Phytochemistry, 1971, Vol. 10, pp. 165 to 169. Pergamon Press. Printed in England.

SOME UNUSUAL ANTHOCYANINS OCCURRING NATURALLY OR AS ARTIFACTS

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(*Received* 10 *March* 1970, *in revised form* 18 *April* 1970)

Abstract—Artifacts appeared during chromatographic purification of anthocyanins in BAW (butanol–acetic acid–water, 4:1:5); they were shown to be produced by the combined action of acetic and hydrochloric acids during concentration of components eluted from the paper. The extent of their formation depended upon the nature of the sugar, e.g. cyanidin 3-glucoside formed an artifact more readily than cyanidin 3-galactoside. These and similar anthocyanins produced using propionic, butyric and valeric acids instead of acetic acid were assumed to be acylated with the appropriate acid. An artifact of similar type occurred also during solvent development (BAW) of strongly acid solutions of pelargonidin 3-glucoside but not of cyanidin or delphinidin 3-monosides. Two naturally occurring anthocyanins of similar properties found in *Cichorium intybus* leaves appeared to be acylated derivatives of the main pigment, cyanidin 3-glucoside.

INTRODUCTION

RECENT reports of the occurrence of acylated anthocyanins in maize seed (*Zea mays*)[1] and apple skin (*Malus*)[2] and of unidentified anthocyanins in some fruits and vegetables,[3] prompts us to record our own observations of some unusual anthocyanins which occur naturally or which can arise as artifacts during experimental manipulation.

RESULTS

During paper chromatography of extracts of *Chaenomeles speciosa* petals, copper beech (*Fagus sylvatica*) leaves, willow (*Salix*) bark,[4] apple (*Malus*) skins and chicory (*Cichorium intybus*) leaves, it was observed that anthocyanins which behaved initially as single bands or components were sometimes accompanied on further purification in BAW by faint additional components of higher R_f values. Although, when isolated, these new anthocyanins behaved as discrete and separate entities in BAW, they nevertheless possessed identical aglycone and sugar components and spectral characteristics as their parent anthocyanins. When applied to paper in acetic acid solution and chromatographed, the artifacts tolerated additions of appreciable amounts of hydrochloric acid (up to 10% HCl in the applied solution or up to about 2% HCl in the BAW solvent itself); above these levels they reverted to their parent anthocyanins. The extent of their formation depended upon the nature of the sugar group, glucose being affected more than galactose. Thus, a single band containing both cyanidin 3-glucoside and cyanidin 3-galactoside (similar R_fs) gave two bands on further chromatography in BAW. The component of higher R_f contained more glucose than galactose; conversely, the major band of normal R_f contained more galactose than glucose. We have now ascertained that these new anthocyanins did not occur naturally in the original plant material studied but were formed as artifacts during experimental manipulation.

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[1] J. B. HARBORNE and G. GAVAZZI, *Phytochem.* **8**, 999 (1969).
[2] I. PAIS and G. GOMBKOTO, *A. Kertesz. Szoleszeti Föiskola Kémi. Tansz.* **31**, 71 (1967).
[3] T. FULEKI, *J. Food Sci.* **34**, 365 (1969).
[4] P. BRIDLE, K. G. STOTT and C. F. TIMBERLAKE, *Phytochem.* **9**, 1097 (1970).

It is standard procedure during preparative chromatography of anthocyanins on thick paper to elute a separated component from the paper with a solvent containing acetic acid (such as methanol, acetic acid, water; MAW) rather than HCl, in order to avoid undue hydrolysis of the less stable acylated anthocyanins and the formation of arabinose which may interfere with subsequent sugar identification.[5] After concentration, the anthocyanin remains dissolved in strong acetic acid. The artifact is formed only if sufficient HCl is also present in the extract undergoing concentration. The mineral acid may be introduced from residual amounts remaining on the paper being eluted, which derive from the crude acid extract of plant material and/or from a preceding separation in a solvent containing HCl. Thus, portions of the single band obtained after chromatography in HOAc–HCl of a crude extract of copper beech leaves were eluted separately with (a) methanol—0·1% HCl and (b) MAW. On subsequent chromatography in BAW extract (a) gave a single band whereas extract (b) containing both HOAc and HCl gave a double band. The artifact was readily produced from a pure anthocyanin such as cyanidin 3-galactoside (which was available from apple skins).[6,7] Treatment at room temp. with HOAc containing 5–30% of conc. HCl resulted in the formation of two bands on subsequent chromatography in BAW. The addition of either acid alone gave no artifact. The two bands also appeared when the excess acids were removed *in vacuo* before chromatography. The additional band was thus formed by the acid treatment rather than during development on the paper. Under these conditions, an appreciable amount of artifact was formed with only 1% conc. HCl in HOAc. The dried anthocyanin when dissolved in methanol streaked to the solvent front with considerable blueing, but exhibited well-defined double banding and comparative stability when dissolved in methanol containing 1–5% HCl.

Although their identity has not yet been proved unequivocally, the behaviour of these new anthocyanins suggests that they may contain sugars acylated with one or more acetate groups. Moreover, the replacement of the acetic acid in the reaction mixture by propionic acid, or *n*-butyric acid, or *n*-valeric acid (solubilized with acetone), but not formic acid, yielded with cyanidin 3-galactoside artifacts of increasing R_fs, suggesting the possible incorporation of the acid anion into less-polar anthocyanin structures. Occasionally a second artifact, weaker but of still greater R_f was observed with acetic or propionic acid. The mobility of both artifacts showed some variation, probably dependent upon the amount of HCl present. Typical values of the ratio (R_f of artifact/R_f of cyanidin 3-galactoside) from three separate BAW chromatograms are given in Table 1. Further, under similar conditions galactose itself formed up to four components, tentatively identified as mono-, di-, tri- and tetra-acetates by the identity of their R_fs (TLC) with those of the hydrolysis products of β-galactose penta-acetate. Our inability, as yet, to demonstrate the presence of intact acetylated sugars among the hydrolysis products of the artifacts is probably due to their instability under the conditions employed (10% HCO_2H, or H_2O_2).[8]

Following these observations it was of interest to ascertain whether "acetylation" of anthocyanins could occur also during development in BAW. Since the solvent itself contains acetic acid, acetylation might arise if the applied solution contained a high concentration of HCl, as is possible after concentration of a methanolic-HCl extract of plant material.

[5] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 14, Academic Press, London (1967).
[6] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 155, Academic Press, London (1967).
[7] B. H. SUN and F. J. FRANCIS, *J. Food Sci.* **32**, 647 (1968).
[8] B. V. CHANDLER and K. A. HARPER, *Australian J. Chem.* **14**, 586 (1961).

Accordingly pelargonidin 3-glucoside[9] was dissolved in aqueous solutions containing varying amounts of HCl, streaked on paper and developed in BAW. Above a certain level of HCl, an additional band of higher R_f was formed, running only a little slower than the acid front. After elution with methanol–0·01 % conc. HCl, the new band ran to the usual R_f of pelargonidin 3-glucoside and possessed its spectrum. A similar but more pronounced artifact was produced when acetic acid in the solvent was replaced by propionic acid, but was not evident when the solvent contained formic acid. Occurrence of the new form of pelargonidin 3-glucoside in BAW depended on the total amount of acid applied to the paper rather than its concentration in the applied solution. It was formed with concentrations as low as 20% of the conc. acid (6% HCl) if repeated applications were made. Since concentrations of acid greater than this may be attainable in a conc. MeOH–HCl plant extract, the formation of artifacts of pelargonidin glycosides during initial separation in BAW would seem a distinct possibility. Extracts containing cyanidin or delphinidin glycosides appear to differ in this respect from those containing pelargonidin glycosides since an artifact formed with cyanidin 3-galactoside only under extreme conditions (in concentrated acid) and could not be produced with delphinidin 3-glucoside. Multiple-zoning or spotting[10] of the types described above have not been reported previously for anthocyanins and are quite distinct from effects observed with some acylated anthocyanins due to conversion to violet-blue anhydro-base forms.[11]

TABLE 1. RATIOS OF (R_f ARTIFACT/R_f CYANIDIN 3-GALACTOSIDE) IN BAW

	Acetate artifacts		Propionate artifacts		Butyrate artifact	Valerate artifact
Chromatogram	1	2	1	2	1	1
1	1·4	—	1·7	—	2·1	3·0
2	2·0	3·8	2·9	5·4	—	—
3	1·7	2·8	2·2	—	—	—

The above findings are pertinent to our observations on chicory anthocyanins. Previous investigation[12,13] has been confined to the blue flowers which we confirm contain delphinidin glycosides. We have now examined the nature of the anthocyanin in young reddish leaves of the recently introduced cultivar "Rosa di Verona". The main component (III) and two minor components (IV and V) of increasing R_f in BAW were purified. Each component contained only cyanidin and glucose (after total hydrolysis), was unsubstituted in the 5-position and non-acylated with phenolic acids (identical low absorption 310–330 nm).[14] III was identified as cyanidin 3-glucoside since it gave cyanidin only on controlled hydrolysis and possessed the same R_f as an authentic sample. IV and V were rather unstable, easily reverting to III and IV respectively. On controlled hydrolysis V gave IV, which then gave III and cyanidin; IV gave III and cyanidin. It appeared therefore that V might be a 3-triglucoside which on hydrolysis yielded a 3-diglucoside (IV) and cyanidin 3-monoglucoside (III).

[9] P. V. NAIR and R. ROBINSON, *J. Chem. Soc.* 1611, (1934).
[10] R. A. KELLER and J. C. GIDDINGS, *Chromatog. Rev.* **3**, 1 (1961).
[11] R. F. ALBACH, R. E. KEPNER and A. D. WEBB, *J. Food Sci.* **30**, 69 (1965).
[12] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 227, Academic Press, London (1967).
[13] J. T. A. PROCTOR and L. L. CREASY, *Phytochem.* **8**, 1401 (1969).
[14] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 17, Academic Press, London (1967).

However the R_fs of the anthocyanins in BAW were in the reverse order of those normally expected for increased glycosidation, and although in the usual order in aqueous-acid solvents, they were not as high as might be expected for di- and tri-glucosides (Table 2).[15] Further, repeated attempts to isolate a di- or a tri-glucoside were unsuccessful; the only sugar which could be identified with certainty after hydrolysis of IV or V with acetic acid or hydrogen peroxide was glucose. Quantitative analysis of IV[16] gave a ratio of glucose/cyanidin of *ca.* 1, indicating that IV was a monoglucoside. The spectra of all three bands were identical in the u.v. region and indicated that IV and V were not contaminated with leucoanthocyanins. The mobility of IV and V in BAW suggested that they were monomeric and not polymeric. While they might contain anomeric forms of glucose,[17,18] a more likely explanation, in view of their similarity to the anthocyanins described previously, was that they consisted of cyanidin 3-glucoside acylated with acids of unknown constitution. Proof that they occurred naturally and not as artifacts produced during manipulation was afforded by chromatography of the crude extract in HOAc–HCl. The three bands so obtained (I–III) corresponded after further purification to III, IV and V respectively in BAW. A surprising feature of these anthocyanins was their stability in the acid conditions prevailing after concentration of the crude MeOH-HCl extracts. In this and in some other respects they were similar to the acylated derivatives of cyanidin 3-glucoside found in *Zea mays*.[1]

TABLE 2. CHROMATOGRAPHIC AND SPECTRAL PROPERTIES OF CHICORY ANTHOCYANINS

	Absorption spectra*				R_f (× 100)† in			
Band	λ_{max} (nm)	$\frac{E_{440}}{E_{vis.max}}$ (%)	$\frac{E_{u.v.max}}{E_{vis.max}}$ (%)	$AlCl_3$ shift	BAW	BuHCL	1% HCL	HOAc–HCl
III	528,282	23	49	+	28	23	05	25
IV	528,282	23	52	+	33	41	08	29
V	528,282	23	51	+	40	56	10	40

* In methanol containing 0·01% conc. HCl.
† Whatman No. 1 paper.

These findings further emphasise the desirability of employing mild conditions of extraction[19] of anthocyanins from plant material in the event that acylated or other anthocyanins are present which are less acid-tolerant than those in chicory or *Zea mays*. The formation of artifacts on purification can be minimized by replacing acetic acid in the eluting solvent by formic acid or by restricting the use of MAW to elution after the final purification, when little HCl is present. However, mineral acid in MeOH–HCl elution solvents, which could be used at the intermediate purification stages may still give rise to artifacts if the paper contains residual acetic acid derived from a preceding solvent.

[15] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 32, Academic Press, London (1967).
[16] J. B. HARBORNE, *Biochem. J.* **74**, 262 (1960).
[17] W. PIGMAN and H. S. ISBELL, *Advan. Carbohydrate Chem.* **23**, 11 (1968).
[18] S. E. ANGYAL, *Angew Chem.* **8**, 157 (1969).
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EXPERIMENTAL

Chromatography

The following solvents were used (all v/v): BAW, *n*-BuOH–HoAc–H_2O (4:1:5); BuHCl, *n*-BuOH–2N HCl (1:1); HOAc, H_2O–HOAc (98:2); HOAc–HCl, HOAc–conc. HCl–H_2O (15:3:82); HCl, H_2O–conc. HCl (97:3).

Purification of Anthocyanins

Cichorium intybus, a concentrated MeOH–HCl extract of young leaves of 1-yr growth, after removal of chlorophyll with ether, was streaked on Whatman No. 3 paper and irrigated for 48 hr or more with BAW. Five bands separated, numbered I–V from the origin, in amounts decreasing in the order III > IV > V > I = II; the latter bands I and II were very faint and were not examined further. The remaining bands were purified further by successive chromatography in HOAc–HCl and BAW, with intermediate elution from the paper with MAW. Young leaves of the second years' growth were treated similarly but in addition the crude extract was chromatographed in HOAc–HCl.

Acetylation of Galactose

Galactose penta-acetate was partly hydrolyzed by heating a 4% solution in acetic acid—conc. HCl (9:1) for 2·5 min at 80°. Silica-gel TLC (toluene 85–methanol 15, 3 runs) showed the presence of four spots (R_f 0·08, 0·14, 0·19 and 0·30) in addition to the penta-acetate itself (R_f 0·65), which were located by the ferric hydroxamate reaction.[20] The intermediate spots were assumed to represent galactose mono-, di-, tri- and tetra-acetates respectively, since spots of identical R_f were obtained by heating D+ galactose (1·6%) with the acid mixture under identical conditions. In the cold, galactose formed compounds corresponding to the three lower acetates with a trace of the tetra-acetate, depending upon the time of reaction. The stability of the penta-acetate was tested under the conditions under which H_2O_2 (± NH_3) is used to split off the sugar in the anthocyanin 3 position[8] — H_2O_2 alone (1 hr) gave the tetra-acetate; NH_4OH alone (few min boiling in methanol) gave a mixture of the four lower acetates; H_2O_2 + NH_3 gave only traces of the mono-acetate. The artifact itself did not give the ferric hydroxamate reaction (which was rendered less sensitive due to some darkening after the treatment with alkaline hydroxylamine). Hydrolysis with H_2O_2 (−NH_3) or 10% formic acid did not yield positive identification of sugar-acetates.

Acknowledgements—Thanks are due to C. S. Gundry for growing the chicory plants and others, Dr. A. Pollard for advice and The British Council for a scholarship for one of us (S.S.T.).

[20] M E. Tate and C. T. Bishop, *Can. J. Chem.* **40**, 1043 (1962).