-layer chromatography on cellulose (Fig. 2).

Although chloroform–acetic acid–water, v/v 3:1:1 (CAW*) (cf. Ref. 13), was not capable of developing anthocyan pigments, it was found that if *n*-pentanol–methanol–water, v/v 10:7:5, was used to modify the atmosphere inside the chromatographic jar, the CAW* solvent system separated the acylated pigments from *V. cinerea* into four well-defined bands (I, II_A, II_B, II_C; Fig. 1). The derivatives of malvidin and peonidin have higher R_f values than the corresponding derivatives of cyanidin, petunidin and delphinidin and the *p*-coumaric acid derivatives are faster than the corresponding caffeic acid derivatives. When mineral acid was avoided in the extraction and separation procedures, much larger relative amounts of the acylated pigments, in particular those in bands II_A and II_B, were isolated using CAW.*

Separations by this chromatographic technique are strongly dependent on the atmosphere-modifying solution used. Only a slight movement of pigment on the paper occurs if *n*-pentanol alone is used, and chlorophyll moves near the solvent front. With methanol alone the acylated pigments migrate in a single broad band, chlorophyll still moving in front of the pigment. With water the pigment mixture again moved as a single band but with chlorophyll remaining at the origin. Attempts to simplify the system by modifying the CAW* developing solvent and omission of the atmosphere-modifying solution were not successful. The R_f values, separation, and compactness of the acylated pigment bands can be controlled by adjusting the composition of the pentanol–methanol–water mixture. Increasing the methanol content produces larger R_f values and better separation but the bands are not as sharp. Increasing the water content results in sharper band definition but R_f values are reduced slightly. If the water to methanol ratio is greater than 1:1, the solution becomes two phase and gives poor separations. The total dependence of the CAW* system upon the composition of the atmosphere for separation is an advantage in that it is possible to effect very subtle separations which are not otherwise possible. The composition used here was determined experimentally to give optimum separation of the acylated pigments in *V. cinerea*. It gave completely reproducible results over a period of several months. The length of time, normally 1–4 hr, the chromatogram is equilibrated in the modified atmosphere before development is started is not too critical.

A second solvent front, not associated with a pH transition, always appears as the chromatogram develops with the CAW* solvent system. This solvent front migrates to the area of band III–IV, just above the first acylated band II_C, in 15–18 hr.

Pigment extracted from the skins with methanol does not separate effectively with the CAW* solvent system because of large amounts of chloroform-insoluble material present. *n*-Pentanol containing about 5 per cent acetic acid was far more effective as a solvent for extraction of acylated pigments. The non-acylated anthocyanin-3-monoglucosides were removed before chromatography in some runs by washing the pentanol extracts with water. Considerable loss of the acylated pigments also occurred due to water solubility, however, with much greater loss of the pigments in bands II_A and II_C than in bands I and II_B.

After final purification the pigment from each of the four acylated anthocyanin bands was subjected to alkaline hydrolysis and the anthocyanins (Table 4) and acylating acids (Table 5)

[13] C. F. VAN SUMERE, G. WOLF, H. TENCHY and J. KINT, *J. Chromatog.* **20**, 48 (1965).

investigated. Malvidin and peonidin 3-monoglucosides were found from bands I and II_A. Band II_C gave petunidin, cyanidin and delphinidin-3-monoglucosides, while band II_B gave all five glucosides as hydrolysis products. The relative amounts of the various anthocyanin-3-monoglucosides in the acylated form are roughly comparable to the relative amounts of

TABLE 4. ANTHOCYANINS FROM ALKALINE HYDROLYSIS OF ACYLATED PIGMENT BANDS FROM *V. cinerea*

Material	$R_f \times 100$ AHW*	$R_f \times 100$ PAW*
V. Cinerea		
Band I	53, 55	58, 62
Band II_A	53, 56	57, 62
Band II_B	29, 41, 45, 53, 55	27, 45, 50, 57, 61
Band II_C	29, 40, 46	28, 45, 50
Known 3-glucosides of		
Delphinidin	30	28
Petunidin	41	46
Cyanidin	46	50
Malvidin	53	57
Peonidin	55	62

* Determined by TLC on cellulose (MN-300) plates.

TABLE 5. ACID MOIETIES FROM ALKALINE HYDROLYSIS OF ACYLATED PIGMENT BANDS FROM *V. cinerea*

Acid	$R_f \times 100$ 15% Acetic acid*	$R_f \times 100$ PAW* (2:1:1)	$R_f \times 100$ CAW† (4:1:1)	Fluorescence Air dried	Fluorescence NH_3 fumed	Color, DSA reagent
V. Cinerea						
Band I	57	94	56	None	Blue	Yellow
Band II_A	—	—	—	—	—	—
Band II_B	58, 45	93, 82	55, 16	Blue	Blue, pale green	Yellow, buff
Band II_C‡	57, 45	93, 80	56, 17	Blue	Blue, pale green	Yellow, buff
Knowns						
p-Coumaric	57	93	55	None	Blue	Yellow
Caffeic	45	81	16	Blue	Pale green	Buff
Ferulic	54	89	76	Blue	Blue	Brown

* Determined by TLC on cellulose (MN-300) plates.
† Determined by TLC on cellulose (MN-300), silica gel (1:1) plates.
‡ Acids detected in trace amounts only.

the anthocyanins present in the non-acylated form. Hydrolysis of band I gave only *p*-coumaric acid; band II_B gave *p*-coumaric and caffeic acids; band II_C gave trace amounts only of *p*-coumaric and caffeic acids, insufficient to account for the total amount of acylated pigment present; while band II_A gave no evidence of any cinnamic acid derivative.

The nature of the acids present in the acylated pigments was also investigated spectroscopically. The conventional u.v. spectra, A, and the difference spectra, B, for the acylated

pigments from *V. cinerea* bands I and II_A, are shown in Figs. 3 and 4, respectively. The difference spectra were recorded with a solution of malvidin-3-monoglucoside in the reference beam. The difference u.v. spectrum of band I (Fig. 3) shows a strong absorption at 312 nm confirming the presence of *p*-coumaric acid[14] and consistent with one molecule of acid per

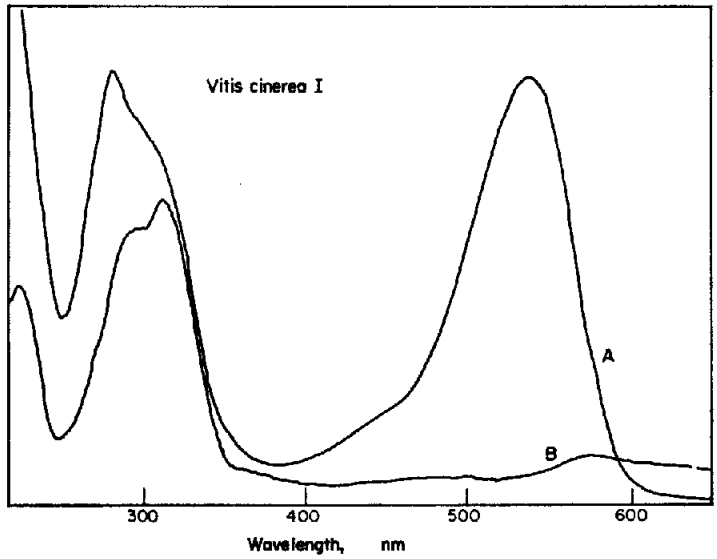

FIG. 3. ABSORPTION SPECTRA OF *V. cinerea* BAND I.
Curve A, methanol in reference beam; curve B, methanol solution of malvidin-3-glucoside in reference beam.

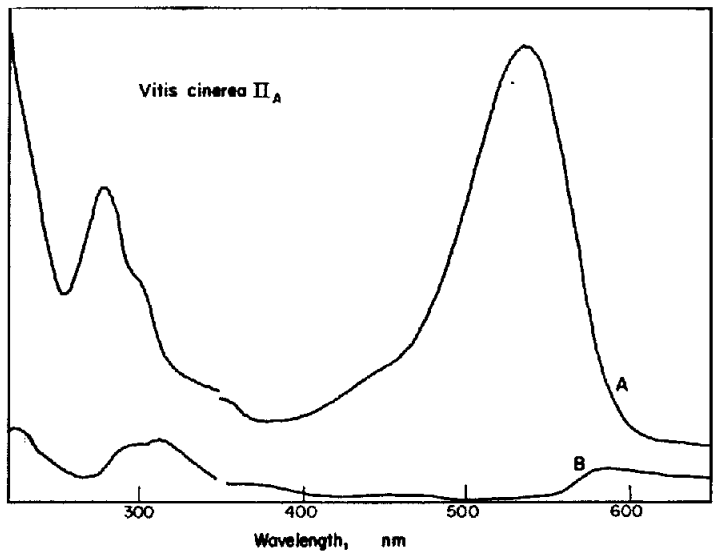

FIG. 4. ABSORPTION SPECTRA OF *V. cinerea* BAND II_A.
Curve A, methanol in reference beam; curve B, methanol solution of malvidin-3-glucoside in reference beam.

[14] J. B. HARBORNE, *Biochem. J.* 70, 22 (1958).

molecule of anthocyanin. The difference spectrum of band II_A (Fig. 4) shows essentially a complete lack of absorption over the entire u.v. visible region, indicating further that it is unlikely that the acylating acid is a cinnamic acid derivative. The difference spectrum of band II_B pigment shows, as expected, an absorption at 320 nm which is between the values expected for *p*-coumaric (310 nm) and caffeic (328 nm) acids. The difference spectrum of band II_C pigment shows only trace amounts of *p*-coumaric and caffeic acids present as acylating acids.

Band I, the second most abundant acylated band, is composed of an approximately 5:1 mixture of malvidin and peonidin 3-monoglucosides acylated with *p*-coumaric acid. This mixture can be separated using a two-phase atmosphere-modifying mixture (*n*-pentanol–methanol–water, v/v 10:5:7) in conjunction with the CAW* developing solvent. The malvidin derivative surprisingly moves ahead of the peonidin derivative. From hydrolysis studies and the position of the band on the chromatogram it is evident that the majority of the pigment in band II_B is composed of the *p*-coumaric acid derivatives of cyanidin, petunidin and delphinidin 3-monoglucosides together with smaller amounts of malvidin and peonidin 3-monoglucosides acylated with caffeic acid. By the same reasoning the caffeic acid in band II_C would be expected to be combined with the cyanidin, petunidin and delphinidin 3-monoglucosides found as hydrolysis products. The trace of *p*-coumaric acid found from this band is presumably the result of a trace contamination of band II_B. Investigation of the acylating acid in the pigment from band II_A, the most abundant acylated pigment present and the additional acylating acid from the pigment in band II_C will be presented later.

EXPERIMENTAL

Plant Material

All of the fruit from the specimen vine N20 VI, *Vitis cinerea* Engelmann, was harvested in the fall of 1967, placed in polyethylene bags, and immediately stored at −20°.

Chromatographic Procedures

Paper chromatograms were run on Whatman No. 3 MM or No. 1 sheets, descending. TLC, developed by upward migration of solvent for 15 cm, were run on 0·25 mm cellulose (MN-300, Brinkmann) plates for pigment separations, on 0·5 mm Silica Gel-G (Merck) buffered with 0·3 M sodium acid phosphate for analysis of sugars,[15] and on a 0·25 mm 1:1 mixture of cellulose and silica gel-G for separation of acids.[13]

Sugars were sprayed with Partridge's reagent[16] and acids with diazotized sulfanilic acid (DSA).[17]

General solvent systems utilized were: BAW, *n*-butanol–acetic acid–water, (4:1:5, v/v), upper phase;[18] PAW, *n*-pentanol–acetic acid–water (2:1:1, v/v);[7] CAW, chloroform–acetic acid–water (4:1:1, v/v), lower phase,[13] CAW*, chloroform–acetic acid–water (3:1:1, v/v), lower phase; AHW, acetic acid–conc. HCl–water (25:3:72, v/v);[19] FHW, formic acid–conc. HCl–water (5:2:3, v/v);[19] Forestal, acetic acid–conc. HCl–water (30:3:10, v/v);[19] S_I, *n*-butanol–acetone–water (4:5:1, v/v);[15] S_{II}, *n*-butanol–methanol–water (5:3:1, v/v);[15] 15% aq. acetic acid; 0·6% aq. citric acid;[1] 1% aq. HCl.

Spectral Analyses

Purified pigment samples in MeOH containing 0·01% HCl, to give absorptivities of 0·80–0·95 at the visible maximum,[14] were used. Shifts were recorded 1 min after addition of $AlCl_3$ (3 drops of 5% w/v in EtOH). Difference spectra were recorded using as reference methanolic malvidin-3-glucoside, adjusted to give the same absorptivity as the sample at the visible maximum.

[15] V. S. OVODOV, E. V. EVTUSCHENKO, V. E. VASKOVSKY, R. G. OVODOVA and T. F. SOLOVEVA, *J. Chromatog.* **26**, 111 (1967).
[16] S. M. PARTRIDGE, *Nature* **164**, 443 (1949).
[17] R. N. AMES and H. K. MITCHELL, *J. Am. Chem. Soc.* **74**, 252 (1952).
[18] E. C. BATE-SMITH, *Nature* **161**, 835 (1948).
[19] J. B HARBORNE, *J. Chromatog.* **2**, 581 (1959).

Investigation of Non-Acylated Anthocyanins

Isolation of pigments. The frozen berries were washed (H_2O), peeled, the skins washed in H_2O, blotted dry and refrozen. The frozen skins (40 g) were powdered under liquid N_2 and extracted with MeOH (3 × 200 ml) in the cold (−20°) for 24 hr. One drop of conc. HCl was added to the combined extracts, the extract was concentrated under reduced pressure at 30–35° to *ca.* 40 ml and the concentrate centrifuged to remove all solid matter.

Chromatographic separation of pigments. The pigment concentrate was streaked onto Whatman No. 3 MM papers (approx. 5 ml. per sheet) and washed by downward migration of 1% aq. HCl for 12 hr.[20] The chromatograms were partially dried, the pigment mixture eluted (MeOH) and after concentration restreaked on Whatman No. 3 MM papers. PAW (27 hr) gave the separations indicated in Fig. 1. The non-acylated pigments were eluted as bands III–IV, V, VI, and VII and purified by rechromatography. Band III–IV was separated into two bands by rechromatography with BAW for 24 hr. The last traces of *n*-pentanol were removed as a water–pentanol azeotope under reduced pressure. Each of the purified pigments was redissolved in MeOH and evaporated to dryness before storage at −20° under N_2.

Two-dimensional thin-layer chromatography. About 5 μl of pigment concentrate was spotted on a 0·25 mm cellulose TLC plate. The plate was developed with AHW in the first direction and PAW in the second direction for 15 cm (Fig. 2). Photodensitometric measurements of the spots were recorded with a Photovolt Recording Densitometer, Model 525 (Photovolt Corp., New York), using a 1 × 20 mm slit and a 525 nm filter.

Acid hydrolysis. Purified pigment (5–6 mg) (bands III–VII) was refluxed in 2 N HCl (3 ml) for 60 min under N_2 at 100°. The aglycones were extracted with *n*-pentanol (1 × 3 ml; 2 × 1 ml), taken to dryness (H_2O was added to facilitate removal of the *n*-pentanol as an azeotrope), the aglycones taken up in MeOH and examined by TLC and on Whatman No. 1 paper.

The aqueous hydrolysate was neutralized (Amberlite IR4B resin OH) for 2–3 hr and the resin eluted with H_2O until free from sugar (Tes-tap, Eli Lilly & Co.). The eluate was vacuum concentrated and chromatographed, along with appropriate markers, on Whatman No. 1 paper and on silica gel-G TLC plates using the S_I and S_{II} solvent systems.

Partial acid hydrolysis. Partial hydrolyses of the purified pigments (3–5 mg) from each of the anthocyanin bands (III–VII) were performed by the method of Abe and Hayashi.[8] Aliquots were examined on cellulose TLC using AHW.

Alkaline degradation. The purified pigment (5–6 mg) from each anthocyanin band (III–VII) was refluxed 1 hr in 15% $Ba(OH)_2$ under N_2.[21] The products were isolated and chromatographed, along with appropriate reference compounds, on Whatman No. 1 paper with BAW and on cellulose TLC plates with PAW and 15% acetic acid.

Investigation of Acylated Anthocyanins

Isolation of pigments. Approximately 500 g of frozen berries were washed with *n*-hexane (3 × 200 ml) to remove wax from the skins. The skins were then removed and powdered under liquid N_2 as described above. *Ca.* 70 g of powdered skins were extracted with 250 ml of *n*-pentanol and acetic acid (20:1 v/v) at room temperature with stirring for 12 hr. The extract was filtered through glass wool, a 1·5-fold vol. of H_2O added, and evaporated to dryness at 25°. The dried pigment was dissolved in 35 ml of MeOH, centrifuged to remove any suspended material and kept at −20°.

Chromatographic separation of acylated pigments. Approximately 1·5 ml of pigment extract was applied to a sheet of Whatman No. 1 paper, which was cut into 9-in. wide sheets for development. Each 12 in.^{2} all-glass developing tank was lined with two sheets of Whatman No. 1 paper between the walls and the solvent trough rack. An atmosphere modifying solution (110 ml) composed of *n*-pentanol–methanol–water (10:7:5 v/v) was sprayed on the lining sheets and allowed to equilibrate for *ca.* 1 hr. Two 9-in. papers were suspended in the jar about 3 in. from the wall and development with CAW* carried out for 18 hr. (Attempts to run more than two papers in each 12 in. tank resulted in badly skewed pigment bands with poor resolution.) After each run it was necessary to clean each tank completely and to dry the lining papers before re-use.

The chromatograms were air dried and the four acylated pigment bands (I, II_A, II_B, II_C) were cut out and eluted with MeOH. The eluates were evaporated at 25° and the dried pigments dissolved in MeOH and further purified by rechromatography on Whatman No. 3 MM paper using 30% acetic acid. The purified bands were eluted with MeOH and evaporated. The last traces of acetic acid were removed by washing the dried pigment with Et_2O (10 × 2 ml). Between each washing the pigment was redissolved in methanol and re-evaporated to dryness.

Alkaline hydrolysis of pigments. Approximately 8 mg of purified pigment from each acylated pigment band was hydrolyzed with 10% KOH solution.[22] After acidification the reaction mixture was evaporated to dryness under vacuum and the solid residue extracted with *n*-PrOH (3 × 1 ml). The PrOH extract was evaporated

[20] B. H. Koeppen and D. S. Basson, *Phytochem.* **5**, 183 (1966).
[21] D. Y. C. Lynn and B. S. Luh, *J. Food Sci.* **29**, 735 (1964).
[22] R. F. Albach, R. E. Kepner and A. D. Webb, *J. Food Sci.* **30**, 69 (1965).

to dryness and the residue redissolved in MeOH. Portions of this solution were examined on 0·25 mm cellulose TLC plates with AHW and PAW. Another portion was examined on a 0·25 mm cellulose–silica gel (1:1) TLC plate with CAW. An additional portion of each MeOH extract was evaporated to dryness, extracted with Et_2O (3 × 1 ml) which was evaporated to dryness, and the residue redissolved in MeOH. This solution was examined on 0·25 mm cellulose plates using PAW or 15% acetic acid.

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