Phytochemistry, 1971, Vol. 10, pp. 1591 to 1593. Pergamon Press. Printed in England.

ZONE ELECTROPHORETIC INVESTIGATION OF ANTHOCYANINS*

YOSHINOBU ÔSAWA, MASAKO KOIZUMI and NORIO SAITÔ
Chemical Laboratory, Meiji-Gakuin University, Shirokane, Tokyo, Japan
and
TAKESHI KAWAI
Meiji Gakuin Higashimurayama Senior High School, Higashimurayama, Tokyo, Japan

(Received 16 *February* 1970, *in revised form 26 May* 1970)

Abstract-Cellulose acetate film electrophoresis has been carried out with anthocyanins, and related compounds. Most pigments migrate towards the cathode, except the blue protocyanin and the red pigment of Chrysanthemum which are anionic. Commelinin separates into four spots, one slightly anionic (major) and three cationic congeners, and the genuine blue pigment of *Gentiana* seems to resemble the three latter compounds. *Platycodon* and *Viola* were shown to contain at least two pigments, both of them, like the *Hydrangea* blue pigment, exhibiting expectable electrophoretic behavior. The violet petals of *Centaurea* contain a red and a blue pigment. These findings provide convincing proof that the anthocyanins *in situ,* have a flavylium cation structure.

INTRODUCTION

THERE have so far been many proposals concerning the chemical structure of anthocyanin pigments, since the excellent work of Willstätter *et al.*,[1] who showed that cyanin from *Centaurea cyanus* L. has an oxonium cation structure. Some of the structures recently put forward on the basis of analytical studies on isolated crystalline pigments.[2-4] Owing to the lack of conclusive evidence, however, the question of their exact structure still remains to be elucidated.

Zone electrophoresis using filter paper has been tried on several of anthocyanins. In acetate buffer[2,5,6] the pigments do not move far and so borate buffer has been more commonly used.[7,8] Such electrophoretic behavior, as is well known, is not due to the net charge of the original pigment molecule, but due to the formation of charged borate complexes.

Recently, the present authors have used cellulose acetate film in acetate buffer and shown that the red anthocyanins have a flavylium cation structure.[9]

This paper deals with the electrophoretic analysis of red, violet, blue anthocyanins and some other related compounds in more detail.

* A part of this work was presented at the 34th Annual Meeting of the Botanical Society of Japan, Tokyo (1969).

[1] R. WILLSTÄTTER and A. E. EVEREST, *Liebigs* Ann. *Chem.* 401, 189 (1913).
[2] N. SAITÔ, K. HIRATA, R. HOTTA and K. HAYASHI, *Proc. Jap. Acad. 40, 516* (1964).
[3] N. SAITÔ, *Meno. Seitoku Junior College Nutrition* 1, 29 (1965).
[4] K. TAKEDA, N. SAITÔ and K. HAYASHI, *Proc. Jap. Acad. 44,352 (1968).*
[5] E. BAYER, *Angew. Chem. 78,* 834 (1966).
[6] N. SAITÔ and K. HAYASHI, SC. *Rep. T. K. D.*, Sect. B, 12, 39 (1965).
[7] N. ISHIKURA and K. HAYASHI, *Botan. Mug. Tokyo* 76, 6 (1963).
[8] M. SHIBATA, K. YAMAZAKI and N. ISHIKURA, *Botan. Mug. Tokyo 78, 299 (1965).*
[9] *Y.* ÔSAWA, N. SAITÔ, M. KOIZUMI and T. KAWAI, *Proc. 34th Ann.* Meet. *Bot. Soc. Japan,* **44** (1969); Y. ÔSAWA, M. KOIZUMI, N. SAITÔ and T. KAWAI, *Meiji-Gakuin Rev.* 159,187 (1970).

RESULTS AND DISCUSSION

Within the pH range from 3·8 to 5·8, in general, the mobility of pigments at lower pH values is greater than that at higher ones except in a few cases with anionic pigments. The relative electrophoretic mobility, however, did not change with pH. Accordingly, the results obtained only at one pH value are summarized and shown in Table 1. From this, it is evident that red and violet flower pigments *(Dahlia variabilis* DESF., *Rosa multiflora* Thumb., awobanin, *Fuchsia hybrida* Voss. and *Melastoma candidum* D. Don var. *Nobotan Makino.),* with the exception of that from *Chrysanthemum,* show mobility toward the cathode. Protocyanin *(Centaurea cyanus* L.) migrates markedly toward anode (the violet petals of *Centaurea* contain red and blue pigments corresponding to cyanin and protocyanin, respectively). Commelinin separates into four spots, one slightly anionic (major) and three cationic pigments, and the *Gentiana* blue pigment moves a little toward cathode. The pigments of *Platycodon* and *Viola* give both blue and violet spots, and these as well as the *Hydrangea* blue pigment are cationic.

TABLE 1. ELECTROPHORETIC PROFILES OF ANTHOCYANINS AND RELATED COMPOUNDS

Sources	Aglycones	(−) 2 1 0 1 1 2 cm (+)
Red pigments:		
Dahlia variabilis DESF.	Pelargonidin	
Dahlia variabilis DESF.	Cyanidin	
Rosa multiflora Thumb.*	Cyanidin	
Chrysanthemum ***morifolium*** Ramat. var. ***Sinensis*** *Makino.*†	Cyanidin	
Commelina communis L. (Awobanin)	Delphinidin	
Fuchsia *hybrida* Voss	Malvidin	
Dahlia *variabilis* DESF. (Cyanin-Cl)	Cyanidin-Cl	
Fuchsia *hybrida* Voss. (Malvin-Cl)	Malvidin-Cl	
Dahlia variabilis DESF.	Pelargonidin-Cl§	
Dahlia cariabilis DESF.	Cyanidin-Cl§	
Violet pigments:		
Melastotna *candidum* D. Don var. ***Nobotan Makino.***	unknown	
Platycodon glaucus Nakai.	Delphinidin:;	
Viola ***tricolor*** L. var. *hortensis DC.*‡	Delphinidin¶	
Centaurea cyanus L.	Cyanidin	
Blue pigments:		
Centaurea cyanus L. (Protocyanin)	Cyanidin	
Commelina communis L. (Commelinin)	Delphinidin	
Gentiana ***scabra*** Bunge var. ***Buergeri Maxim.***	Delphinidin	
Hydrangea macrophylla Seringe var. ***Otaksa*** *Makino.*	Delphinidin	
Others:		
Quercitrin	Quercetin	
Cu-chlorophyllin		

* cv. "Crimson glory". † cv. "Sayohime". ‡ cv. "Purple queen". § Aglycones were spotted. ¶ Violet and blue spots are shown by black and white circles, respectively. $\mu = 0{\cdot}05$ acetate buffer, pH 42, 10 v/cm, 45 min at *ca.* 5".

It seems most likely that the flavylium cation of anthocyanins is mainly responsible for the cationic property of these pigment molecules. The sugar residue in the anthocyanins does not contribute to the positive charge since quercitrin and rutin scarcely move. The fact that no mobility was found with aglycones, is possibly due to their insolubility in the acetate buffer used. Also red and violet pigments free from organic acids show almost the same mobility as those containing them. Neither does the substitution pattern of the B-ring affect the electrophoretic property, for there are hardly any differences in mobility between pigments having varying number of hydroxyl and/or methoxyl groups.

The red pigment of ***Chrysanthemum,*** which migrates toward the anode, has been reported to be a new type of anthocyanin,[10] the structural analysis of which is in progress at present. It may be suggested that the pigment is a kind of metal complex, or that it is associated with a strongly anionic, unknown moiety. The reason why the blue pigments are anionic in nature is still obscure. It has been shown that the mobility of a metal-free red pigment from protocyanin[6] (free from Fe(III), Mg(II), peptide and carbohydrate) becomes larger when ferric chloride is admixed, but, Mg(II) had no effect. It seems possible, therefore, that the negative charge of protocyanin is due to anionic complex formation with the metal through the adjacent cis dihydroxy groups of B-ring and/or sugar residues. However, mixtures of cyanin or malvin with ferric chloride did not migrate towards the anode. Therefore, if anionic complex formation does occur in protocyanin, it must be a complicated one.

Commelinin was separated into four well-distinctive spots on electrophoresis, only the major spot having a weak anionic nature (Table 1). This may be due to variations in the mode of combination of Mg(II), awobanin and flavocommelin which are combined in a molar ratio of 1: 4 : 4 in commelinin. The blue pigments of ***Gentiana*** may be formed from varying complexes in a similar way. On the other hand, the violet flowers of ***Platycodon*** and ***Viola*** were shown electrophoretically to contain at least two pigments, ***i.e.*** a violet and a blue one. This agrees with chromatographic findings reported earlier." The blue compounds, like the blue anthocyanin of ***Hydrangea,*** may have the same flavylium cation structure as most of the red anthocyanins.

MATERIALS AND METHODS

Pigment samples were prepared as follows: fresh petals were mixed with crushed dry-ice and ground into a powder, which was extracted with acetone–EtOH (2: 1, v/v) (1·8 ml/g). After squeezing the suspension through cloth, the resulting solution was treated with *iso*-amyl alcohol. The aqueous layer was removed, and concentrated at 40" ***in vacuo***. The crude pigment samples were obtained by centrifugation of the concentrate at 15,000 rev/min for 60 min.

Authentic specimens, pelargonin and cyanin, were obtained from ***Dahlia*** by the method reported already.[2,3] Commelinin was prepared following Hayashi et ***al.***[12]

Electrophoresis was run by the use of cellulose acetate film, 'Separax' (1 x 10 cm) under the following conditions: buffer; μ = 0·05 acetate buffer, pH 3·8, 4·2, 4·5, 4·8, 5·2 and 5·8. Operation voltage; 10 V/cm, for 45 min at ca. 5".

TLC, which was used for the measurement of molecular size, was carried out by a method presented elsewhere.'[3]

[10] K. KAWASE, Y. TSUKAMOTO, N. SAITÔ and Y. ÔSAWA, ***Proc. 33rd Ann. Meet. Bot. Soc. Japan 57 (1968);*** K. KAWASE, Y. TSUKAMOTO, N. SAITÔ and Y. ÔSAWA, ***Plant Cell Physiol.*** 11, 349 (1970).
[11] Y. ÔSAWA and N. SAITÔ, ***Abstract. Paper,*** 11th ***Internation. Bot. Congress,*** p. 164 (1969); Y. ÔSAWA and N. SAITÔ, ***Meiji-Gakuin Rev. 156,l*** (1970).
[12] K. HAYASHI, Y. ABE and S. MITSUI, ***Proc. Jap. Acad. 34,373 (1958).***
[13] ***Y.*** ÔSAWA, N. SAITÔ and K. ISHIZUKA, ***Proc.*** 33rd ***Ann. Meet. Bot. Soc. Japan 57 (1968).***