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PIGMENTS OF CENTROSPERMAE—I.
BETACYANINS FROM *PHYLLOCACTUS HYBRIDUS* HORT. AND *OPUNTIA FICUS-INDICA* MILL

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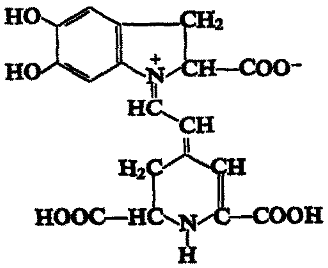
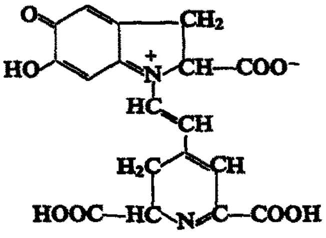
Abstract—Four betacyanins are present in the flowers of *Phyllocactus hybridus* Hort. Of these pigments, two were shown to be betanin and isobetanin, the remaining two being other glucosides of betanidin and isobetanidin, respectively. Only the first two betacyanins are present in the fruits of *Opuntia ficus-indica* Mill.

INTRODUCTION

BETACYANINS are sap-soluble pigments which as yet have been found only in plants belonging to the *Centrospermae*.[1-4] On account of the difficulty in isolating them, investigation of these nitrogenous compounds has proceeded extremely slowly.[5] The recent isolation of betanin in crystalline state from *Beta vulgaris*,[6-9] however, has changed this situation. By acid hydrolysis, this yields, along with glucose, a mixture of hydrochlorides of two compounds which can be differentiated by electrophoresis and chromatography, and are called *betanidin* and iso*betanidin*, respectively.[10] For betanidin two alternative formulas were


(I)


(II)

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[1] H. REZNIK, *Z. Botan*, 43, 499 (1955).
[2] H. REZNIK, *Planta*, 49, 406 (1957).
[3] T. J. MABRY, A. TAYLOR and B. L. TURNER, *Phytochemistry* 2, 61 (1963).
[4] W. RAUH and H. REZNIK, *Bot. Jb.* 81, 95 (1961).
[5] A. S. DREIDING, *Recent Developments in the Chemistry of Natural Phenolic Compounds*, p. 194, Pergamon Press, London (1961).
[6] H. WYLER and A. S. DREIDING, *Helv. Chim. Acta* 40, 191 (1957).
[7] H. WYLER, G. VINCENTI, M. MERCIER, G. SASSU and A. S. DREIDING, *Helv. Chim. Acta* 42, 1696 (1959).
[8] O. T. SCHMIDT, P. BECHER and M. HUEBNER, *Chem. Ber.* 93, 1296 (1960).
[9] M. PIATTELLI and L. MINALE, *Rend. acc. sci. fis. e mat.* (*Soc. nazl. sci. Napoli*) 29, 80 (1962).
[10] H. WYLER and A. S. DREIDING, *Helv. Chim. Acta* 42, 1699 (1959).

suggested.[11,12] According to our experiments, Formula II is favoured,[9] and thus the glucosyl residue in betanin is linked to the hydroxyl group in position 6.*

Phytolacca decandra L. is the only other plant belonging to the *Centrospermae* from which it has been possible to obtain the major pigment in crystalline form;[13] the betacyanin obtained in this way has been shown to be identical to betanin. From 15 other species belonging to the *Centrospermae* the main betacyanins were isolated in small quantities and partially characterized.[14] In all cases the aglycones obtained by acid hydrolysis were shown, by paper electrophoresis and paper chromatography, to be a mixture of betanidin and isobetanidin.

In order to study the distribution of betacyanins and their taxonomic implications, it is necessary to isolate and characterize as many pigments of this class as possible. The present paper deals with betacyanins from two plants of the family *Cactaceae*, namely, *Phyllocactus hybridus* Hort. and *Opuntia ficus-indica* Mill.

RESULTS

Four red-violet pigments (A, B, C and D) were isolated from methanolic extracts of *Phyllocactus hybridus* Hort. flowers by chromatography on columns of polyamide. The four pigments were shown to be homogeneous by paper electrophoresis, and their spectra and electrophoretic behaviour showed them to be betacyanins. One of these substances (A), present in larger amounts, was crystallized and identified as betanin by comparing its

TABLE 1. PROPERTIES OF BETACYANINS IN FLOWERS OF *Phyllocactus hybridus* HORT. AND IN FRUITS OF *Opuntia ficus-indica* MILL

Compounds	Total betacyanin, %	λ_{max} mμ in water	E_b^*		Hydrolysis with almond emulsin†	Acid hydrolysis products‡
			pH 2·4	pH 4·5		
Betanin	—	538	1	1	+	b, i, g
Isobetanin	—	538	0·93	0·93	+	i, g
Unknowns§						
A	60	538	1	1	+	b, i, g
B	32	538	0·93	0·93	+	i, g.
C	7·5	538	1	1·25	−	b, i, g
D	1·7	538	0·93	1·14	−	i, g
A1	79	538	1	1	+	b, i, g
B1	21	538	0·93	0·93	+	i, g

* Migration in paper electrophoresis relative to betanin.
† Hydrolysed (+); not hydrolysed (−).
‡ Key: b = betanidin, i = isobetanidin, g = glucose.
§ A–D from *P. hybridus*; A1, B1 from *O. ficus-indica*.

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* *Added in proof:* In a recent paper H. WYLER *et al.* (*Helv. Chim. Acta* **46**, 1745 (1963)) gave conclusive evidence in favour of Formula I.

[11] H. WYLER and A. S. DREIDING, *Helv. Chim. Acta* **45**, 638 (1962).

[12] T. J. MABRY, H. WYLER, G. SASSU, M. MERCIER, I. PARIKH and A. S. DREIDING, *Helv. Chim. Acta* **45**, 640 (1962).

[13] H. WYLER and A. S. DREIDING, *Helv. Chim. Acta* **44**, 249 (1961).

[14] H. WYLER and A. S. DREIDING, *Experentia* **17**, 23 (1961).

properties (infra-red and ultra-violet spectra, X-ray diagram and its behaviour on electrophoresis and chromatography) with those of an authentic sample. The other three pigments could not be isolated in sufficient amounts for complete characterization. Pigment B proved to be identical to a betacyanin also isolated in small quantities from *Beta vulgaris*, in which it makes up about 3 per cent of total pigment. This betacyanin had already been observed in *Beta vulgaris* by other authors and named isobetanin.[7, 13] On acid hydrolysis A and C yielded mixtures of betanidin and isobetanidin in a ratio of approximately 7:3, whereas B and D yielded isobetanidin only. All four pigments also yielded glucose. Though C and D were not affected by almond emulsin, A (betanin) and B (isobetanin) were quickly hydrolysed yielding betanidin (free of isobetanidin) and isobetanidin, respectively. This shows that they are β-glucosides, a conclusion which disagrees with earlier German workers,[8] who reported that emulsin had no effect on betanin.

Two betacyanins were found in the fruits of *Opuntia ficus-indica* Mill., but only one (A1) was obtained in crystalline state and was identified as betanin. The other (B1) was shown to be identical to isobetanin. The distribution of betacyanins in the flowers of *Phyllocactus hydridus* Hort. and in the fruits of *Opuntia ficus-indica* Mill., and their properties, are summarized in Table 1.

DISCUSSION

Previous authors have always obtained mixtures of betanidin and isobetanidin by acid hydrolysis of betacyanins;[10, 13, 14] the relationship between these two substances has not been fully clarified so far, but it is likely that they are isomers. According to Wyler and Dreiding,[5, 10] an equilibrium mixture is obtained from either betanidin or isobetanidin in acid and in alkaline solution (in the absence of oxygen). We have observed that although a partial transformation of betanidin into isobetanidin occurs under the conditions used for the acid hydrolysis, the reverse transformation does not take place. The fact that mixtures of the two aglycones are obtained by acid hydrolysis of the pigments A and C is ascribed to a partial transformation of betanidin into isobetanidin by the action of the acid. However, betanidin is not transformed into isobetanidin in the conditions used for the enzymic hydrolysis of the betacyanins and under these conditions A and B yield only betanidin and isobetanidin respectively. These results show therefore that in the *Centrospermae* betacyanins derived from both betanidin and isobetanidin occur, and B and D appear to be the first isolated betacyanins which have isobetanidin as the aglycone.

EXPERIMENTAL

Authentic Pigments

An improved modification of our previous procedure[9] was used to obtain both betanin and isobetanin from *Beta vulgaris*. Aqueous extracts of *Beta vulgaris*, adjusted to pH 3, are passed onto a column of acid resin (Dowex 50W-X2). Under these conditions betanin and isobetanin were bound to the resin non-ionically, whereas small amounts of other betacyanins (prebetanin and isoprebetanin[7]) were removed by thoroughly washing with 0·1% hydrochloric acid. Betanin and isobetanin were eluted with water; the eluate, concentrated to a small volume, was chromatographed on polyamide, as described below.

Authentic samples of betanidin and isobetanidin were isolated from the mixture of hydrochlorides of the two aglycones obtained by acid hydrolysis of betanin by chromatography on polyamide using 0·1% hydrochloric acid as the eluent. Their properties agreed completely with those reported in the literature.[7]

Materials

Polyamide (polyhexamethylene adipamide) powder was prepared by precipitating the polymer (100 g) from a solution in formic acid (750 ml) by slow addition of a water–ethanol mixture (50:50, v/v; 1000 ml). The product was washed with distilled water until acid free and stored wet. Almond emulsin was purchased from Mann Research Laboratories.

Paper Chromatography and Paper Electrophoresis

Chromatograms were run at room temperature by the descending technique on Whatman No. 1 paper. Electrophoretograms were run for about 1 hr in a horizontal apparatus, using either a pyridine-formate buffer (0·05 M, pH 4·5) or formic acid (0·1 M, pH 2·4), the potential gradient being 16 V/cm.

Spectra

Ultra-violet spectra were determined on a Unicam SP 500 spectrophotometer. Infra-red spectra were determined (KBr pellets) on a Perkin–Elmer model 13C spectrophotometer.

Extraction and Isolation of Betacyanin from Phyllocactus hybridus *Hort.*

Fresh petals of *Phyllocactus hybridus* Hort. (1 kg), previously washed with light petroleum 40–70°, were ground in methanol (1 l.) in a Waring blendor for 15 sec. The homogenate was heated on a steam-bath and filtered through two layers of cheese-cloth; the extraction was repeated three times and the combined filtrate after centrifuging were concentrated *in vacuo* to 500 ml. The solution, after standing overnight at 4°, was centrifuged and the supernatant concentrated *in vacuo* in a rotatory evaporator and finally adsorbed onto Whatman cellulose powder. This powder was placed on top of a cellulose column (5 × 30 cm), and after removal of the yellow pigments (betaxanthins[1, 2, 15]) by washing with methanol (7 l.), the betacyanins were eluted with water (500 ml). The eluate was adjusted to pH 3 with N HCl and the total betacyanin fraction was adsorbed onto a column of Dowex 50W-X2, (H^+ form, 2·5 × 20 cm). The column was washed with 0·1 % hydrochloric acid (2 l.) and the washings discarded. The red–violet pigments were eluted with water (1 l.), and the eluate was taken to dryness under reduced pressure, thus obtaining 150 mg of crude betacyanins. Further purification and separation of the pigments were effected on six columns of polyamide (5 × 30 cm, flow rate of about 200 ml/hr). Elution with 0·1 % hydrochloric acid gave four fraction (A, B, C and D) which emerged from the columns respectively after about 1·3 l., 1·8 l., 2·7 l. and 3·7 l. The resolved bands were purified by resin treatment as above. The solutions were made up to a known volume and their adsorbivities measured at 538 mμ. The relative percentages shown in Table 1 were calculated on the assumption that all betacyanins have the same $E_{1\%}^{1\,cm}$ of betanin ($E_{1\%}^{1\,cm} = 1120$).

Fraction A was concentrated to a small volume (10 ml) and allowed to stand overnight at 4°; the crystals were filtered off, washed with water (2 ml) and dried to constant weight (11·2 mg) in a vacuum desiccator. X-ray diagrams, ultra-violet and infra-red spectra of the compound were determined and shown to agree with those of authentic betanin. The unknown gave a single spot after electrophoresis at pH 2·4 and 4·5 when mixed with betanin. Further identification was made by hydrolysing the compound as described below (see Table 1).

After evaporating to dryness, fraction B, C and D gave three other betacyanins which were not present in sufficient amount for crystallization. In paper electrophoresis at pH 2·4 and

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[15] M. PIATTELLI and L. MINALE, *Rend. acc. sci. fis. e mat.* (*Soc. nazl. sci. Napoli*) (in press).

4·5, B migrated to the same extent as isobetanin; co-chromatography on polyamide (0·1% hydrochloric acid as eluent) showed no separation. Properties of the pigments C and D are summarized in Table 1.

Extraction and Isolation of Betacyanins from Opuntia ficus-indica *Mill.*

Mature red-violet fruits (2 kg) of *Opuntia ficus-indica* Mill. were macerated in 500-g batches with water in a blendor. The homogenate was heated at 70° and squeezed through several layers of cheese-cloth. The extract was cooled to 10°, adjusted to pH 3 with N hydrochloric acid and allowed to stand overnight at 4°. The brown precipitate was removed by centrifuging and the clear supernatant purified on a column (6 × 35 cm) of resin as before. The eluted betacyanin fraction was taken to dryness under reduced pressure. Chromatography of the residue (200 mg) on six polyamide columns (5 × 30 cm) using 0·15% sodium acetate as the eluent, gave two bands (retention volumes 0·7 l. and 1·8 l. respectively). The separate bands were purified with resin and the eluate of the first band was concentrated to a small volume (about 10 ml) at 40° under reduced pressure and allowed to stand overnight at 4°; the crystals formed were collected, washed with water and dried in a vacuum desiccator (15·7 mg). X-ray diagrams, ultra-violet and infra-red spectra of the product agreed with those of authentic betanin.

No crystals could be obtained from the second fraction. When mixed with reference isobetanin this pigment gave a single spot after electrophoresis at pH 2·4 and 4·5, and chromatography on a polyamide column (0·9 × 40 cm) gave a single peak.

Acid Hydrolysis of Betacyanins

A solution of the pigment (1 mg) in 22% HCl (1 ml) was heated at 80° for 5 min. The hydrolysate was evaporated to dryness *in vacuo*, and excess HCl was removed by adding a small amount of water to the residue and evaporating to dryness four successive times. The residue was finally dissolved in water (0·5 ml) and the aglycones identified by paper electrophoresis (pH 2·4 and 4·5). The remainder of the solution was applied to a small column (1 × 10 cm) of polyamide, and sugars were eluted with water. The eluate was taken to dryness, dissolved in water (0·1 ml) and analysed for carbohydrates by chromatography.

The solvent systems used, were n-BuOH:CH_3COOH:H_2O (12:3:5), isoPrOH:H_2O (4:1), $CH_3COOC_2H_5$:CH_3COOH:H_2O (14:3:3), n-PrOH:$CH_3COOC_2H_5$:H_2O (7:1:2) $CH_3COOC_2H_5$:pyridine:H_2O (73:18:9). The sugars were detected on air-dried chromatograms by aniline phosphate.

Enzymic Hydrolysis of Betacyanins

To a solution of the betacyanins in sodium acetate buffer (pH 5), almond emulsin (1 mg in 0·1 ml of water) was added and the mixture was kept at 37°. Electrophoretic analysis (pH 2·4 and 4·5) of the reaction mixture and of an enzyme-free control solution were made at 15-min intervals during 3 hr.