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ELECTRON SPIN RESONANCE STUDIES ON ANTHOCYANINS

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Abstract—The ESR characteristics of anthocyanins and their congeners have been determined using both crystalline and non-crystalline preparations. For the latter, a micro-technique has been proposed. The results show that red and violet pigments specifically exhibit a distinct signal at g = 2·057 being different from blue anthocyanins. It has been suggested that the g = 2·057 signal arises from the unpaired electron of pyrylium ring. Comparison of ESR spectra of isolated anthocyanins with those in fresh petals has further shown that there is no difference in the g = 2 region of ESR spectra and that the appearance of the g = 2·003 signal is thus not due to anthocyanins alone.

INTRODUCTION

THE ISOLATION of anthocyanins in a crystalline form without the use of mineral acid has been carried out by Hayashi *et al.* with considerable success [1–9]. It has been shown that these crystal-line preparations ("genuine" anthocyanins) have, without exception, almost the same visible light absorption spectra as those of materials in fresh petals.[10]

However, the structure of these crystalline anthocyanins is still not resolved. In the case of blue anthocyanins, which have been shown to have a metallo-complex structure by analytical studies, it has not been decided how the metal(s) is joined to the free anthocyanins. Electron spin resonance (ESR) studies were therefore undertaken to throw some further light on these questions. Since the isolation of many "genuine" anthocyanins is, unfortunately, rather difficult, part of this work has been carried out using non-crystalline specimens. Furthermore, the ESR characteristics of these isolated anthocyanins has been compared with those in *in vivo* using fresh petals.

RESULTS AND DISCUSSION

ESR Study of Crystalline Preparations

ESR spectra of crystalline anthocyanins and their congeners are as shown in Fig. 1. It is evident from these results that the red and violet anthocyanins (pelargonin and cyanin) of *Centaurea cyanus* L. and *Dahlia variabilis* DESF. respectively show a distinct signal at g = 2·057 as well as at g = 2·003. In cyanin chloride, on the other hand absorption at g = 2·057 in ESR spectra is absent, but the red pigment prepared from cyanin chloride by removal of

[1] K. HAYASHI, Y. ABE and S. MITSUI, *Proc. Japan Acad.* **34**, 373 (1958).
[2] S. MITSUI, K. HAYASHI and S. HATTORI, *Proc. Japan Acad.* **35**, 169 (1959).
[3] K. HAYASHI, N. SAITÔ and S. MITSUI, *Proc. Japan Acad.* **37**, 393 (1961).
[4] N. SAITÔ, S. MITSUI and K. HAYASHI, *Proc. Japan Acad.* **37**, 485 (1961).
[5] K. TAKEDA and K. HAYASHI, *Proc. Japan Acad.* **38**, 161 (1962).
[6] N. SAITÔ, K. HIRATA, R. HOTTA and K. HAYASHI, *Proc. Japan Acad.* **40**, 516 (1964).
[7] N. SAITÔ and K. HAYASHI, *Sci. Rep. Tokyo Kyoiku Daigaku* **12**, 39 (1965).
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[9] K. TAKEDA and K. HAYASHI, *Proc. Japan Acad.* **46**, 449 (1965).
[10] N. SAITÔ, *Phytochem.* **6**, 1013 (1967).

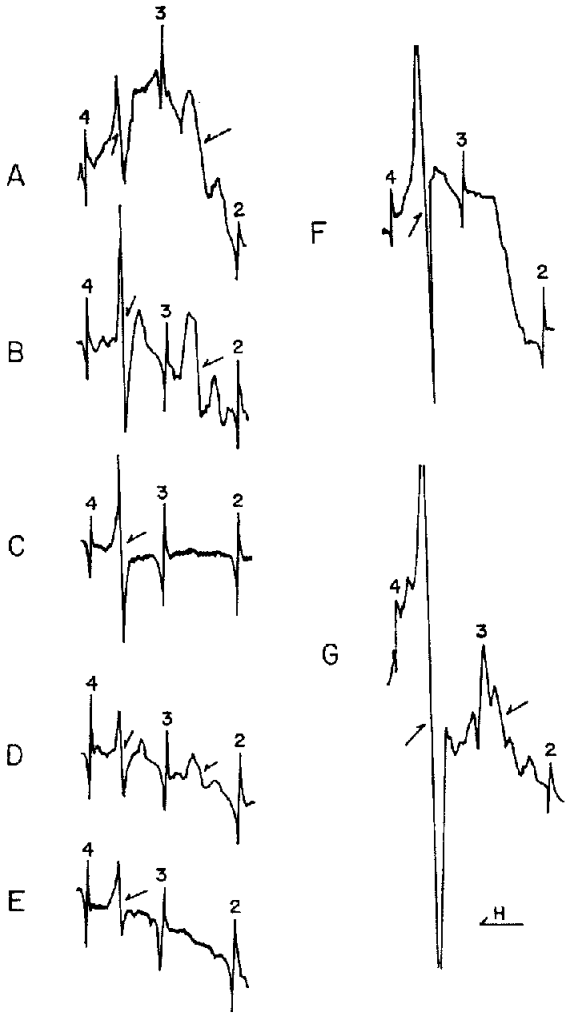


FIG. 1. ESR SPECTRA DETECTED BY NORMAL METHOD IN CRYSTALLINE ANTHOCYANINS AND THEIR CONGENERS.

The three sharp signals 2, 3 and 4 are from Mn^{2+} marker. The arrow located between 2 and 3 indicates the g = 2·057 signal, and the g = 2·003 signal is observed between 3 and 4. A, *Centaurea cyanus* L., pelargonin; B, *Dahlia variabilis* DESF., cyanin; C, *Dahlia variabilis* DESF., cyanin chloride; D, red pigment from cyanin chloride; E, *Centaurea cyanus* L., protocyanin; F, *Commelina communis* L., commelinin; G, metal-free pigment brought about from commelinin.

chloride ion gives this g = 2·057 signal. In the case of the blue anthocyanins such as protocyanin and commelinin, only the g = 2·003 signal is observed in g = 2 region. The metal-free pigment formed from commelinin by treatment with oxine gives signals at both g = 2·003 and 2·057.

ESR Study of Non-Crystalline Specimens

ESR spectra of non-crystalline anthocyanins and their congeners have been examined by the use of a microtechnique in which the pigment was first separated by filter paper chromatography. The ESR were then determined directly on the paper. As shown in Fig. 2, filter

paper is diamagnetic. The ESR spectra detected either by the microtechnique or on crystalline specimens correspond quite well.

On the basis of the above experiment the microtechnique was applied to the detection of ESR spectra of a number of other non-crystalline specimens. In Fig. 3, it will be seen that the "genuine" red and violet anthocyanins of *Salvia splendens* Ker., *Dahlia variabilis* DESF. and *Senecio cruentus* DC. show the distinct signal at g=2·057 in addition to the one at g=2·003.

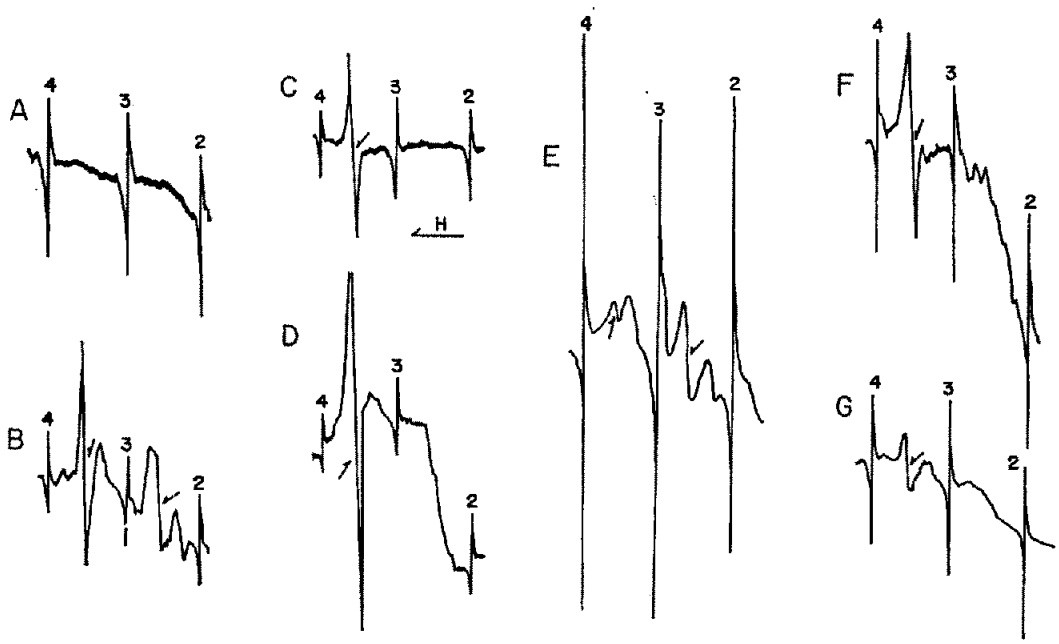


FIG. 2. COMPARISON OF ESR SPECTRA MEASURED BY THE TWO TECHNIQUES.
The three sharp signals 2, 3 and 4 are due to Mn^{2+} as marker. The arrow located between 2 and 3 indicates the g = 2·057 signal, and the g = 2·003 signal is observed between 3 and 4. A, filter paper; B and E, *Dahlia variabilis* DESF., cyanin; C and F, *Dahlia variabilis* DESF., cyanin chloride; D and G, *Commelina communis* L., commelinin; B, C and D, by normal technique with crystalline preparations; E, F and G, by the microtechnique on filter paper.

Conversely the chlorides of pelargonin and cyanin do not exhibit the g=2·057 signal, nor do the blue pigments of *Gentiana scabra* Bungei varl Buergeri Maxim., *Commelina communis* L. and *Pharbitis hederacea* CHOIS.

Properties of the isolated anthocyanins and their congeners are summerized in Table 1.

On the whole, one can reasonably account for these findings on the assumption that the absorption at g=2·057 in ESR spectra in "genuine" red and violet anthocyanins arises, although its existence has still to be confirmed, from the unpaired electron of their pyrylium ring. That is, the lack of definite signal at g=2·057 in the anthocyanin chlorides might be interpreted by the disappearance of this unpaired electron due to bond formation. The red pigment prepared by removal of chloride ion from cyanin chloride as expected again exhibits the g=2·057 signal. On the other hand, the blue pigments presumably do not give the signal at g=2·057 because the formation of a complex with metal(s) leads to the disappearance of unpaired electron, of pyrylium ring. The metal-free pigment from commelinin shows an analogous ESR spectrum to that of the "genuine" red and violet pigments. Thus, it is suggested

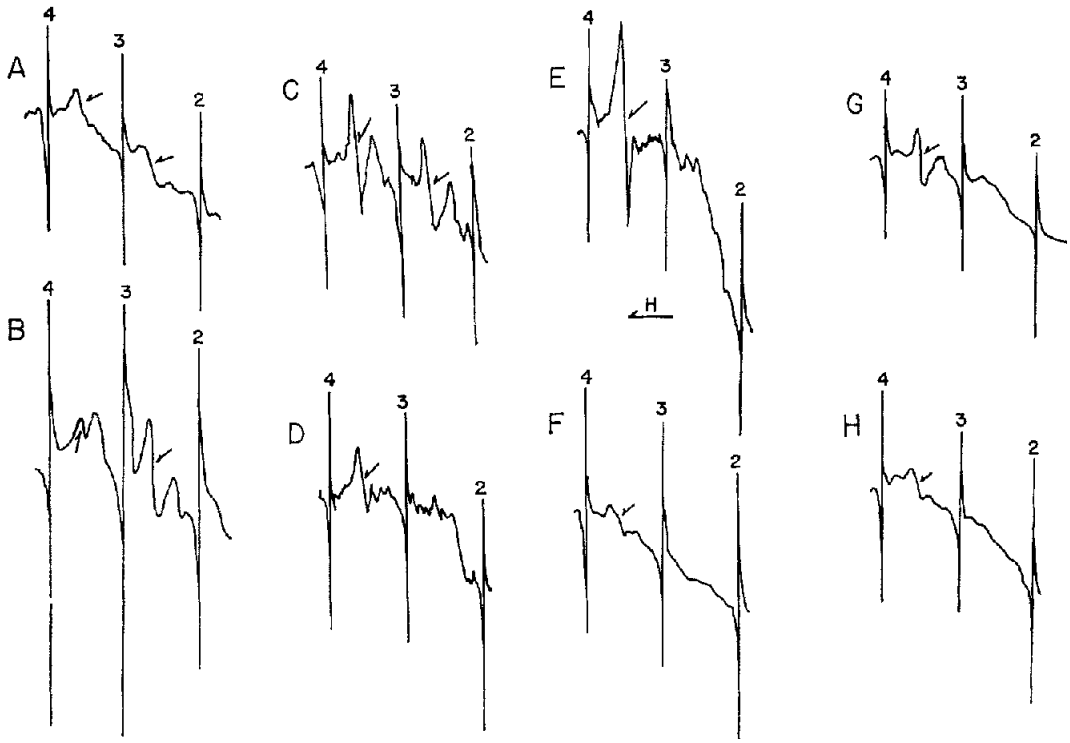
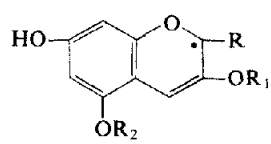


FIG. 3. ESR SPECTRA MEASURED BY THE MICROTECHNIQUE.
The three sharp signals 2, 3 and 4 are due to Mn^{2+} as marker. The arrow located between 2 and 3 indicates the g = 2·057 signal, and the g = 2·003 signal is observed between 3 and 4. A, *Salvia splendens* Ker., scarlet; B, *Dahlia variabilis* DESF., purplish-red; C, *Senecio cruentus* DC., purplish-red; D, *Dahlia variabilis* DESF., pelargonin chloride; E, *Dahlia variabilis* DESF., cyanin chloride; F, *Gentiana scabra* Bunge var. Buergeri Maxim., blue; G, *Commelina communis* L., blue; H, *Pharbitis hederacea* CHOIS., blue.

that the flavylium moiety of red and violet anthocyanins can be represented by I and in the blue ones metal(s) or unknown substance be joined by coordinated covalent bonds to free anthocyanins through the unpaired electron of their pyrylium rings.



I

R = Aryl
R_1 & R_2 = H or sugar residue

ESR Study on Fresh Petals

ESR spectra of fresh petals such as *Cosmos bipinnatus* CAV., *Torenia Fournieri* Linden, *Gentiana scabra* Bungei var. Buergeri Maxim. and *Chrysanthemum morifolium* RAMATUELLE are illustrated in Fig. 4. It will be observed that the red and violet petals show the signal at g = 2·057 unlike the blue and white ones.

TABLE 1. SOME PROPERTIES OF ISOLATED ANTHOCYANINS AND THEIR CONGENERS USED IN THIS STUDY

Figures correspond.	Plant species	Flower colours	Major antho-cyanidins	R_f values I*	R_f values II†	R_f values III‡	No. of free radicals	Amount of samples used (mg)
1-A	*Centaurea cyanus* L.	Red	Pelargonidin				$1{\cdot}89.10^{13}$	17·97
1-B	*Dahlia variabilis* DESF.	Purplish-red	Cynanidin				$4{\cdot}89.10^{13}$	23·52
1-C	*Dahlia variabilis* DESF.	Purplish-red	Cyanidin-Cl				$4{\cdot}02.10^{13}$	24·99
1-D	(Red pigment dechlorinated from cyanin chloride)	(Red)	Cyanidin				$2{\cdot}04.10^{13}$	6·04
1-E	*Centaurea cyanus* L.	Blue	Caynidin				$1{\cdot}80.10^{13}$	3·47
1-F	*Commelina communis* L.	Blue	Delphinidin				$1{\cdot}05.10^{13}$	6·79
1-G	(Metal-free pigment from commelinin)	(Reddish-purple)	Delphinidin				—	13·70
3-A	*Salvia splendens* Ker.	Scarlet	Pelargonidin	0·85	0·66	0·77		0·07
3-B	*Dahlia variabilis* DESF.	Purplish-red	Cyanidin	0·91	0·69	0·79		1·05
3-C	*Senecio cruentus* DC.	Purplish-red	Delphinidin	0·60	0·49	0·57		0·02
3-D	*Dahlia variabilis* DESF.	Scarlet	Pelargonidin-Cl	0·79	0·82	0·81		0·06
3-E	*Dahlia variabilis* DESF.	Purplish-red	Cyanidin-Cl	0·83	0·82	0·80		0·07
3-F	*Gentiana scabra* Bunge var.Buergeri Maxim.	Blue	Delphinidin	0·85	0·62	0·50		0·01
3-G	*Commelina communis* L.	Blue	Delphinidin	0·91	0·71	0·77		0·02
3-H	*Pharbitis hederacea* CHOIS.	Blue	Delphinidin	0·42	0·62	0·54		0·06

* BuAc/*n*-BuOH/EtOH/water (1:1:1:3).
† *iso*-Propanol/EtOH/water (1:2:2).
‡ 50% EtOH.

In comparison with isolated anthocyanins, it is now apparent that there is no difference in the g=2 region of ESR spectra between the isolated "genuine" anthocyanins and those present *in vivo* ones, and that the appearance of the g=2·003 signal is obviously not due to anthocyanins alone.

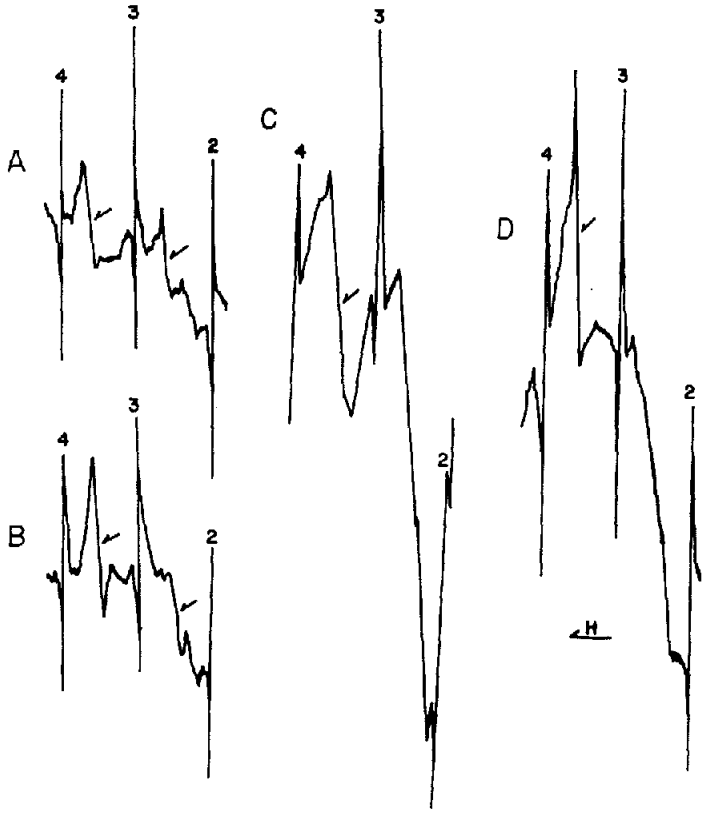


FIG. 4. ESR SPECTRA MEASURED AT LIQUID NITROGEN TEMPERATURE ON SOME FRESH PETALS. The three sharp signals 2, 3 and 4 are due to Mn^{2+} marker. The arrow located between 2 and 3 indicates the g=2·057 signal, and the g=2·003 signal is observed between 3 and 4. A, *Cosmos bipinnatus* CAV, pink; B, *Torenia Fournieri* Linden, violet; C, *Gentiana scabra* Bungei var. Buergeri Maxim, blue; D, *Chrysanthemum morifolium* RAMATUELLE, white.

MATERIALS AND METHODS

Crystalline preparations of genuine anthocyanins and their congeners were prepared by previously reported methods.[1–9] Some were obtained through the courtesy of Professor K. Hayashi.

The ESR spectra in the crystalline preparations of several genuine anthocyanins, their chlorides and the red pigment produced by removal of chloride ion from cyanin chloride were examined by normal techniques at room temperature by use of JEOL JES-3BS-X type ESR Spectrometer.

For measurement on non-crystalline specimens, the following micro-technique was employed; the pigments of fresh petals were partially purified without using mineral acid. The crude pigments thus obtained were applied to conical paper-chromatograms[11] using the

solvent systems: *iso*-propanol/ethanol/water (1:2:2), or *n*-butanol/ethanol/butylacetate/water (1:1:1:3). The resulting coloured bands were cut out, eluted and re-chromatographed using 50% ethanol. The required single spot was cut out, curled and pushed into the sample tube 5 mm in diameter. The tube was evacuated down to 10^{-3} mm Hg. ESR spectra were then recorded as above.

For the examination of intact petals, the fresh materials were measured at liquid nitrogen temperature.

[11] Y. Ôsawa, *Nature* **180**, 705 (1957).