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PIGMENTS OF CENTROSPERMAE—II.
DISTRIBUTION OF BETACYANINS

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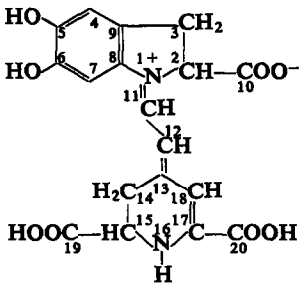
Abstract—The distribution of betacyanins in thirty-seven plants, including examples from seven families of the order Centrospermae, has been investigated by using an automatic method of analysis on polyamide column. A large number (forty-four) of betacyanins, most of them previously undescribed, have been observed and the spectrophotometric and electrophoretic properties of these pigments have been determined. When sufficient amounts were available, the betacyanins were subjected to acid hydrolysis; analysis of the hydrolysate has shown that they are all derived from betanidin and isobetanidin.

INTRODUCTION

RED-VIOLET pigments called betacyanins[1] (formerly regarded as nitrogenous anthocyanins)[2] and corresponding yellow ones, for which the name betaxanthins has been proposed,[3] occur in many plants of the order Centrospermae.[3–7] Up to the present, only the betacyanins have been extensively investigated. Betanidin, the aglycone of the red-violet pigment betanin isolated originally from *Beta vulgaris*,[8–11] has been shown to have the structure I,[12,13] quite different from that of the anthocyanins or other flavonoids. Treatment of betanidin with an



FORMULA I

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acid or a base (in the absence of oxygen) effects a partial change of configuration at C-15; the product of transformation being isobetanidin.[13,14]

There is no known case of the coexistence in the same plant of betacyanins and anthocyanins; other classes of flavonoid pigments are common in the betacyanin-containing species.[4,5] Betacyanins are present in flowers and fruits, and often in roots, stems, bracts and other parts of plants as well. They may also form in pathological conditions such as insect injury or malnutrition. The function of these pigments in the plant is unknown, except that when present in flowers they may have a role, like the anthocyanins, in insect or bird pollination.

Even before the structure of these pigments was clarified, their systematic significance had been evaluated by several authors. At first betacyanins were found in eight families[3–5] (Amaranthaceae, Basellaceae, Cactaceae, Chenopodiaceae, Mesembryanthemaceae, Nyctaginaceae, Phytolaccaceae and Portulacaceae) usually grouped in the order Centrospermae. More recently Rauh and Reznik[6] observed betacyanins in Didieraceae, while Mabry *et al.*[7] found them in *Stegnosperma halimifolium* of the family Stegnospermaceae. This family had previously been considered by Hutchinson as belonging to the order Pittosporales[15] although most taxonomists include it in Phytolaccaceae. Among the families of the classically constituted Centrospermae only the Caryophyllaceae and Illecebraceae apparently lack betacyanins. These families do, however, contain anthocyanins, and it has been proposed by Mabry *et al.*[7] that they be considered as a phyletic group related but not belonging to the order Centrospermae, the latter being reserved for the betacyanin-containing families.

These significant taxonomic results, relying solely on the demonstration of the presence or absence of betacyanins, invite a more thorough investigation of the relationship between the betacyanin patterns and the botanical classification of plants of the Centrospermae at lower systematic level. Such research involves the analysis of the betacyanins from a very large number of species belonging to all the genera under investigation; furthermore, it is necessary to examine the different plant parts separately. Unfortunately, paper chromatography, so often used for chemotaxonomic studies, gives poor results with betacyanins; more useful data are obtained by paper electrophoresis. Nevertheless, even this technique is unsatisfactory for systematic work, since different betacyanins often migrate to the same extent (see Table 1). Moreover, it should be noted that when crude extracts are analysed, the presence of extraneous material makes resolution worse still. Nor is it suitable to follow Mabry *et al.*[7] in adopting the purification method proposed by Pucker *et al.*,[8] since this method causes the partial esterification of the betacyanins, making interpretation of the results even more difficult.

In order to study the distribution of these pigments, it was clear that a better separation procedure must be devised. Since strongly acid cation exchange resin and polyamide powder had proved very useful, the first for the purification and the second for the separation of the betacyanins,[11,16] we used them in the present work for routine analyses of betacyanins. The method has been made automatic and permits the analysis of a betacyanin mixture in under 24 hr.

In addition to the possibility of applying this method to the taxonomic study of Centrospermae, it may be profitably used for the screening of a large number of plants in order to select those from which certain of the betacyanins can be isolated for structural elucidation.

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TABLE 1. PROPERTIES OF BETACYANINS

Pigment	λ_{max} in water (mμ)	Column 1		E_b‡		Aglycones obtained by acid hydrolysis§
		R_v* (ml)	R_{vb}† (ml)	pH 4·5	pH 2·4	
1 Amarantin	536	49·2	0·64	1·16	1·00	b, i
2 Isoamarantin	536	57·0	0·74	1·16	1·00	i
3 Betanin	538	76·5	1·00	1·00	1·00	b, i
4 Isobetanin	538	90·0	1·18	0·93	0·93	i
5 Iresinin I	538	105·0	1·38	1·17	1·00	b, i
6 Iresinin II	538	111·5	1·44	1·17	1·00	n
7 Phyllocactin	538	126·5	1·63	1·25	1·00	b, i
8 Celosianin	544–546	145·5	1·90	1·26	1·11	b, i,
9 Isophyllocactin	538	153·0	2·00	1·14	0·93	i
10 Isocelosianin	542–544	159·0	2·08	1·26	1·11	i
11 Bougainvillein I	538–540	164·0	2·15	0·96	0·94	b, i
12 Bougainvillein II	540–542	187·0	2·45	0·90	0·92	i
13 Gomphrenin I	535–537	195·0	2·55	1·00	0·96	b, i
14 Betanidin	542–546	206·0	2·70	1·00	0·70	
15 Bougainvillein III	540–542	209·5	2·75	1·00	0·94	b, i
16 Bougainvillein IV	540–542	220·5	2·88	0·96	0·81	n
17 Isobetanidin	542–546	226·0	2·95	0·87	0·70	
18 Gomphrenin II	536–538	230·0	3·00	0·91	0·89	i
19 Oleracin I	534–536	234·0	3·06	0·80	0·72	n
20 Oleracin II	534–536	250·0	3·26	0·80	0·72	n
21 Gomphrenin III	536–538	252·5	3·30	0·91	0·89	n
22 Bougainvillein V	540–542	257·5	3·37	0·84	0·81	b, i
23 Bougainvillein VI	544–546	281·0	3·68	0·84	0·81	i
24 Bougainvillein VII	544–546	293·0	3·82	0·84	0·81	b, i
25 Gomphrenin IV	540–542	293·0	3·82	0·80	0·72	b, i
26 Prebetanin	540–542	301·0	3·95	1·34	1·78	n
27 Bougainvillein VIII	544–546	306·5	4·01	0·84	0·81	b, i
28 Isoprebetanin	540–542	315·0	4·14	1·21	1·78	n
29 Bougainvillein IX	544–546	317·0	4·15	0·84	0·81	n
30 Gomphrenin V	542–544	317·0	4·15	0·80	0·72	b, i
31 Bougainvillein X	544–546	335·0	4·37	0·84	0·81	b, i
32 Gomphrenin VI	542–544	335·0	4·37	0·80	0·72	b, i
33 Rivinianin	541–543	341·0	4·48	1·34	1·78	n
34 Bougainvillein XI	544–546	348·0	4·55	0·84	0·81	i
35 Gomphrenin VII	542–544	348·0	4·55	0·80	0·72	i
36 Bougainvillein XII	548–550	359·0	4·70	0·66	0·52	b, i
37 Gomphrenin VIII	540–542	359·0	4·70	0·80	0·72	n
38 Bougainvillein XIII	549–551	377·0	4·90	0·66	0·52	b, i
39 Bougainvillein XIV	550–552	386·5	5·05	0·66	0·52	n
40 Bougainvillein XV	544–546	413·0	5·30	0·51	0·37	b, i
41 Bougainvillein XVI	544–546	441·0	5·78	0·51	0·37	n
		Column 2				
		R_v* (ml)	R_{vb}† (ml)			
42 Mesembryanthemin I	540–542	93·0	4·25	0·48	0·28	n
43 Mesembryanthemin II	540–542	136·0	6·16	0·48	0·28	n
44 Mesembryanthemin III	540–542	166·0	7·55	0·48	0·28	n

* Absolute retention volume.

† Retention volume relative to betanin.

‡ Migration in paper electrophoresis relative to betanin.

§ Key: b = betanidin, i = isobetanidin, n = no sufficient amount of the pigment was available for the acid hydrolysis.

TABLE 2. NATURAL OCCURRENCE OF BETACYANINS

No.	Plant	Plant parts*	Amarantin	Isoamarantin	Betanin	Isobetanin	Iresinin I	Iresinin II	Celosianin	Isocelosianin	Gomphrenin I	Gomphrenin II	Gomphrenin III	Gomphrenin IV	Gomphrenin V	Gomphrenin VI	Gomphrenin VII	Gomphrenin VIII		
	Amaranthaceae																			
1	*Alternanthera versicolor* Seub.	lv	+++	++	+															
2	*Amaranthus caudatus* L.	in	+++	++																
		lv	+++	+		+														
		st	+++	+																
3	*A. graecizans* L.	in	+++	++																
4	*A. hybridus* L.	lv	+++	++		+														
5	*A. hypocondriacus* L.	in	+++	++																
6	*A. retroflexus* L.	in	+++	+																
7	*A. tricolor* L.	rt	+++	−	+	+														
8	*A. tricolor* cv. "Re del fuoco"	lv	+++	++																
9	*Atriplex hortense* L.	lv	++	+	+				+++											
10	*Celosia cristata* L.	in	+++	++					+++	+										
11	*C. cristata* cv. "Toreador"	in	+++	++																
12	*C. plumosa* cv. "Forest fire"	in	+++	++																
13	*Gomphrena globosa* L.	in	++	++	++				+++		+++	++	++	+	+++	+++	++	++		
		lv	+	+																
14	*Iresine lindenii* Van Houtte	lv	+		+		+++	+++												
15	*I. lindenii* var. formosa	lv	++				+++													
16	*I. herbstii* Hook	lv	++				+++													

No.	Plant	Plant parts*	Betanin	Isobetanin	Isophyllocactin	Phyllocactin
	Cactaceae					
17	*Opuntia ficus-indica* Mill.	fr	+++	++		
18	*O. bergeriana* A. Weber	fl	+++	+		
19	*O. vulgaris* Mill.	fr	+++	++		
20	*Phyllocactus hybridus* Hort.	fl	++	++	+	+

No.	Plant	Plant parts*	Amarantin	Isoamarantin	Betanin	Isobetanin	Isophyllocactin	Phyllocactin	Celosianin	Isocelosianin	Prebetanin
	Chenopodiaceae										
21	*Beta vulgaris* L. var. rubra	rt	++	+	+++	++			+		+
22	*Chenopodium album* L.	lv	++	+	+++	++					
23	*Ch. amaranticolor* Coste	lv	++	+	++	+					
24	*Kochia scoparia* Schrad.	lv	++		+++	+	+	++	+		
25	*Salsola soda* L.	st	++	++	++	+			+++		
26	*Spinacia oleracea*	pt	++		+	+		+	+++	+	

No.	Plant	Plant parts*	Betanin	Isobetanin	Betanidin	Isobetanidin	Mesembryanthemin I	Mesembryanthemin II	Mesembryanthemin II	Mesembryanthemin I
27	Mesembryanthemaceae (Aizoaceae)									
28	*Mesembryanthemum conspicuum* Haw.	fl	+++	+++	+++	+	+++	+++	+	
29	*M. edule* L.	fl	+++	+++	+++	+	+++	+++	+	
	M. floribundum Haw.	fl	+	+						

No.	Plant	Plant parts*	Betanin	Isobetanin	Betanidin	Isobetanidin	Bougainvillein I	Bougainvillein II	Bougainvillein III	Bougainvillein IV	Bougainvillein V	Bougainvillein VI	Bougainvillein VII	Bougainvillein VIII	Bougainvillein IX	Bougainvillein X	Bougainvillein XI	Bougainvillein XII	Bougainvillein XIII	Bougainvillein XIV	Bougainvillein XV	Bougainvillein XVI
30	Nyctaginaceae																					
31	*Bougainvillea fastuosa*	br					+++		++													
32	*B. glabra* var. *sanderiana*	br					+++	++	++	+	+	+	++	++	+	++	++	+++	+++	+	+++	+++
33	*Mirabilis jalapa* L.	fl	+++	+																		

No.	Plant	Plant parts*	Betanin	Isobetanin	Betanidin	Isobetanidin	Rivinianin
34	Phytolaccaceae						
35	*Phytolacca decandra* L.	fr	+++	++	+		
	Rivinia humilis L.	fr	+++				

No.	Plant	Plant parts*	Betanin	Isobetanin	Betanidin	Isobetanidin	Olefacin I	Olefacin II	Mesembryanthemin II	Mesembryanthemin III
	Betanin									
	Isobetanin									
	Betanidin									
	Isobetanidin									
	Olefacin I									
	Olefacin II									
	Mesembryanthemin II									
	Mesembryanthemin III									

No.	Plant	Plant parts*	Betanin	Isobetanin	Betanidin	Isobetanidin	Olefacin I	Olefacin II	Mesembryanthemin II	Mesembryanthemin III
36	Portulacaceae									
37	*Portulaca oleracea* L.	st	+++	++	++	+	+++	++	++	+
	P. grandiflora Hook	fl	+++							
		st	+++							

* Key: br = bracts, fl = flowers, fr = fruits, in = inflorescences, lv = leaves, pt = petioles, rt = roots, st = stems.

Since the $E^{1\%}_{1cm}$ is known only for betanin and betanidin, both absolute and relative amounts of the pigments cannot at present be calculated from the peak areas. Therefore, the data on betacyanin distribution in the plants examined are presented in semiquantitative form.

Peak areas are classified as small (+), medium (++) and large (+++).

Betacyanins present in trace amounts (peak area $< 1\%$ of the total area) are not indicated.

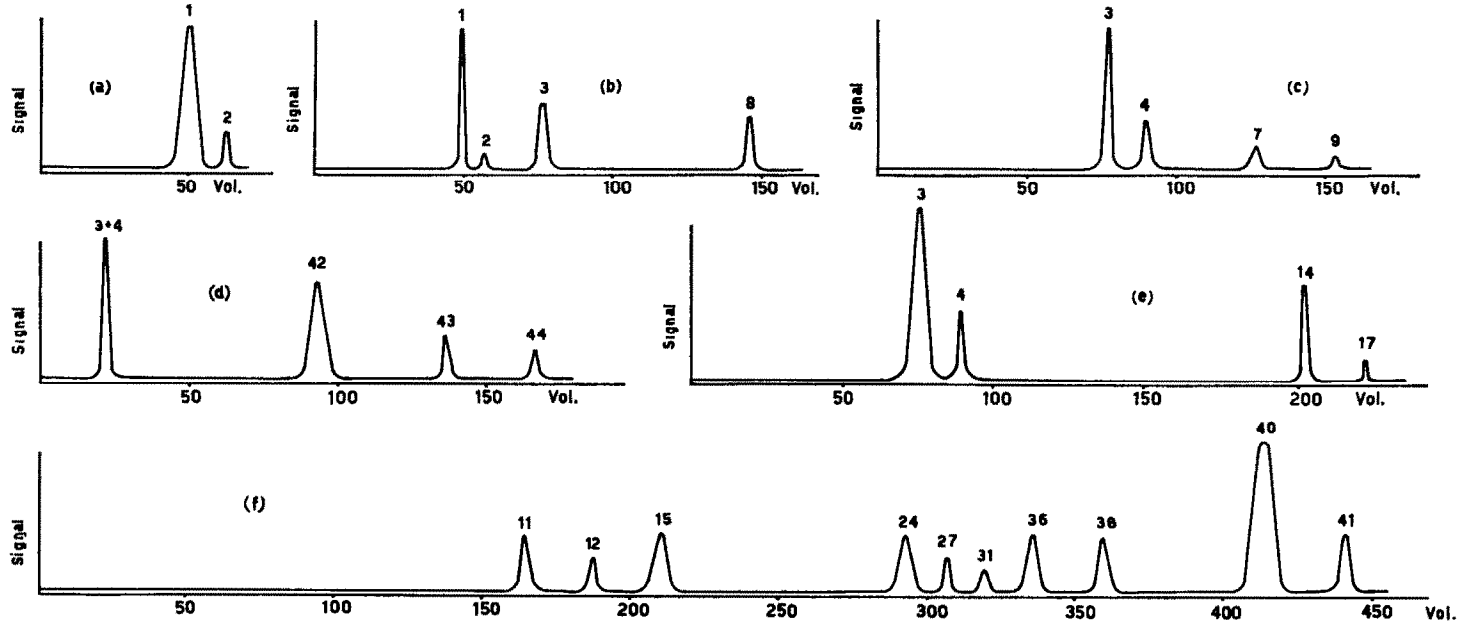

FIG. 1. CHROMATOGRAMS ON POLYAMIDE COLUMN OF BETACYANINS DERIVED FROM: (*a*) LEAVES OF *Amaranthus caudatus* (Table 2; 2), (*b*) LEAVES OF *Chenopodium amaranticolor* (Table 2; 23), (*c*) FLOWERS OF *Phyllocactus hybridus* (Table 2; 20), (*d*) FLOWERS OF *Mesembryanthemum floribundum* (Table 2; 29), (*e*) FLOWERS OF *Portulaca grandiflora* (Table 2; 37), (*f*) BRACTS OF *Bougainvillea glabra* (Table 2; 31). Chromatograms (*a*), (*b*), (*c*), (*e*), (*f*) were run on column 1; chromatogram (*d*) was run on column 2. The numbers on the peaks refer to the betacyanins listed in Table 1.

RESULTS

Thirty-seven plants were studied, including examples from seven families of Centrospermae (see Table 2).

The betacyanins were extracted with water from homogenated tissues, purified by non-ionic absorption on a strongly acid resin, and chromatographed on a column of polyamide (40 × 0·9 cm; column 1), using increasing concentrations of methanol in aqueous citric acid as the developing solvent. To prevent interconversion of betanidin and isobetanidin derivatives, and hydrolysis and further degradation of the pigments, it proved necessary to carry out the whole procedure at 5°. In a few specimens, pigments with retention time longer than 24 hr on column 1 were observed; they gave sharper peaks on a shorter column (30 × 0·9 cm; column 2), using a 50:50 v/v mixture of 10% aqueous citric acid and methanol as the developing solvent. Under these conditions, there was an overlapping of the pigments having a shorter retention time, making a second analysis on column 1 necessary. The eluate of either column, after continuous monitoring of the betacyanin concentration level, was collected in fractions of 8 ml. The resolved bands were freed from citric acid by resin treatment; electrophoretic mobilities and u.v. spectra of all the isolated pigments were determined. When a specific betacyanin was available in sufficient amounts, it was subjected to acid hydrolysis and the hydrolysate was examined for betanidin and isobetanidin.

The properties of 44 betacyanins isolated in the course of the present work are summarized in Table 1; most of them have not been described previously. Distribution of the betacyanins in the plants examined is shown in Table 2. Some typical chromatograms on polyamide columns are shown in Fig. 1.

DISCUSSION

Chromatography on polyamide gives far better results than the techniques previously used for betacyanin analysis. Its limitations are due to the large number of betacyanins which exist, probably even greater than the number found so far by analysing only thirty-seven species. In none of the individual species examined, did a pigment separated by polyamide chromatography prove to be inhomogeneous by paper electrophoresis. Nevertheless, as can be seen from Table 1, pigments having identical retention volume on polyamide column, but distinguishable by electrophoresis, were found in two different species, viz. *Bougainvillea glabra* and *Gomphrena globosa* (Bougainvillein VII–Gomphrenin IV; Bougainvillein IX–Gomphrenin V; Bougainvillein X–Gomphrenin VI; Bougainvillein XI–Gomphrenin VII; Bougainvillein XII–Gomphrenin VIII; see Table 1). A more frequent case is that of pigments, separable by polyamide chromatography, which have the same electrophoretic behaviour (for example, the seven Bougainvillein V–XI). In the light of this, the results reported by previous authors must be considered with reserve since many seemingly pure betacyanins may in fact be complex mixtures. For example, Wyler and Dreiding's so-called 'Gomphrenin', which was thought to make up 80% of the betacyanin content of the inflorescences of *Gomphrena globosa*,[3] is actually a mixture of numerous pigments (Gomphrenin IV–VIII; see Table 1). On the other hand, it should be kept in mind that insufficient resolution of betacyanins by paper electrophoresis cannot account for all the discrepancies between our results and those of previous workers. Some of these differences depend on different betacyanin composition in individual plants of the same species, differences which are a result of external and internal factors (nutritional conditions, stage of development, and so on) whose influence has not yet been investigated. This is the case with *Kochia scoparia*. Wyler and Dreiding found 95% of its betacyanin total to be made up of betanin,[3] whereas we found, in addition to betanin, large amounts of phyllocactin. Since this pigment has electrophoretic mobility

markedly different from that of betanin, it could not have been overlooked by the Swiss workers. The intra-specific variation of the betacyanin pattern within a species also results from the comparison of data concerning two specimens of *Bougainvillea glabra* collected at different stations during the same period (see Table 2, Nos. 31 and 32).

From the results summarized in Table 1, it appears that all the betacyanins subjected to acid hydrolysis gave either isobetanidin or a mixture of betanidin and isobetanidin. In a previous communication[16] it was shown that, while the isobetanidin glycosides examined yielded only isobetanidin by acid hydrolysis, the betanidin glycosides gave a mixture of betanidin and isobetanidin, as a result of the partial isomerization of the aglycone. From these facts it can be readily assumed that all the betacyanins which have been subjected to acid hydrolysis are derived either from betanidin or isobetanidin. This finding suggests that the differences among the betacyanins involve the glycosidic pattern and/or the configuration at C-15 of the aglycone rather than the basic structure.* Additional work is necessary to ascertain which of the described pigments differ only in the configuration at C-15. So far, we have observed that betanin, amarantin, phyllocactin and celosianin are reversibly transformable into isobetanin, isoamarantin, isophyllocactin and isocelosianin, respectively. Since the first four pigments yielded a mixture of betanidin and isobetanidin by acid hydrolysis while the other four yielded isobetanidin, it is to be deduced that the two terms of every pair of mutually transformable compounds have the same relationship one to the other as betanidin to isobetanidin, i.e. they differ in configuration at C-15.

It is still an open question whether both betanidin and isobetanidin derivatives are synthesized by the plant or whether one or the other are primary products of metabolism, the remaining ones being secondary products due to epimerization of the aglycone. However, it is probable that both types of pigments are present in plant tissues, for we have adopted experimental conditions that would prevent isomerization from taking place. The fact that in a few plants (e.g. *Atriplex hortense*, *Chenopodium amaranticolor*, *Salsola soda*) some betanidin glycosides occur unaccompanied by their corresponding isomers, whereas the opposite was never observed, might indicate that the betanidin pigments are the primary products and the isobetanidin pigments are formed by isomerization.

As for the possible taxonomic significance of betacyanin distribution in Centrospermae, the number of plants so far examined is admittedly too small to permit any general conclusions to be drawn. The following provisional assumptions, however, can be made:

(*a*) in some species the betacyanin pattern does not depend on the tissue which is examined (e.g. *Amaranthus caudatus* and *Celosia plumosa*), whereas in other species the different plant parts have different betacyanin composition (e.g. *Gomphrena globosa* and *Portulaca gradiflora*).

(*b*) within a single genus, the patterns of different species may be very similar (e.g. in *Amaranthus* or *Opuntia*,) or completely different (e.g. in *Portulaca*). This makes it difficult, if not impossible, to assign a given species to a particular genus;

(*c*) due to the wide difference in the betacyanin pattern of genera belonging to the same family, it is often impossible to assign a given species to a particular family.

EXPERIMENTAL

Collection of Plant Material

The plants listed in Table 2 were collected from April to October 1963 in the Naples area, with the exception of *Salsola soda* (No. 25) and *Bougainvillea glabra* (No. 32) which were col-

* It cannot be excluded at the present time that some other modification of the structure of the aglycone such as esterification of the carboxyl groups, might account for the different betacyanins.

lected near Pescara and on the island of Ischia respectively. In order to prevent post-harvest changes, the plant material was worked up as soon as possible.

Extraction of Plant Material

The fresh plant material was ground in 4–6 vol. of cold water in a Waring blender. The homogenate was heated at 70° for 5 min and filtered through several layers of cheese-cloth; the extraction was repeated until no more colour was removed. The combined extracts were adjusted to pH 3 by addition of N HCl and centrifuged. The clear supernatant was passed onto a column of Dowex 50W-X2 (H^+ form, 1·5 × 10 cm). Under these conditions the betacyanins were bound to the resin non-ionically. After washing of the column with 0·1% HCl (200 ml)* the betacyanins were slowly eluted with water. Absorption and elution was performed at 5–10°.

The volume of the eluate was reduced *in vacuo* at 30° until a 0·1-ml sample, after dilution to 10 ml, had an optical density (540 mμ) ranging from 0·5 to 1. Aliquots (1 ml) of the concentrated solution were used for analysis of betacyanins on polyamide column(s).

Analysis of Betacyanins on Polyamide Column

1. *Material.* In the course of the present work two types of polyamide (polyhexamethylene adipamide) powder, differing in particle size, were used:

Type *a*, prepared by precipitating the polymer (100 g) from a solution in formic acid (750 ml) by slow addition (2 hr) of water–ethanol mixture (50:50, v/v; 1000 ml).

Type *b*, of smaller particle size, was prepared by precipitating the polymer (100 g) from a solution in formic acid (1000 ml) by rapid addition of ethanol (3000 ml).†

The polyamide powders were washed with distilled water until acid free and stored wet.

2. *Apparatus.* A schematic diagram of the automatic analyser is shown in Fig. 2. The details are as follows: (i) Polyamide columns. Two heavy-walled jacketed chromatograph tubes of 0·9 cm i. d. with heights of 50 cm (column 1) and 40 cm (column 2) were uniformly packed to a height of 40 and 30 cm respectively, with a 50:50 w/w mixture of the two types of polyamide powder. The columns were maintained at 5° throughout analysis. (ii) Proportionating pump (Technicon Instrument Corporation, Chauncey, New York). (iii) The manifold was constructed according to Fig. 2, with 0·045, 0·035 and 0·030 in. (i. d.) tygon tubes for the diluent, the nitrogen and the eluent respectively. (iv) A Technicon colorimeter fitted with a 6-mm light path flow cuvette was used with interference filters which transmitted maximally at 540 mμ. (v) The recorder was a Speedomax type G, model S (Leeds and Northrup Co., U.S.A.). (vi) The gradient device was a 550-ml twelve-chambered Autograd (C. Erba) similar to that described by Peterson and Sober[17]. (vii) A C. Erba fraction collector was used.

3. *Procedure.* At the conclusion of a run, as indicated by the absence of pigments on the column, the following procedure was started:

(Column 1) after equilibration of the column with 5% aq. citric acid, the solution was removed from above the polyamide surface with a suction line, and the sample (1 ml), prepared as above, was applied. After absorption of the sample under gravity flow and a

[17]E. A. Peterson and H. A. Sober, *Anal. Chem.* **31**, 857 (1957).

* A few pigments (prebetanin, isoprebetanin and rivinianin) were eluted in part during the acid washing of the resin. The fraction of the washing containing these pigments was taken to dryness *in vacuo* and the residue added to the main betacyanin fraction.

† Type *b* polyamide powder gave a better resolution of the betacyanins but had too high a resistance to flow.

small wash of about 0·5 ml of 5% aq. citric acid, the space above the column was filled with 5% aq. citric acid. The top of the column was connected to the gradient device the chambers of which contained 23 ml of 10% citric acid and 23 ml of increasing concentrations of methanol in water: the first eight containing 0, 4, 6, 8, 10, 12, 14, and 18 ml respectively and the last four 23 ml of absolute methanol. The pump was then started.

(Column 2) no equilibration was necessary at the conclusion of a run. After addition of the sample, the top of the column was connected to a reservoir containing 250 ml of a 50:50 v/v mixture of 10% aq. citric acid and methanol.

At the exit from the column (column 1 or column 2), the eluate (0·32 ml/min) was diluted with water (0·82 ml/min) and segmented with nitrogen (0·42 ml/min). This diluted sample stream was continuously monitored, and collected in 8-ml fractions. The resolved bands were

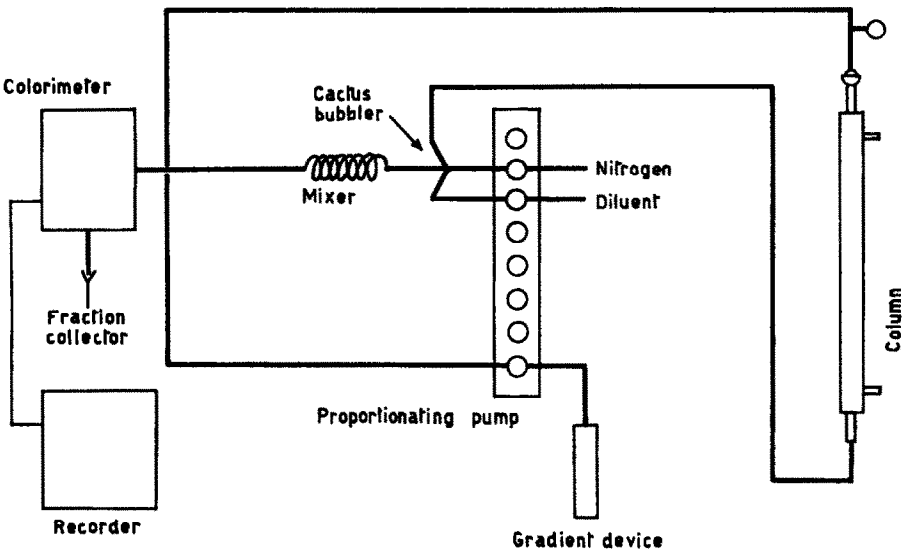

FIG. 2. DIAGRAM OF APPARATUS.

purified on a column (1·5 × 5 cm) of resin as before. Electrophoretic mobilities at pH 2·4 and 4·5 and u.v. spectra were measured; when sufficient material was available, acid hydrolysis was performed.

Paper Electrophoresis

Electrophoretograms were run for about a 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 of 16 V/cm.

Spectra

Ultra-violet spectra were determined on Unicam SP 500 spectrophotometer.

Acid Hydrolysis of Betacyanins

The acid hydrolysis of the betacyanins was performed as described in a previous paper.[16] The aglycones were identified by paper electrophoresis at pH 2·4 and 4·5.

Isomerization of Betacyanins

A solution of betanin (1 mg) in 5% aq. citric acid (1 ml) was allowed to stand at room temperature for 36 hr and then chromatographed on column 1. Betanin and isobetanin in a 6:4 ratio were present as well as traces of betanidin and isobetanidin. Under the same experimental conditions isobetanin was partially transformed into betanin (betanin:isobetanin ratio 4:6; traces of betanidin and isobetanidin were also present). Similarly, amarantin, phyllocactin and celosianin were reversibly transformed into isoamarantin, isophyllocactin and isocelosianin respectively.