BIOCHEMICAL GENETICS OF THE ANTHOCYANINS OF BARLEY (*HORDEUM VULGARE* L.)

by

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B.Sc. (Hons.) Agr., Delhi University, 1954

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We accept this thesis as conforming to the required standard

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Division of Plant Science

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FACULTY OF GRADUATE STUDIES

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DHARAM BIR MULLICK

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ABSTRACT

Patterns of flavonoid and melanic pigmentation were studied by visual and qualitative chemical means in 20 varieties of cultivated barley. Two new techniques, one manual and one chemical, were developed which made possible the study of the pigments in the separate tissues of the caryopsis. The observation that anthocyanins are localized in the spermoderm and not in pericarp is an example of the usefulness of the techniques. Environmental modifications of pigmentation patterns in varieties and in individual plants are large and biochemical and genetical analyses of patterns are thereby greatly complicated. For example, pelargonidin derivatives were regularly produced in field grown plants but not in greenhouse grown plants. Genes which control pigment production and disappearance during development add to the complexity of pigment patterns. Colors which are visually very similar may be biochemically dissimilar. Leucoanthocyanins occur in aleurone and endosperm but do not occur at any stage in pericarp, spermoderm, or other maternal tissues. In aleurone, which is alkaline, anthocyanins occur as anhydro bases. In aleurone, also, and in certain other tissues, such as the hood veins of the black variety Gatami, anthocyanins may occur as pseudo bases. In young aleurone tissue, anthocyanins are largely free and minor amounts are tissue bound; later in development most are tissue bound. At any stage of development anthocyanins in aleurone are difficult to extract because the nucellar epidermis, alone or with the aleurone envelop, is highly impermeable to most solvents.

Biochemical differentiation of anthocyanins, using accepted techniques of extraction and processing, and using paper chromatography, was undertaken for varieties, lines and tissues. Support and extension of earlier genetical study is given. Biochemical phenotypes could be differentiated from basal leaf sheath extracts when visual phenotypes could not be. In varieties such as Black Hulless, anthocyanins from aleurone were largely delphinidin and petunidin derivatives while those from spermoderm were largely derivatives of cyanidin and peonidin. Anthocyanin patterns from different maternal tissues of a single plant were similar but some seasonal

variations were recorded. Anthocyanin patterns in varieties, with very different breeding such as Gopal and Black Hulless, even when grown under a variety of environments, were similar and pointed to parallel genetical control of color. Three new anthocyanins with novel characteristics were found in young, but not in mature, caryopses; an outstanding property was their rapid movement on chromatopaper when BAW was the solvent. Whether or not their frequent disappearance was due to inherent instability or was a normal feature of development was uncertain. In some varieties and tissues at least it was established that cyanidin derivatives form first and pelargonidin and/or peonidin derivatives form later. Qualitative associations in development of polyphenols, anthocyanins and melains were noted. In future, studies of anthocyanins in development would be greatly aided if the experimental barley was grown in constant environments.

Detailed characterization of the anthocyanins of barley was made difficult by the complexities of the color patterns and by their instability when commonly accepted processing procedures were used. Most of the anthocyanins of barley were complex and split readily into simpler components. Ninety-three isolates were obtained from basal leaf sheaths and caryopses; some isolates split into as many as 5 components while others did not split. The anthocyanidins of 63 anthocyanin isolates, run against known anthocyanidins, were identified by Rf values obtained in 7 solvents and by spectra; 39 isolates were hydrolyzed for sugar analyses and 25 were hydrolyzed partially for structural elucidation. Many anthocyanins were crystallized and many were rather fully characterized. Many of the anthocyanins of barley are new and had not been reported for other species.

New techniques were devised to achieve anthocyanin stability and to enable work with micro amounts of tissue from single hybrid plants. It was shown that evaporation to dryness of extracts in 1% methanolic HCl, a common procedure, is a very serious, but not the single, cause of anthocyanin degradation; certain anthocyanins show a degradative spectral peak ca. 360 mu attributed provisionally to that of chalcones and most show splitting. Clamping and sewing of chromatobands directly to new paper avoided elution and flash evaporation in methanolic HCl. Modification of existing spectral technique also permitted the examination of even very weak anthocyanin

bands on paper. Employment of very weak (0.03%) methanolic HCl in procedures was useful. Although the problems of anthocyanin lability were reduced by modifying older procedures and devising new techniques, they were not eliminated. Evidence was obtained finally which showed that anthocyanins are best extracted in neutral methanol as pseudo bases rather than, as is customary, in acidified methanol, as flavylium salts.

Not only were techniques devised for anthocyanic stability, they were also devised for handling micro extracts from single plants. Time and materials were conserved in techniques for hydrolysis, concentration, purification and characterization on paper. Complementary equipment for banding and hydrolysis on paper was developed. New solvents for anthocyanidin chromatography greatly aided characterization. Hydrolyses of eluates both from anthocyanins and blank chromatopaper yielded glucose, galactose, xylose and arabinose; a method to remove paper-derived sugar artefacts from chromatographically purified anthocyanins is presented. Additionally, it was found that glycosidic hydrolysis of anthocyanins occurs even on chromatopaper and that the so-called irreversibly adsorbed anthocyanins of many investigators are anthocyanidins. Moreover 'glycosidic' hydrolysis of certain barley anthocyanins, even in crystalline state, to simpler anthocyanins, is the basis of anthocyanin splitting and explains the appearance of the complex chromatoband patterns. Other investigators have also found in other species complex patterns but have assumed them to be in vivo patterns.

If biochemical responses to gene action and biogenesis of anthocyanins are to be studied precisely in barley, procedures must avoid artefact production during extraction and processing and yield anthocyanins in their in vivo state.

PUBLICATIONS

D.B.Mullick, D.G.Faris, V.C.Brink, and R.M.Acheson. Anthocyanins and anthocyanidins of the barley pericarp and aleurone tissues. Canad.J.Plant.Sci. 38:454-456 (1958)

D.B.Mullick. The physiology and genetics of the pigments of barley. II. Studies of the anthocyanin pigments. M.S.A. thesis, University of British Columbia (1959)

D.B.Mullick and V.C. Brink. Spectral studies of anthocyanins and anthocyanidins. I. An addition to the ultra-violet spectra of anthocyanins and anthocyanidins. Canad.J.Biochem. (in press)

D.B.Mullick. Spectral studies of anthocyanins and anthocyanidins. II. Spectrophotometric identification of the pseudo base of synthetic cyanidin. Canad.J.Biochem. (in press)

D.B.Mullick and V.C.Brink. Localization of anthocyanins by peeling the investments of the barley caryopsis. Crop Science (in press)

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INTRODUCTION

In 1955 Dr. D.G. Hamilton, Research Branch, Canada Department of Agriculture, Ottawa, stated his concern over the variations in the color occurring in genetically uniform lines and varieties of Canadian barleys. Inasmuch as colors were used as varietal markers and were significant in barley malts and were, therefore, important to breeders, maltsters, brewers, and others concerned with the very large internal and external trade in barley, Dr. Hamilton felt that an intensive study of the nature of color, largely attributable to anthocyanin pigments, was warranted. He gave his support to a study in the Division of Plant Science at the University of British Columbia; exploratory studies were the subject of two Master's theses by Faris (1956) and Mullick (1959) and of one paper by Mullick, Brink, Faris and Acheson (1958).

Through the offices of Dr. Hamilton (C.D.A.) and Dr. Wiebe (U.S.D.A.), a small but diversified collection of varieties and isolines was assembled in 1958. The genetics of some color variations had been studied in barley prior to the initiation of this study but virtually no work relating the pigment genetics to biochemistry and physiology was extant; perhaps the only other contributions came in 1961 when cyanidin-3-arabinoside was isolated both from barley husks (Metche and Urion, 1961), and barley corn (Urion and Metche, 1961).

With the long series of brilliant investigations of anthocyanins by Wilstatter, Onslow, and the Robinsons of several decades ago, with those of today by the laboratories of Harborne, Neish, Geissman and others and with the related genetical studies of Haldane, Scott-Moncrieff, and

Emerson as background, it was hoped, that biochemical study would not be detailed or prolonged and that the nature of the genetical controls in pigment development could be accorded intensive study. It was early discovered that knowledge of pigments gained from flower colour studies could not be applied in simple fashion to barley colour studies and the evolution of new techniques and their application became a major part of the research.

1. REVIEW OF LITERATURE

Barley, the oldest of the common cereals, has the widest habitat range. It grows beyond the Arctic Circle where, in summer, the soil thaws no more than a few inches below the surface and on the tropical plains of India. High on Ethiopian mountain slopes, barley may ripen beside pools of water which freeze nightly. It grows on the lower delta of the Nile where salt water is found at depths of a little more than a foot. In Tibet it occupies large acreages on plains at 15,000 feet and may grow, occasionally, on higher adjacent Himalayan slopes. It is an important though rarely the major crop in nearly every agricultural region.

Barley, as has been mentioned, is a very old crop and has played an important role in the development of neolithic culture in the Old World. The problem of its origin interests archaeologists, anthropologists, agronomists and geneticists. Its importance to the human race has grown steadily and it has emerged as one of the leading cereals of the world.

Barley, like maize, is a diploid species, for which a wide range of mutations has been described. The crop is the subject of a number of books, monographs, and extensive reviews; among these are the works of H.V. Harlan (1957), Smith (1951), Weaver (1943), Aberg and Wiebe (1946, 1948), Derr (1911), Beaven (1947), Hunter (1952), Takahashi (1955) and Nilan (1964). Thousands of scholars have made contributions to our knowledge of barley. Takahashi (1943) records 1300 articles published by 1941 dealing with general morphology, classification, genetics, cytology, plant breeding, diseases, physiology, cultivation, livestock feeding, brewing, and chemical and physical properties of the grain. Smith (1951) brought certain sections of the bibliography up to date in 1950 and Nilan (1964) to date in 1962. The first international congress on barley genetics (Barley Genetics, 1964) indicates something of the growing scientific interest in the crop.

Until 1959 (Mullick, M.S.A. Thesis), there had been no serious attempt to bring together the relevant literature on colors and pigments of barley and, in particular, on the water-soluble anthocyanin pigments. Relevant literature since 1958 is briefly cited below.

Biogenesis has been considered by Bogorad (1958) and Neish (1960). The organic chemistry of flavonoids and related compounds is the subject of several books and reviews, notably those by Wawzonek (1951), Blank (1958), Geissman (1962, 1963), Swain and Bate-Smith (1962), Gore, Joshi, Sunthankar and Tilak (1962) and Goodwin (1965). The chromatography of flavonoid pigments has been reviewed by Harborne (1959a, 1959b). Relevant reviews on the enzyme biochemistry of phenolic compounds and acetogenins have been edited by Pridham (1963), Harborne (1964), Richards and Hendrickson (1964). Anthocyanogens in malting and brewing have been discussed by Cook (1963) and Pollock (1963).

2. THE BARLEY COLLECTION

From some 60 varieties and isogenic lines grown on the University Farm, a few were selected to provide suitable color diversity for the purposes of this study. Most varieties and lines had been carefully selected for agronomic and genetical characters for ten years; the most useful are listed in Table 2.I.

TABLE 2.I
LIST OF BARLEY VARIETIES MOST COMMONLY USED

Name	Plant Breeder's Color	Original Source
Gatami	black	C.D.A., Ottawa
Lion	black	C.D.A., Ottawa
Kwan	blue	C.D.A., Ottawa
Montcalm	blue	C.D.A., Ottawa

Name	Plant Breeder's Color	Original Source
Trebi	peculiar blue	C.D.A., Ottawa
Black Hulless	purple	C.D.A., Ottawa
Gopal (C.I.1091)	bright purple	U.S.D.A., Aberdeen, Id.
Golden Pheasant (C.I.2488)	white	U.S.D.A., Aberdeen, Id.
Hanna	white	C.D.A., Ottawa
C-54-55	white & near* white	C.D.A., Ottawa
Atlas(C.I.4118)	blue	U.S.D.A., Aberdeen, Id.
Compana	white	U.B.C., Vancouver, B.C.
C.I. 5628	purple	U.S.D.A., Aberdeen, Id.
C-54-22	pale blue	C.D.A., Ottawa
Deficiens(C.I.2225)	white	U.S.D.A., Aberdeen, Id.
Ethiops	white & near white	C.D.A., Ottawa
Kama-Ore.(C.I.694)	near white	U.S.D.A., Aberdeen, Id.
Kitchen	black	C.D.A. Ottawa
Orange lemma (C.I.5649)	white & near white	U.S.D.A., Aberdeen, Id.
Orange lemma, 57-AB-1390	white & near white	U.S.D.A., Aberdeen, Id.
Normal lemma, oo 57-Ab-1394	white & near white	U.S.D.A., Aberdeen, Id.
Vantage	white	U.S.D.A., Aberdeen, Id.
31-bl bl-13	white	U.S.D.A., Aberdeen, Id.
33-Bl Bl-13	blue	U.S.D.A., Aberdeen, Id.
36-bl bl-21	white	U.S.D.A., Aberdeen, Id.
36-Bl Bl-21	blue	U.S.D.A., Aberdeen, Id.
71-pr pr-10	white	U.S.D.A., Aberdeen, Id.
71-Pr Pr-10	purple	U.S.D.A., Aberdeen, Id.
5090-2-3	grey & near white	C.D.A., Ottawa
5090-10-4	white & near white	C.D.A., Ottawa
5090-15-1	white	C.D.A., Ottawa
5423-4	dark blue or grey	C.D.A., Ottawa
5424-7	dark blue or black	C.D.A., Ottawa
5425-8	grey	C.D.A., Ottawa
5428-2	black	C.D.A., Ottawa

*'Near white' is colloquially referred to as 'dirty white' by plant breeders.

3. LOCALIZATION OF ANTHOCYANINS BY PEELING THE INVESTMENTS OF THE BARLEY CARYOPSIS

The interest of geneticists and plant breeders in the anthocyanin pigments of the caryopsis of barley (Hordeum vulgare L. Emend, Lam.) is shown in the reviews by Smith (1951), Nilan (1964) and Aberg and Wiebe (1948). The interest of the maltster, brewer and cereal processor in anthocyanins and the related anthocyanogens has been stated by Pollock (1963) and by Morgan et al. (1964). To analyze or to correlate the many observations relating to these compounds is difficult for the reason that their localization in the complex of tissues investing the caryopsis is seldom attempted. The technique and observations in the present study may simplify or clarify genetical and chemical analysis.

Thin sectioning and pearling, followed by histochemical study, are obvious means of localizing flavonoid pigments but Mullick (1959) found them lacking in precision. Pollock et al. (1955) "decorticated" malting barley by immersing grains in 50% sulfuric acid for three hours. Morgan

et al. (1964), peeled graminaceous grains by a lye treatment. In all of these procedures anthocyanins diffuse from the outer coverings and suffer degradation.

3.1. MATERIALS AND METHODS

In most barleys at maturity, as Harlan (1920) points out, lemma and palea are bonded chemically to the caryopsis but may be scraped away. At dough stage the bonding is weak or non-existent. It is often convenient to use naked or hulless varieties. A diagram of a barley caryopsis is shown in Fig. 4.1.

Three tissue layers can be peeled manually and successively from the caryopsis surface at dough stage. Excision of the embryo, easily accomplished with a thumbnail, leaves the outer investments loose in the scutellar region and provides a good starting point for peeling. Tissues at dough stage peel easily. Tissues at flint state maturity peel after soaking grains for four days in water at near freezing temperatures; accompanying chemical change is minimal.

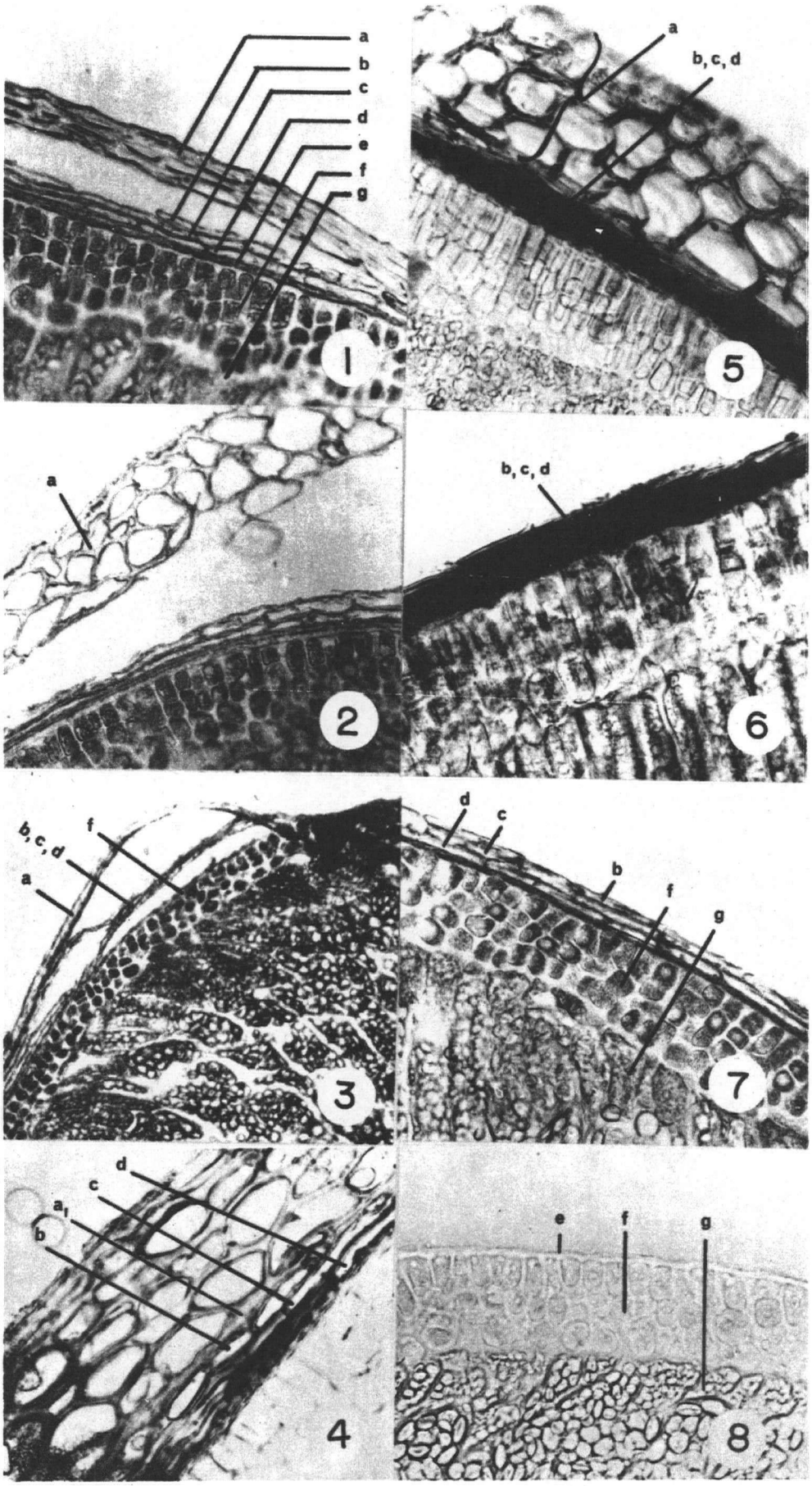
Although the histology of the barley caryopsis has been studied by a number of workers, including Brown (1907, 1909), Mann and Harlan (1915) and Collins (1918), it is not possible from their work to relate with any certainty the peelable layers of the caryopsis to the specific tissues they name. Obviously clarification was needed. First attempts to achieve correspondence used sections, obtained freehand or from sliding or rotary microtomes. Three types of sections were compared: (a) those obtained without peeling; (b) those with the first or outer layer peeled and (c) those with the second and third layers removed. The sections were mounted

in glycerol: 3N hydrochloric acid (5:1) and photographed. Later, better sections were obtained by supporting caryopses in a matrix of Lab-Tek O.C.T. (range 0°C to -15°C) and sectioning at 16 microns in an International Harris Cryostat with the chamber at -20°C. The sections obtained were mounted in 1 drop of aqueous 1% hydrochloric acid followed by a drop or two of 3N hydrochloric acid: glycerol (1:1). Other water soluble mounting media tried were not satisfactory.

3.2. OBSERVATIONS AND DISCUSSION

Photographs of sections of the outer tissues of the mature caryopsis are presented in Figs. 311 to 3.8. Seven layers are marked in Fig. 3.1. The outermost, and most prominent, is non-chlorophyllous and consists of four to seven cell layers of parenchyma and inner and outer epidermis. It is equivalent to the pericarp of Collins (1918). The morphology of the pericarp seen in Figs. 3.1 to 3.5 as tissue 'a', changes substantially even during the later stages of development. There is also some variation in the appearance of the pericarp in a single caryopsis at maturity for, when the parenchyma cells are large and blocky in the region of the furrow, they are thin and elongate over the dorsal surface of the caryopsis. Separation occurs in the tissue designated by Collins (loc. cit.) as the inner epidermis of the pericarp (Fig. 3.4,a_1). In some caryopses and in certain regions of a single caryopsis it may be a well defined cell layer, but in others it may be reduced to a band of poorly defined, elongated cell walls. The pericarp is in contact with the outer embryo face and covers the other caryopsis tissues. By the early dough stage, the pericarp is papery and colorless, although Mann and Harlan

Figs. 3.1 to 3.8
Showing outer tissues of the barley caryopsis, pigment localization and stages in removal of tissues by hand. Fig. 3.1 pericarp (a) exfoliating; tissues derived from integuments (b), (c), and from the nucellus (d); aleurone envelope (e); aleurone layer, 3 to 5 cells thick (f), endosperm (g). Fig. 3.2 blocky cells of the pericarp seen in the area of the furrow compare with elongated cells in Fig. 3.1. Fig. 3.3 exvoliation of pericarp (a), at the innermost cell layer of the pericarp and exfoliation of tissues of integumentary origin (b), (c), from tissues of nucellar origin (d), and nucellar tissue from aleurone (f). Fig. 3.4 shows clearly inner epidermis (a1), of pericarp where separation occurs. Fig. 3.5, intense development of anthocyanin pigment in spermodern (b), (c), (d), beneath the pericarp (a), which is characteristically colorless. Fig. 3.6, pericarp peeled manually to leave pigmented spermodern (b), (c), (d). Fig. 3.7 similar to Fig. 3.6 but shows the cell structure of the spermodern. Fig. 3.8 aleurone envelope (e), aleurone (f), endosperm (g), after peeling pericarp and spermoderm.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(1915) found chlorophyll in the pericarp parenchyma during ovule development. Occasionally acidification produces brown, non-anthocyanin spots in pericarp peeled from mature kernels. The slight anthocyanin pigmentation, occasionally seen in mature pericarp tissues, may simply be the result of diffusion from, or attachment of, fragments of underlying tissue.

A chlorophyllous layer, b, which remains after the pericarp is peeled is seen in Fig. 3.7, as well as in Fig. 3.2, 3.4 and most clearly in situ in Fig. 3.1. As the caryopsis approaches maturity, the cells, at least three deep, become elongate and peeling is difficult. At early dough stage the tissues b, c and d split readily and may be manually peeled as two layers. Sectioning, followed by a twist of the sections, may split the tissues, as shown in Fig. 3.3. The double layered tissue, for convenience, was named "spermoderm," a term used by Brown (1907) for a nearly equivalent tissue. In hulless varieties such as 'Black Hulless' with a 'purple grain,' the spermoderm is heavily pigmented, so much so that, in sectioned material, cell walls are often obscured, as in Figs. 3.5 and 3.6, by the diffusing anthocyanins. Under the location our conditions present the spermoderm of varieties closely invested by lemma and palea is colorless, whereas in some varieties if the tissue is genetically competent, the spermoderm over the area not fully covered by lemma and palea may be pigmented.

The two peelable layers, designated in Figs. 3.1 to 3.8 as b, c, and/or d, generally overlie the aleurone except where the aleurone faces the embryo and appears to be referable to the 'nucellar epidermis' and 'tegmen' of Collins (loc. cit.). Both tend to face a limited area of embryo and both tend to parallel one another in development and physiology. It seemed, therefore, appropriate to treat these tissues collectively in

chemical studies and to name the associated tissues 'spermoderm' as did Brown in 1907.

Underlying the spermoderm is a non-cellular layer clearly shown in Figs. 3.1, 3.7 and 3.8 of sections from the variety, Black Hulless which, herein, is named the aleurone envelope. Beneath the envelope is the aleurone layer, 3 to 5 cells in thickness and sharply delimited from the endosperm. The aleurone layer is shown clearly in Figs. 3.1 and 3.7. In Fig. 3.8 the aleurone envelope and cells are shown after the pericarp and spermoderm have been removed manually. The aleurone envelope appears to have been overlooked in histological studies; it is importantly involved in permeability for its impedes the movement of anthocyanins, and water and many other solvents. That it originates in the aleurone is indicated by the fact that it does not occur where only the pericarp or spermoderm face the embryo. It is well known that the aleurone cells have triploid nuclei, that they display xenia and that they are highly proteinaceous. The tissue designated in Figs. 3.1 to 3.8 as (f), and exposed as the peelable tissues are removed, is easily recognized as aleurone. The aleurone of the Black Hulless variety, sections of which are shown, is heavily pigmented and blue. In other varieties, provided the tissue is genetically competent, the aleurone pigmentation develops whether the lemma and palea are bonded to the caryopsis or not and independently of deep pigmentation in the spermoderm.

Following the peeling of pericarp, spermoderm and aleurone tissues as separate entities, it has been possible to establish the aleurone anthocyanins as distinctly different from those of the spermoderm and to show that the anthocyanins of aleurone are of two general types, one readily extracted from the tissue by water and alcohol and the other

protein bound and not extractable without sonic or other means of partitioning. By peeling and placing the separate tissues of the caryopsis in an acid medium, it has been possible to accurately place individuals in color classes in segregating populations and to classify lines and varieties accurately by color (Sec. 4; Appendix: Tables 27.I to 27.XX). Finally, the technique has made possible a definitive chemical study of the anthocyanins of the barley caryopsis.

3.3. SUMMARY

Three tissue layers namely, pericarp, spermoderm and aleurone may be peeled manually and successively from the caryopsis of barley directly at the dough stage, or at the flinty stage by first soaking in water for four days. Correspondence of the peelable tissues to those named by histologists several decades ago was determined by sectioning with a cryostat. The technique has made possible a definitive chemical study of the anthocyanins and anthocyanogens of barley and precise grain color classifications of lines and varieties.

4. FIELD OBSERVATIONS OF COLOR PATTERNS

A common sense early step in the study of color variation in barley is to make comparative observations of color distribution and development in different genotypes. Another early and logical step is to survey intravarietal variation under different climatic and edaphic conditions. The malting trade had undertaken a casual survey of color variation in standard varieties in Western Canada, therefore effort was early directed towards ocular study of intervarietal patterns at one location viz., the University Farm. It was expected that such studies would provide a rational basis for color sampling in biochemical and physiological phases and, possibly provide clues to biogenetical or biochemical relationships.

From much of the genetical literature the impression was gained that many color patterns in barley were strongly expressed. From the malting trade and comments from breeders, on the other hand, the impression was gained that expressivity varied considerably. Color patterning did

indeed respond sensitively to variations in environment and variations associated with planting date and season complicated later studies.

4.1. A SHORT REVIEW OF RELEVANT LITERATURE

Flavonoid pigmentation in the barley plant may appear in the basal leaf sheath (rarely, other leaf sheaths) leaf tips (occasionally), auricles, nodes, internodes, glume awns, lemma awns, pericarp, aleurone, and endosperm (occasionally). Vegetative plant parts, such as basal leaf sheaths, may be red, yellow or white. Spike and grain colours may be various, viz., black, brown, gray, blue, purple, red, buff, orange, near-white or white (Takahashi and Yamamoto, 1950; Smith, 1951).

Harlan (1914) was the first to suggest that the red and blue pigments were anthocyanins. This observation was amply confirmed by chemical studies by Mullick, Faris, Brink and Acheson (1958) and Mullick (1959). The black and brown pigments of barley were believed by Harlan (1914) to be melanins. Lewicki (1929) and Buckley (1930) supported this view. Nonetheless, the chemistry of the black and brown pigments has not been accorded sufficient study to permit a definite statement about their nature. Yellow and orange pigments are probably flavonoids and may be similar or identical to the yellow saponarin, and lutonarin, and its 3-methy ether, isolated from barley leaves by Seikel and her associates (1957, 1959, 1962). Leucoanthocyanins were isolated from malt by Harris (1956), from malt husk by Harris (1958) and from grains of certain barley varieties by Mullick *et al.*, (1958). Four leucoanthocyanins originally detected by Harris (1956) have since been designated as cyanigen I, cyanigen II, delphinigen I and delphinigen II (Reynolds, Atterton, Kirsop

and Pool, 1961).

A number of compounds, possibly related biogenetically to the flavonoid pigments of barley, have been identified, viz., +(d)-catechin by Harris (1958), vanillic, ferulic and p-coumaric acids by Harris (1958) and by Sumere et al., (1958) herniarin, sinapic acid, scopoletin, coumarin, umbelliferone, aesculetin, p-hydroxybenzoic acid, o-hydroxycinnamic acid, syringic acid and chlorogenic acid by Sumere et al., (1958).

In some species, such as alfalfa, Medicago sativa, two pigment systems, quite unrelated chemically, one carotenoid and one flavonoid, occur. In barley, flavonoid colors (purple, blue, red, yellow and white) and melanin or melanin-like colors (black, brown, red, grey and off-white) occur, but whether or not they are involved in one or two related chemical systems has not been established. Color inheritance studies, which are many, and which might be expected to shed some light on the chemical relationships, have not been very helpful.

If anthocyanins are produced, in part, from 'residues' of aromatic amino acids after deamination, as suggested by several investigators, such as Geissmann and Hinreiner (1952) and Kandler (1958), then it is conceivable that relations of the flavonoid and melanic systems may not be distant.

The flavonoid and putative melanic pigments of barley are useful to distinguish varieties. Aberg and Wiebe (1946, 1948) and Wiebe and Reid (1961) especially, have employed them to great advantage in the taxonomy of mature plants. The extension of their color studies to stage by stage observation in the growing plant appeared to be a useful addition to their work and a useful preface for biogenetic studies. Also it was believed that stage by stage study might aid in finding reasons for the

variable expressivity of color genes.

4.2. METHODS

The studies in this section report direct observations of color changes taking place in the several plant parts and tissues which develop flavonoid pigments.

4.2.1. Changes of color were recorded for the parts and tissues of all available barley varieties and isolines during the 1958, 1959 and 1960 growing seasons. Observations were made in the field on the University Farm. Color patterns were also carefully studied in greenhouse grown material, particularly during the winter of 1961-62.

Although very desirable, but because the study was of a general nature, the successive stages of pigment development were not recorded from continuous observations of color changes taking place in a single tissue during development. The continuous color changes in the same tissue of the grain, particularly, pericarp, spermoderm and aleurone, cannot be observed because the tissues overlie one another, and importantly because the tissues are peeled off manually (Sec. 3) prior to observations. Two criteria were used to determine the successive stages of pigment development, one for the plant tissues, and the other for the spike and grain tissues:

4.2.1.1. Stages at which observations were made on plant tissues, such as, basal leaf sheaths, auricles, nodes etc., are presented in terms of color development rather than in terms of more customary physiological age.

For example, the stage designated as 'I', irrespective of the part or tissue studied is a stage where little or no flavonoid pigment is apparent, stage II, a stage where some color is apparent, stage III, a first stage of major color development, stage IV, a stage of further color modification, and stage V, a final stage which may depending on tissue, be a colorless or colored stage. Differences in planting dates, and in tiller initiation and placement make such an approach to stage classification nearly mandatory.

4.2.1.2. The pigments in the tissues of the spike or grain develop during kernel maturation. It was observed that in hulls the stages of pigment development mentioned in 4.2.1.1 are correlated with the stages of kernel maturation as shown in Table 4.I. Accordingly, the spikes after

TABLE 4.I.
CHANGES IN HULL COLOR[1] AND STAGES OF KERNEL MATURATION

Color Variants	Stage of Kernel Maturation[2]				
	I	II	III	IV	V
Purples	G	P & G	deep P	P & slightly Y	dull PY
Whites	G	GY	Y	Y-BrY	dull Y
Dirty Whites	G	G-GY	YG-Y	dull Y	dull Y
Blues	G	GY	YG	Y	dull Y
Blacks	G	GY-GBk	BkY-BkG	Bk	dull Bk

[1] Colors were recorded after removing the hulls from the caryopsis.

[2]
I. Early soft dough stage.
II. Late soft dough stage.
III. Early hard dough stage.
IV. Late hard dough stage.
V. Early flinty stage.

harvesting were arranged in sequence of kernel maturation and the successive stages of color development in different tissues ascertained. Thus, the observations of pigment development in the spike and grain tissues were made largely in terms of the physiological age. The observations on the grain tissues, namely, perticarp, spermoderm, and aleurone were made by manual peeling (Sec. 3). The pigment development in the spike and grain tissues of the color variants noted in Appendix (Tables 27.1 to 27.XX), was recorded generally up to late hard dough stage (IV), and occasionally up to early flinty stage (V), because the grain tissues past stage IV cannot be peeled easily.

4.2.2. Direct observation of color can be easily made in basal leaf sheath, node, auricle, awn tip, glumes, lemma and palea. Tissues of the grain may be several, their pigmentation may not be parallel and their pigment patterns may be superimposed. In most barley varieties close to maturity the lemma and palea are united with the outer pericarp (Harlan, 1914) but these bracts may be scraped off with some difficulty. Coverage of the caryopsis by lemma and palea is not always uniform, and pigmentation may occur only over the parts of the caryopsis thinly or incompletely covered by lemma and palea; in other words, grain color may, in some circumstances, be seen through lateral 'slits', between lemma and palea. Slit width may vary with genotype or with plumpness or fill of the grain.

4.2.3. The designation of a color continues to be a highly subjective matter especially for those colored surfaces which cannot readily be analyzed spectrographically. A customary procedure is to match by trial and error, using several observers, the colored surface to be named with one to be found in standardized color charts such as those produced by

Ridgway or the Royal Horticultural Society. Colored plant surfaces are very difficult to match for great variations are commonly induced by co-pigmentation, light source and incidence, topography and character of surface, etc. For the most part, therefore, the simplest of color designation and a few adjectives to qualify the designations were employed. A list of abbreviations used in this section and also throughout the thesis is included in Table 4.II. However, because of their taxonomic importance, the aleurone colors were determined carefully by comparison with Ridgway's color charts (Table 4.III). The designations used are given.

TABLE 4.II
A LIST OF ABBREVIATIONS

A, Abs	absorption	'nin	anthocyanin
b	bright	O	orange
Bk	black	P	purple
Bl	blue	p	pale
Br	brown	Peo	peonidin
C	colorless, also anthocyanin-free	Pelar	pelargonidin
Cr	cream	Pk	pink
C-3-G	Cyanidin-3-glucoside	R	red
Cya	Cyanidin	R_f	The R_f value in the Tables unless otherwise specified will always be stated as R_f x 100
D	dull		
d	deep		
dk	dark	SF	solvent front
Delph	delphinidin	SW	short wave
'din	anthocyanidin	syn	synthetic
F	fades	T	trail
f	fluorescence	t	traces
G	green	UV	ultraviolet
g	gray	V	visible light; violet in Appendix I, and Fig. 11.2.
l	light		
LW	long wave		
M	melanic (blackened) in Sec. 4 and Appendix I; magenta in the rest of the thesis	Vio	violet
		v	very
		W	white
		wk	weak
mu		Y	yellow
Mv	mauve		

TABLE 4.III. [1]
RIDGWAY'S COLORS USED TO CHARACTERIZE ALEURONE PIGMENTS

Color	Abbreviation
Begonia rose (I)	1
Maize yellow (IV)	2
Prussian blue (IX)	3
Light blue violet (X)	4
Tyrian rose (XII)	5
Dull citrine (XVI)	6
Cream (XVI)	7
Naples yellow (XVI)	8
Buffy citrine (XVI)	9
Straw yellow (XVI)	10
Serpentine green (XVI)	11
Olive lake (XVI)	12
Honey yellow (XXX)	13
Chamois (XXX)	14
Deep olive buff (XL)	15
Dark olive buff (XL)	16
Buffy brown (XL)	17
Tea green (XLVII)	18
Vetiver green (XLVII)	19
Pale green blue gray (XLVIII)	20
Dark Medici blue (XLVIII)	21
Bluish slate black (XLVIII)	22
Deep olive gray (LI)	23
Olive gray (LI)	24
Light olive gray (LI)	25

[1] Roman numeral in parenthesis indicate Plate number in Ridgway's Color Charts (1912).

An effort was made to estimate ocularly pigment concentration or depth of color in the aleurone layers of kernels. Kernels were divested of their outer layers and placed in acidified methanol. A sample of the blue aleurone variety, Montcalm, in late hard dough stage was used as a standard and given a grade of (+). Thus kernels of a 'black' variety such as Gatami which has an aleurone heavily pigmented with anthocyanins, at maturity, graded 5 and at learly flinty stage 0.5+.

4.2.4. Qualitative chemical tests were employed to extend or to clarify ocular observations of pigmentation, and were simply as follows:

4.2.4.1. Anthocyanins: All tissues at all stages of development and regardless of their color were placed in methanol containing 1% concentrated hydrochloric acid.

4.2.4.2. Leucoanthocyanins: Appropriate anthocyanin-free tissues were directly hydrolyzed in 3 N hydrochloric acid (Bate-Smith 1954).

4.2.4.3. Melanins: The colors which are believed to be associated with melanin or melanin-like compounds in barley are 'dirty-white', (off-white), (near-white), browns and blacks. There are no simple tests for melanins.

4.2.4.4. Flavonoid Compounds which are not Anthocyanins: Non-anthocyanin flavonoid compounds impart color, particularly yellow color, to some barley tissues. Their chemical relationships to anthocyanins are, in general, close but their presence is not always so obvious as that of the anthocyanins. Their occurrence was determined for most tissues by a series of simple tests: Tissues were exposed to ammonia fumes as suggested by Geissman (1955) and many others. Results are not always easy to assess, especially when tissues contained anthocyanins. Tissues first extracted with 1% conc. hydrochloric acid-methyl alcohol and then exposed to ammonia fumes or immersed in sodium hydroxide often gave better yellow or yellow-green colors characteristic of the non-antho-flavonoid compounds. The reduction test (Geissman, 1955) for flavones, flavanones, and/or flavonals which employs magnesium and hydrochloric acid was often

used. Polyphenols were detected with the diazotized sulfanilic acid test (Albright, Larson and Deiss, 1953) and the potassium ferricyanide-ferric chloride test of Barton, Evans and Gardner (1952). Freshly prepared reagents used in these two tests were generally sprayed on chromatograms; the chromato-solvent used was butanol-acetic acid-water (4:1:5).

4.3. OBSERVATIONS

4.3.1. General Observations of Plant Colors

4.3.1.1. Basal leaf sheaths.

Nearly all varieties and lines of barley developed color in the basal leaf sheath. Anthocyanin colors, always bluish reds, developed from the soil surface vertically and continued usually to an inch or two of the leaf blade as well. The anthocyanin colors in different stocks cannot be differentiated. The isogenic line, 33-bl bl-13, was the only stock in our collection which did not produce color at any time in the sheaths. In summer, under our field conditions, only 'Kwan', a so-called blue variety, 'Lion', a black, 'Hanna', a white, 'C-54-55', a near white, and 'Gopal', a purple variety, colored intensely; under late fall and winter field conditions nearly all barleys colored intensely in the basal region.

Age of plant, time of tiller initiation, shading and season influenced the coloring of the basal leaf sheath. An individual tiller located peripherally and developing late, might develop anthocyanin colors at the base almost from the time of initiation, whereas neighbouring tillers on the same plant might remain colorless for some time before

coloring. The basal leaf sheath at the first colorless stage did not contain leucoanthocyanins. Sheaths of tillers located centrally in the crown are often colorless when thoseperipherally located are colored. Color develops first in the leaf sheath veins and spreads gradually to interveinal areas. Color disappears as leaf sheaths age; yellowing, browning, occasionally reddening, death and drying occur sequentially.

4.3.1.2. Auricles and collar.

In nearly all varieties, except, notably 33-b1 b1-13, some color occurs at some time in some leaves in the region of the union of blade and sheath. Although the appearance of color is variable there are sequential patterns worth noting. For example, color invariably develops in basal leaf sheaths before it appears in the auricles of leaves more highly placed. Just as in the basal leaf sheaths, the auricles are, at first, cream or yellow, then color develops, and fades. In the strictly vegetative shoot, color develops in the lowermost auricles, veins first, and as intensification occurs, color appears successively in the auricles above. However, as the plant becomes reproductive, and the flag leaf develops, the uppermost auricles become deeply colored, and color in the lowermost auricles fades. The most suitable time for emasculation and artificial pollination was, it was noted, in most varieties, when color reached a maximum in the auricles of the flag leaf. The color in all auricles, in general, fades, or, at best, only the veins retain the color at maturity.

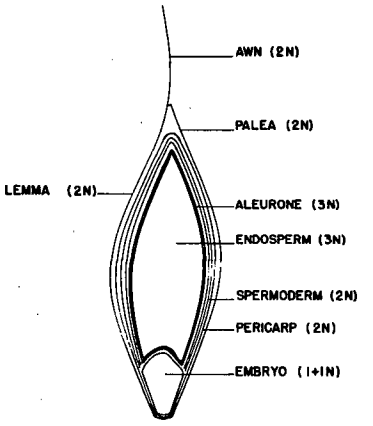
4.3.1.3. Nodes.

In general, but perhaps not in every line, nodes do not color until they have emerged from the confining leaf sheaths. If part of a node is covered by a leaf sheath it will not color; artificial removal of the sheath results in color production (C-54-55). This behaviour of nodes was also noted by Aberg and Weibe (1948).

4.3.2. Detailed Observations of Spike and Grain Colors

The plant colors discussed above are of limited taxonomic value, because their expressivity varies from year to year and from location to location (Aberg and Wiebe, 1948). In contrast to the plant colors, the grain colors have been used in taxonomic classification since Harlan (1914), because they are less variable than plant colors. Acccordingly, the patterns of pigment development in different tissues of the spike were given detailed attention. Inasmuch as the color changes are records of changing biochemical events, each tissue was accorded a careful study. The data for color development in the grain tissues of twenty varieties, were recorded during the 1959 growing season. The data are numerous and it has been necessary to place much of it in consolidated from in Appendix (Sec. 27).

The color changes in the following section have been discussed by plant part, viz., awn tips, glumes, etc. (see Fig. 4.1) and by plant breeders' color classes, viz., purples, blacks, whites, etc. Color pattern change is somewhat easier to perceive using this procedure. The procedure accents color change within a color class, e.g. the purples, and may overlook important differences between varieties or isolines within a color class.



LONGITUDINAL SECTION OF A BARLEY GRAIN
(DIAGRAMMATIC)

Fig. 4.1.
Longitudinal section of a barley grain showing the location and extent of the principle tissues (diagrammatic).

4.3.2.1. Awn Tips.

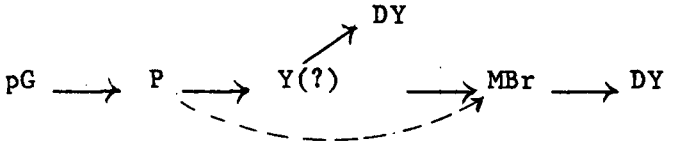
Awns and related structures, such as hoods, are pale green before they emerge from the boot. Soon after the awns protrude, in most lines, coloring begins in the tips. The anthocyanin synthesis takes place not at the tip per se, but about 1-2 mm below the tip. The color of the tip, for example, in Lion, is yellowish white when the 1-2 mm region below the tip is purple and rest of the awn is green. Color commonly intensifies to brilliant reds and purples and spreads downwards. About the time of syngamy, the anthocyanin colors, which are at a maximum in the tips, begin to fade, and yellow or melanic coloration appears in most of the varieties. In 33-b1 b1-13 anthocyanin colors do not develop; in most of the blue, white and black barleys anthocyanin coloration is ephemeral, as it is usually gone before the grain is 'in milk'. In a number of barleys, particularly purple lines, color persists to 'late dough'. The extent to which anthocyanin colors extend below the tip

varies considerably with genotype and environment.

(a) Purples (Appendix: Tables 27.I to 27.IV).

In the case of purple varieties, Black Hulless and Gopal (Appendix, Tables 27.I and 27.II), red-purple color gradually increases until the end of soft dough stage and then fades by the hard dough stage. The red-purple color is gradually replaced by yellow.

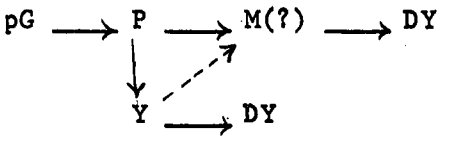
In the other two purple lines, namely 71-Pr Pr-10 and C.I.5628 (Appendix: Tables 27.III, and 27.IV) purple gradually fades and brown (melanic) pigment appears in the tips. The brown are replaced later by yellowish colors. It appears that the transition of the red-purple (anthocyanin) stage to the brown (melanic) stage is separated by a very short-lived yellow stage. The pattern of pigment development in the awn tips of purple lines may be generalized as follows:



(b) Whites, Near-Whites and Blues (Appendix: Tables 27.V to 27.XVII

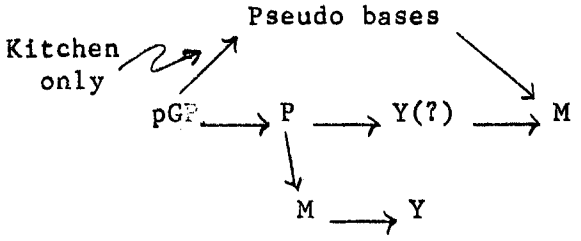
The color patterns of awn tips of whites, near-whites and blues are similar. The awn tips are usually purple until soft dough; gradually anthocyanin colors disappear and, in many cases, brownish melanic color appears which usually persist until the end of hard dough stage. Later yellowish colors appear. In some cases, where the appearance of anthocyanin colors in the awn tips, immediately after emergence, is ephemeral, they are replaced by yellow pigments during the soft dough stage. Whether

the melanic colors appear in between cannot be stated. The pattern of color development in these lines, then is generalized as follows:



(c) Blacks (Appendix: Tables 27.XVIII to 27.XX)

The awn tips of the black varieties, Lion and Gatami (Tables 27.XVIII, and 27.XIX) show ephemerally anthocyanin colors in early stages. These colors degrade and a melanic black pigment appears in the tips. However, in certain cases, before the black pigment appears, a yellowish pigment develops. The yellowish pigments, however, constitute a distinct developmental phase prior to the development of brownish, and occasionally black melanic colors in purple varieties. The black variety Kitchen (Table 27.XX) is hooded. The color of hoods is green during early stages of development. When hoods are dipped in solvent, a bright red color appears in the veins of the hoods indicating the presence of pseudo bases of anthocyanins in nature. Later, as chlorophyll degrades, melanic colors gradually appear. These are, in turn, replaced by dull yellow pigments. Pigment development in the black mutants appears to be:



4.3.2.2. Awn-remainder (Awns minus Awn tip)

Awns, within the boot, are, in all stocks, pale green. Immediately after emergence from the boot awns gradually become deeper green. Anthocyanin colors first appear in the awn tips and gradually travel downwards until the whole awn is more or less pigmented. In a few cases, the anthocyanins may develop in the awn tips but not in the awn-remainder.

(a) Purples (Appendix: Tables 27.I to 27.IV)

In purple varieties, Black Hulless and Gopal, by the end of soft dough stage, as chlorophyllous colors degrade in awn tips, purple color travels gradually downward until the entire awn becomes purple. A greenish yellow color seems to be an intermediate during this transition. The general sequence of color development in the purple varieties appears to be as follows:

$$pG \longrightarrow G \longrightarrow GY(?) \longrightarrow P \longrightarrow DY$$

(b) Whites, Near-whites and Blues (Appendix: Tables 27.V to 27.XVII)

Whether the tips are purple, melanic or yellow, the color of the awn-remainder is initially green and gradually changes to greenish yellow and, finally, to yellow:

$$pG \longrightarrow G \longrightarrow GY \longrightarrow Y \longrightarrow DY$$

(c) Blacks (Appendix: Tables 27.XVIII to 27.XX)

The behaviour of the awn-remainder and interveinal region of the hoods is similar. They are green in earlier stages, then greenish yellow

and finally black. The black color begins at the tips and gradually travels to the base. Whether the black color undergoes further modifications on maturity of the tissue could not be ascertained:

$$pG \longrightarrow G \longrightarrow GY \longrightarrow M \longrightarrow ?$$

4.3.2.3. Glumes (Appendix: Tables 27.I to 27.XX)

The pattern of color development in glume tips and glume-remainder parallels closely that of the awn-tips and awn-remainders of the same variety.

4.3.2.4. Rachis

Rachis of the purple varieties, Black Hulless and Gopal, develops profuse anthocyanin pigments. Detailed records of color changes are not available.

4.3.2.5. Hulls (Lemma and Palea)

Barley hulls consist largely of lemma which is bonded to the pericarp of the caryopsis in most varieties at maturity. Color patterns in a given variety relate closely to those of other tissues of the inflorescence. Changes are not necessarily coincidental, and changes by area, e.g., veins, interveins, etc., with the tissue were recorded.

(a) Purples (Appendix: Tables 27.I to 27.IV)

The color of hulls until soft dough stage is green. By the end of this stage, anthocyanin colors appear in hull <u>interveins</u> around the base of lemma awns. Color <u>in</u> the veins, however, develops later about the beginning of the hard dough stage. Once developed, the color deepens usually until late hard dough stage; later on it fades, and in certain strains, it is replaced by a dull yellow color during flinty stage:

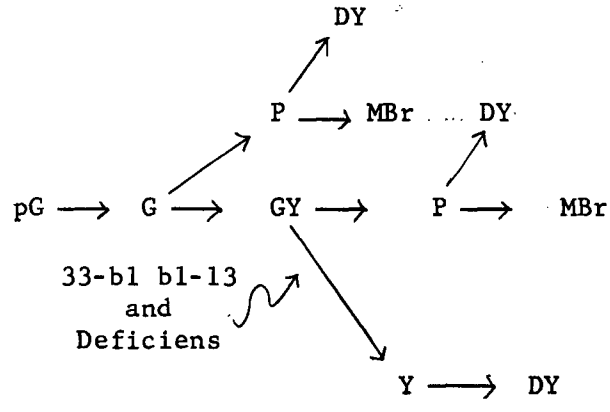
$$pG \longrightarrow G \longrightarrow ? \longrightarrow P \longrightarrow DY$$

(b) Whites (Appendix: Tables 27.V to 27.X)

The interveins of the true white varieties do not develop anthocyanic or melanic colors. The characteristic changes in their color are as below:

$$pG \longrightarrow G \longrightarrow GY \longrightarrow Y \longrightarrow DY$$

Anthocyanin colors develop in the veins of 36-bl bl-21, Golden Pheasant, Vantage and C-54-55 by early hard dough stage; color fades gradually so that by the beginning of flinty stage, the color of the veins becomes either melanic brown or dull yellow. Deficiens and 33-bl bl-13 do not develop anthocyaninic color. The sequence of colors in veins is as below:



(c) Near-Whites and Grays (Appendix: Tables 27.XI, and 27.XII)

The veins of both the lines studied viz., 5090-2-3 and 5090-10-4, develop anthocyanins during late soft dough stage, or early hard dough stage and ultimately turn yellow during later stages of growth, e.g.

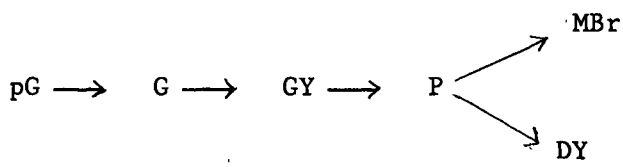
pG → G → GY → P → Y

No anthocyanic color develops in the interveinal region. Behaviour is therefore similar to that in the white varieties.

(d) Blues (Appendix: Tables 27.XIII to 27.XVII)

Whether the veins develop anthocyanin color or not, the interveinal areas, in general, do not show the cyanic or melanic colors; the behaviour parallels that of the white varieties. Occasionally, in certain cases, e.g. Trebi, the interveinal region is reddish violet during hard dough stage; this is due to the underlying layers of spermoderm which develop anthocyanins around early hard dough stages at and around the slit. The color in the spermoderm, however, fades at maturity.

With the exception of C-54-22, the veins of 36-B1 B1-21, Montcalm, Trebi and 33-B1 B1-13 develop anthocyanin colors during hard dough stage. In some cases, the colors fade and are replaced by yellows or, in others, by melanic browns:



(e) Blacks (Appendix: Tables 27.XVIII to 27.XX)

The interveinal region of the three varieties studied, does not develop anthocyanin colors and the order of pigment changes is as follows:

pG ⟶ G ⟶ GY ⟶ MBr ⟶ Bk

The veins of Gatami and Kitchen do not develop anthocyanin colors. However, in the case of Lion, during early stages of development, when interveins are green, the veins become violet black. The solvent color reaction indicates the presence of anthocyanins. Later, when the interveins become black, the veins continue to turn red in solvent but finally,

at the end of the hard dough stage, anthocyanins disappear from the veins and melanic colors appear.

4.3.2.6. Pericarp (Appendix: Tables 27.I to 27.XX).

The pericarp is a colorless and semi-transparent during the milk stage, translucent during soft dough stage, and opaque during the late stages of kernel maturation. By the flinty stage the pericarp becomes dry and brittle.

The pericarp does not, it appears, develop chlorophyll. It is a colorless tissue (perhaps some yellow or achroleucous flavonoids develop) in white, near white, gray and blue varieties. In black varieties, however, late in development, it becomes melanic black. Pericarp, being colorless, seems to be an excellent tissue for studying melanic precursors which originate from the colorless material.

The pericarp of purple varieties is a colorless membrane and easily separable until early hard dough stage. During this stage, anthocyanins appear to develop only at the tips. This appearance of anthocyanins may be due to limitations of the peeling technique which cannot accommodate finger pressures during the peeling process; because the finger pressure squeezes anthocyanin pigment to the pericarp surface from the anthocyanin rich spermoderm layer. More often the microscopic examination reveals that a few lacerated cells of spermoderm layers may remain adhering to the peeled pericarp. The possibility that some anthocyanins actually develop in these regions of the pericarp tissues cannot be ruled out yet. The details of color changes in different stocks are recorded in Appendix: Tables 27.I to 27.XX.

4.3.2.7. Spermoderm

The spermoderm, largely integumentary and nucellar tissue, is chlorophyll-containing unit early dough. The tissue then becomes greenish yellow; depending on variety, anthocyanin colors may then develop. In some varieties and lines anthocyanin pigmentation is general; in many the pigments develop only at the 'slit' in the region of the palea overlay; in others a mosaic of flavonoid yellows and anthocyanin colors develop, and in some only flavonoid yellows develop. In some black varieties anthocyanin colors may develop briefly at the slit, and then disappear as melanic pigments develop. It is also noted that spermoderm, during dough stage, is a thick and sturdy layer in comparison to the pericarp. However, at flinty stage, it becomes a very thin layer in relation to pericarp.

(a) Purples (Appendix: Tables 27.I to 27.IV)

Late in soft dough stage, the chlorophyll in the spermoderm disappears. Probably greenish yellow pigments appear briefly before the appearance of anthocyanins. The anthocyanin colors in the tissue are always red to purple i.e., the anthocyanins exist as oxonium salts. The anthocyanin colors deepen intensely in this tissue and by flinty stage, the colors appear as very dark purples.

$$C \longrightarrow CY(?) \longrightarrow P$$

(b) Whites (Appendix: Table 27.V to 27.X)

The spermoderm layers of Deficiens, Vantage and 33-bl bl-13 do not develop anthocyanins at any stage of development. They are green until the end of soft dough stage, and, later on, develop yellowish flavonoids:

$$G \longrightarrow GY \longrightarrow Y$$

However, in case of 36-bl bl-21, C-54-55 and probably Golden Pheasant, the lateral regions of spermoderm layer, at the slit between the lemma and palea, develop anthocyanin colors during late soft dough and early hard dough stages. The colors gradually fade and brown colors appear especially at the slit by the end of hard dough stage. The sequence of color changes at the slit are:

$$G \longrightarrow GY \longrightarrow P \longrightarrow MBr$$

(c) Whites and Near-Whites and Grays (Appendix: Tables 27.XI and 27.XII).

No anthocyanic or melanic colors appear. However, the yellow flavonoid colors as usual occur.

(d) Blues (Appendix: Tables 27.XIII to 27.XVII).

The spermoderm layers of 36-Bl Bl-21 and Montcalm, also develop anthocyanin colors at the slit bounded by lemma and palea, which later fade by the end of hard dough stage. In Trebi, anthocyanins do not develop at the slit but rather, occur around the slit. In C-54-22, the antho-

cyanins appear in patches, as a mosaic, from early soft dough to late hard dough. The anthocyanic colors however, disappear by the onset of flinty stage. The behaviour of the blues, thus, is related to that of the whites.

(e) Blacks (Appendix: Tables 27.XVIII to 27.XX).

The sequence of color changes in the spermoderm of three black varieties in somewhat as follows:

$$G \longrightarrow GY \longrightarrow Y \longrightarrow W \text{ or } Cr \longrightarrow gBk$$

Lion develops anthocyanic colors at the slit, which later disappear; Kitchen does not do so, and no information is available for Gatami.

4.3.2.8. Aleurone

The aleurone of barley consists of more cell layers than it does in some other cereals (Sawicki, 1951). It is distinguished sharply from the rest of the endosperm by the relatively large percentage of storage protein which accompanies the storage carbohydrates. The layer interdigitates with the rest of the endosperm and cannot readily be peeled from it. It is perhaps worth recalling that aleurone cells are triploid and intergenerational, in that the 2/3 of the chromosomes are maternal and 1/3 paternal. They are thus genetically different to the foregoing tissues which are maternal in origin. Aleurone cells are not chlorophyllous or melanic at any stage. At soft dough they are nearly white. When anthocyanin colors develop they are always in the blue shades of anthocyanin

color bases or in the yellow shades of other flavonoids. Aleurone layer is a rich source of leucoanthocyanins (Sec. 9).

It is noteworthy that when the aleurones are placed in the acidic solvent, the transition of blue anthocyanins (anhydro base state) to red anthocyanins (flavylium salt state) occurs, unlike other tissues, very slowly, sometimes over a period of hours.

In general, the patterns of pigment development in the tissues other than aleurone were ascertained from a relatively large sample because the tissues were largely accessible for direct visual examination. The peeling technique, being laborious, restricted observations to small samples. Nonetheless, the patterns of pigment development in the spermoderm were dependable from relatively small samples because pericarp is a colorless tissue during development. In contrast to the spermoderm, the developmental patterns in the aleurone could not be ascertained because of the limitations of a small sample, because the overlying spermoderm is a relatively thick layer during development; and because, early in development, the spermoderm is chlorophyllous, and, late in development, in several color variants, deeply pigmented. Inasmuch as the aleurone colors have been used widely as variety markers, and inasmuch as the aleurone colors cannot be determined accurately from unpeeled kernels, the delineation of exact aleurone colors by comparison with Ridgway's color charts (1912) was given special attention.

Although necessary from taxonomic viewpoint, the color delineation at flinty stage could not be undertaken because of the limitations of peeling technique.* However, aleurone colors were delineated as late in

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*The aleurone colors of flinty kernels are now delineated by the method described in Sec.21.

development as practicable. It will be noted from Appendix, Tables 27.I to 27.XX that the aleurone colors of all variants at late hard dough stage (IV), and whenever possible, at early flinty stage (V) were delineated from Ridgway's color charts. The aleurone colors, recorded in Appendix (Sec.27), at early stage of kernel maturation were not delineated from the color charts. The colors determined from Ridgway's charts begin with capital letters in the text. The observations of aleurone pigmentation were made on a very small sample, consisting usually of five kernels because manual peeling is time-consuming.

(a) Purples (Appendix: Tables 27.I to 27.IV)

The color of aleurone in purple varieties is white to yellowish white in early stages. The anthocyanic colors appear generally by the end of soft dough stage, or early hard dough stage. Black Hulless and C.I. 5628 are rich in 'blue' anthocyanins. Particularly, in the case of Black Hulless (Table 27.I), for which adequate observations are available, there is marked deepening of anthocyanin colors towards maturity. The color of Black Hulless aleurone at late hard dough (stage IV), in general, is Prussian Blue (++++) at the tips. During early flinty stage (V), the central regions of aleurone become Dark Medici Blue and the rest of the kernel is Bluish Slate Black (++++++++++).

In the other two purple varieties, Gopal and 71-Pr Pr-10 (Tables 27.II and 27.III) the anthocyanin colors scarcely appear. In Gopal, however, during stage IV, the central regions of aleurone are Naples Yellow whereas a small area around the tips is Light Blue Violet (+++). The color of aleurone at stage V, however, was Deep Olive Buff. It is

interesting that when the buffy aleurones were placed in the acidified alcohol, no anthocyanin color was noted (see Sec. 4.3.2.9). For 71-Pr Pr-10, readings for stage V are not available; during stage IV the aleurone is Cream color and anthocyanin colors are absent.

(b) Whites (Appendix: Tables 27.V to 27.X)

The aleurone of Golden Pheasant (Table 27.VII) is Naples Yellow during stage IV (late hard dough) and Honey Yellow during stage V (early flinty). That of Deficiens (Table 27.VIII) is Maize Yellow during stage IV and Buffy Citrine during stage V. The aleurones of both of these varieties when placed in the acidified solvent, do not develop anthocyanin colors.

The aleurone of 33-b1 b1-13 (Table 27.X) a good white variety, is Maple Yellow during stage III, surprisingly, it develops anthocyanin color (0.1+) when placed in the acidified solvent; however, at stage V the color of aleurone is Honey Yellow or Chamois and the anthocyanin reaction is not given. In the case of 36-b1 b1-21 and C-54-55 (Tables 27.V and 27.VI), small quantities of anthocyanin, like the 33-b1 b1-13, become visible only when the tissues are placed in the acidified solvent.

In the case of Vantage, (Table 27.IX), the situation is interesting insofar as it is classified as a plant breeder's white variety. At stage IV, it is Naples Yellow or Cream color and yet it shows the presence of anthocyanins (+) when placed in the solvent; the aleurone color, at stage V, is Deep Olive Buff or Dark Olive Buff (1.5+) (see Sec. 4.3.2.9.) Enough anthocyanin is present at early flinty stage, and therefore, its classification as a white variety appears to be in error.

(c) Near Whites and Grays (Appendix: Tables 27.XI and 27.XII).

The aleurone of 5090-10-4, (Table 27.X) though Cream colored (0.5+) during stage IV and Honey Yellow (0.3+) during stage V, also develops anthocyanins when placed in the solvent.

The aleurone of 5090-2-3, (Table 27.XII) a gray or near white variety, is Dull Citrine (+++) during stage IV and Dark Olive Buff (1.5+) during stage V. Appreciable anthocyanin is present.

(d) Blues Appendix: Tables 27.XIII to 27.XVII).

In 36-B1 B1-21, (Table 27.XIII) anthocyanins appear early in the soft dough stage. The aleurone color is Tea Green (1.5+) during stage IV and Dull Citrine (0.3+) during stage V.

The aleurone of Montcalm (Table 27.XIV) is Tea Green with patches of Olive Gray (+) during stage IV and Vetiver Green during stage V (0.2+). Similarly, Trebi (Table 27.XV), a peculiar blue variety, is Dull Citrine from the centre to the tip of embryo and Pale Green-Blue-Gray from the centre to the tip opposite the embryo (++) during stage IV and is Deep Olive Gray (0.2+) during stage V. Since the two varieties show little anthocyanin color in aleurone around early flinty stage, they do not appear to be good blue varieties.

The aleurone of 33-B1 B1-13 (Table 27.XVII) is partly Dark Olive Buff and partly Vetiver Green (+++++) during stage IV and Vetiver Green to Olive Lake (0.2+) during stage V (see Sec. 4.3.2.9).

The aleurone of the pale blue line, C-54-22 (Table 27.XVI) is

Tea Green with patches of Light Olive Gray (+) during stage IV and somewhat Vetiver Green during stage V. The color expression of this variety is fairly similar to that of Montcalm, but surprisingly, the intensity of the Vetiver Green color of Montcalm is less than (0.2+) whereas that of C-54-22 is (1.5+) (see Sec. 4.3.2.9).

The development of anthocyanins in aleurone occurs around early hard dough stage. It cannot be related to a particular stage of development because in some varieties, the color appears much earlier.

(e) Blacks (Appendix: Tables 27.XVIII to 27.XX)

The aleurone of Lion (Table 27.XIX) is Cream color (0.05+ to 0) during stage IV and Dull Citrine (anthocyanin absent) during stage V (see Sec. 4.3.2.9). For all practical purposes, the aleurone does not show anthocyanins.

The aleurone of Kitchen (Table 27.XX) develops anthocyanins during stage III. The color of aleurone during stage IV is Naples Yellow in the centre and Serpentine Green at the tips, whereas during stage V, the color is Serpentine Green. Anthocyanins do not appear during stages IV and V, when the tissues were placed in the solvent.

The aleurone of Gatami (Table 27.XVIII) develops anthocyanin colors during stage II; during stage IV the color of aleurone is Deep Olive Gray at the centre and Pale Green Blue Gray at the edges and during stage V, some of the aleurones are Dull Citrine (0.5+).

It is clear from the above results that the aleurone colors vary markedly at different stages of kernel maturation. Whether the variation is developmental, due to genetic segregation or largely environmental

remains to be investigated. It is also abundantly clear that the presence of anthocyanins, particularly in the aleurone tissues, should always be ascertained by the acid reaction; their presence in Cream colored or Naples Yellow aleurones, without the acid treatment, could scarcely have been expected. The necessity of carrying out routine acid treatment of the aleurone tissues becomes all the more apparent from the following section.

4.3.2.9. Relationship of Ridgway's Color Designation to the Concentration of Anthocyanins in Aleurone

It was observed in Section 4.3.2.8 that aleurone of several color variants, generally during hard dough stage, was visibly colorless (anthocyanin-free) as denoted by its colors such as Cream Color, Dull Citrine, Naples Yellow, and so on (see Table 4.IV). Surprisingly, the colorless aleurones showed frequently the gradual development of anthocyanin colors, when they were placed in the acidic solvent. The reasons for the gradual development of color are dealt with in Section 6. However, on the basis of some of the observations reported in Sections 6 and 22 and the others, yet to be compiled, it is concluded that the development of anthocyanin color in the otherwise colorless aleurone is due to anthocyanin pseudo bases, and not due to leucoanthocyanins. The occurrence of anthocyanin pseudo bases in nature suggest that the pH of the colorless aleurone tissues is 'neutral' and that of the blue aleurone tissues relatively alkaline. This suggestion can now be substantiated because a technique for studying the physiological activity of aleurone has been developed recently (Secs. 21 and 22). This issue concerning

TABLE 4.IV
ALEURONE COLOR AND ANTHOCYANIN CONCENTRATION

Variety	Classification of Lines According to Color	Aleurone Color according Ridgway's Chart	Anthocyanin Concentration	Development Stage
Vantage	White	Cream Color	+	IV
5090-10-4	near-White		0.5+	IV
Lion	Black		0.05+	IV
71-Pr Pr-10	Purple		Nil	III
5090-2-3	near-White of Gray	Dull Citrine	+++	IV
Gatami	Black		0.5+	V
36-B1 B1-21	Blue		0.3+	V
Lion	Black		Nil	V
Vantage	White	Naples Yellow	+	IV
36-b1 b1-21	White		0.1+	IV
33-b1 b1-13	White		0.1+	IV
Kitchen	Black		Nil	IV
Golden-Pheasant	White		Nil	IV
5090-2-3	near-White or Gray	Deep Olive Buff	1.5+	V
Gopal	Purple		Nil	V
36-b1 b1-21	White		0.1+	V
Vantage	White	Deep Olive Buff or Dark Olive Buff	1.5+	V
C-54-55	White	Dark Olive Buff	0.2+	V
33-B1 B1-13	Blue	Vetiver Green and Dark Olive Buff	++++++	IV
C-54-22	pale Blue	Vetiver Green	1.5+	V
Montcalm	Blue		0.2+	V

the influence of pH on the in vivo anthocyanin transformations is important both fundamentally, in biogenesis, and industrially in elucidating relationships of beer chill haze to leucoanthocyanins, protein bound leucoanthocyanins, anthocyanins, protein-bound anthocyanins (Sec. 6), and anthocyanin pseudo bases, all of which are related structurally and occur 'side by side' in the aleurone tissue.

Another important observation establishes that the apparent color of the aleurone gives misleading indications concerning the total anthocyanin content as illustrated briefly in Table 4.IV. It will be noted that in some lines, the aleurone color as given from Ridgway's Charts, is identical, but, notwithstanding, significant differences in anthocyanin quantity are observed when the aleurones are placed in acidified solvent. The variations in 'pseudo base' concentrations in the colorless aleurones, namely, Cream Color, Dull Citrine, and Maple Yellow, range from nil, 0.1+, 0.3+, 0.5+ to as high as +++. The aleurone colors, such as Deep or Dark Olive Buff, and Vetiver Green, simulate blue colors of aleurone, and can be mistaken easily for blue anthocyanins, particularly so when the unpeeled kernels are examined. It will be noted from Table 4.IV that, like the colorless aleurone tissues, these colored aleurones also show significant differences in anthocyanin quantity on exposure to the acidic solvent. The variations in anthocyanin concentration, again, range from nil, 0.1+, 0.2+, 1.5+ to as high as +++++. The observations, therefore, suggest that the colors such as Deep or Dark Olive Buff and Vetiver Green, which otherwise simulate blue anthocyanin colors, may not be due to anthocyanins. The variations in anthocyanin concentrations appears largely to be due to anthocyanin pseudo bases.

The observations lead to the conclusion that anthocyanin pseudo

bases occur in nature not only in the hood veins of Kitchen but also in aleurone of many varieties. It is also clear that visual observations on the nature of aleurone pigmentation can be highly misleading, a point that deserves careful attention from taxonomists and breeders.

4.4. DISCUSSION

The studies of pigment development in different parts of the barley plant and in different lines of barley were undertaken to gain familiarity with the outward or visual relationships of anthocyanins, related flavonoids, and melanic pigments prior to undertaking biochemical and genetical studies. The rapid chemical survey test were used to augment the ocular observations. These observations may be rewarding in several ways. In any study which hopes to examine the biochemical basis of gene action, it is essential to determine the nature and duration of pigmentation as the barley plants grow. Conceivably the study might aid in delineating appropriate stages suitable for the study of pigment precursors, substitution experiments, related competing, blocked and coupled reactions, and perhaps, the stage of development at which the 'time genes' become active and release the enzymes for the genesis or transformation of anthocyanins, and related pigments.

Anthocyanin production in general, and, particularly in barley, is greatly influenced by environment (Aberg and Wiebe, 1948). The broad observations on pigment 'ontogeny' show that pigment production in some tissues appears simple, but in others it appears to be complex, with physiological overtones. For instance, given genetic competence, the development

of some pigments were maximal, or restricted to the tissue exposed to direct sunlight. That solar radiation may directly control the expressivity of genes governing anthocyanin production is illustrated by observations on the nodes of C-54-55. The formation of a pigmented ring upon removal of the leaf sheath, which partially encircled the node suggests that, although genes for coloring are present, the wavelengths of light absorbed or filtered by the green sheaths determine the genetic expressivity. Although Emerson long ago reported on the influence of light in relation to certain anthocyanin genes in corn, the mechanism of anthocyanin production in such cases is far from understood. Kandler (1958) showed that upon pretreatment of young black mustard seedlings with chloramphenicol or streptomycin, the protein metabolism was blocked resulting in the accumulation of aromatic amino acids. This accumulation brought about the production of anthocyanins because it was proposed both aromatic amino acids and anthocyanins are derived from the same precursor, shikimic acid. Theoretically, the production of anthocyanins in the node, upon removal of the encircled leaf sheath, may be brought about if the intermediates like aromatic amino acids are made available by the action of sunlight. In this connection it is noteworthy that pigments by acting as filters of radiation (Manning, 1950) may influence the metabolism. For instance, the Russian workers Voskresenskaya (1950) and Kursanov (1954) have found that leaves synthesise mainly carbohydrates in red light but accumulate proteins in blue light.

The influence of light on the anthocyanin development in the node as in other plants, e.g., apples, is localized to the cells which are not protected against direct sunlight. Thus, it seems probable that while the node is covered by the sheath, the solar radiation filtered through the

sheath may promote synthesis of proteins; upon removal of the sheath, the rate of protein synthesis may be reduced resulting in the probable accumulation, or extra availability of aromatic amino acids and eventual synthesis of anthocyanins as suggested by Kandler (1960). The developmental features of anthocyanins at nodes, therefore, warrant extensive studies to gain understanding of the specific components of environment that influence anthocyanin development, and therefore, gene expression.

The report on pigmentation changes as they occur in the separate plant parts such as leaf sheath or awn is incomplete without a statement on associated changes. Associated changes or, as early workers in pigment genetics termed them, linkages, might advance the understanding, which at present is slight, of the role of flavonoid pigments in plants. In degree at least, the flavonoid pigments are chemical entities which can be seen. Not only may color relationships from part to part of the plant be explored but also the relationships of the color may be related to specific processes in development. In Lion, for example, as color in the upper auricles fades it deepens in the awns; also this correlated color change seems to take place as pollination occurs and can be used as a guide in hybridization. Ermolaeva *et al.*, (1948), Bottazi (1950) and Mullick (1959) give other associated changes.

Ocular observations *per se* yield some information and chemical tests extends it. To illustrate, when the colorless young hoods of the black variety Kitchen are dipped in acidified methanol the hood veins turn red. Later chemical studies suggested that colorless pseudobases of anthocyanins existing in the hoods were rapidly transformed to the flavylium salt state in acid media.

In alfalfa and many other plants the conspicuous pigments (chlorophylls excepted, may clearly belong to two systems, one a so-called carotenoid color system and another, a flavonoid color system. In barley the carotenoid system appears to be a minor one but Harlan early in this century differentiated, as important, a flavonoid (anthocyanin) system and a melanin system. Following the color sequence observations a query arose as to whether or not the conspicuous pigments of barley were one system or two. Little is known about the nature of melanins in plants and very little evidence supports the view that the dark pigments of barley are melanins. Perhaps the biochemical relationships of the anthocyanins and melanins are quite intimate through common precursors or perhaps interconversions.

The color sequence studies, at times, suggested that such relationships did exist and that, in metabolism, the anthocyanic and melanic pigments were related. It was observed that, in Lion and other black varieties, as anthocyanins faded melanic pigments appeared although usually after an intervening ochroleucous stage.

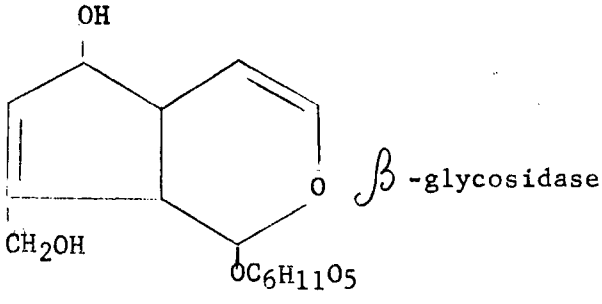
Inheritance studies suggest a relationship between the two pigment classes. Regel (1911) reports two instances of spontaneous mutation in black-chaff barley to white-chaff. Tschermak (1923) from a cross of black-chaff and purple-chaff varieties obtained an F_2 distribution of 12 black-chaff: 3 purple-chaff and 1 white-chaff. In another cross of black-seed and yellow-seed, he obtained black, purple tinged with green, yellow-green, and green segregates. Savinskaja (1941) obtained from crosses of black-seeded and white-seeded varieties, 6 purple, 62 black and 10 white F_2 plants.

In natural selection and in evolutionary sequences the melanins and anthocyanins appear to be associated for it has often been observed that dark-color genes are favored in the low latitudes. In tropical

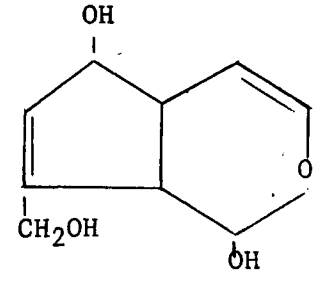
America black and purple maize plants are common in cultivation whereas dilute sun-reds are common in the corn belt.

Speculation therefore in the essential unity of the anthocyanic and melanic pigments may be instructive.

The pigments, generally referred to as plant melanins are basically of two types: those that contain nitrogen and those that are nitrogen-free. The former is defined as "a dark pigment formed during normal ontogeny, or following upon injury, in tissues known to contain the tyrosine-tyrosinase system" (Thompson, 1962b). The nitrogen-free melanins may be due to several reactions: they may originate by enzymatic oxidation of polyphenols to quinones (brownish colors), or light induced polymerization of polyacetylenic and related unsaturated substances to a dark product (Thompson, 1962, a and b). The glycoside aucubin (I) upon hydrolysis with β-glycosidase produces the aglycone aucubigenin (II), which



β-glycosidase



Black product

I

II

upon air oxidation gives a black product (Fujise et al., 1960; Grimshaw et al., 1960; and Haegele et al., 1961). The above glycoside may be responsible for blackening of several plant products (Karrer, 1958).

In the course of work on the purification of barley anthocyanins, it was noted that, under certain conditions, highly purified anthocyanin eluates upon drying in a vacuum oven gave rise to a jet black polymerized product (Sec. 19.3.3.). Whether the anthocyanins polymerize to a black product, in vivo, in a manner similar to that of aucubin remains to be explored.

Although, the chemical nature of barley melanins is unknown if it happens that they are produced from a tyrosine-tyrosinase system, then the biogenetic unity of melanins and flavonoids can be readily seen (Fig. 4.2). It has recently been established that aromatic amino acids (phenylalanine and tyrosine), and flavonoids arise from a common precursor: shikimic acid (Bogorod, 1958; Neish, 1960). The aromatic amino acids are also the precursors of melanins (Thompson, 1962a and b). In barley, the flavonoids develop early in ontogeny and melanins are produced later. Thus, in view of the above biogenetic relationship, it appears possible that, at a certain stage in ontogeny, the aromatic amino acid-ammonia lyases (eg. phenylalanine-ammonia lyase and so on) may be inactivated under the direct or indirect influence of 'time genes', thereby shifting reactions towards melanogenesis.

The value of basal leaf sheaths of barley for studies of flavonoid pigments should be noted. In many varieties they are devoid of chlorophyll but may be white or various reds. As sheaths are removed new ones commonly arise to provide a continuous supply of material of the same genetical constitution, a matter of importance in hybrid plants where

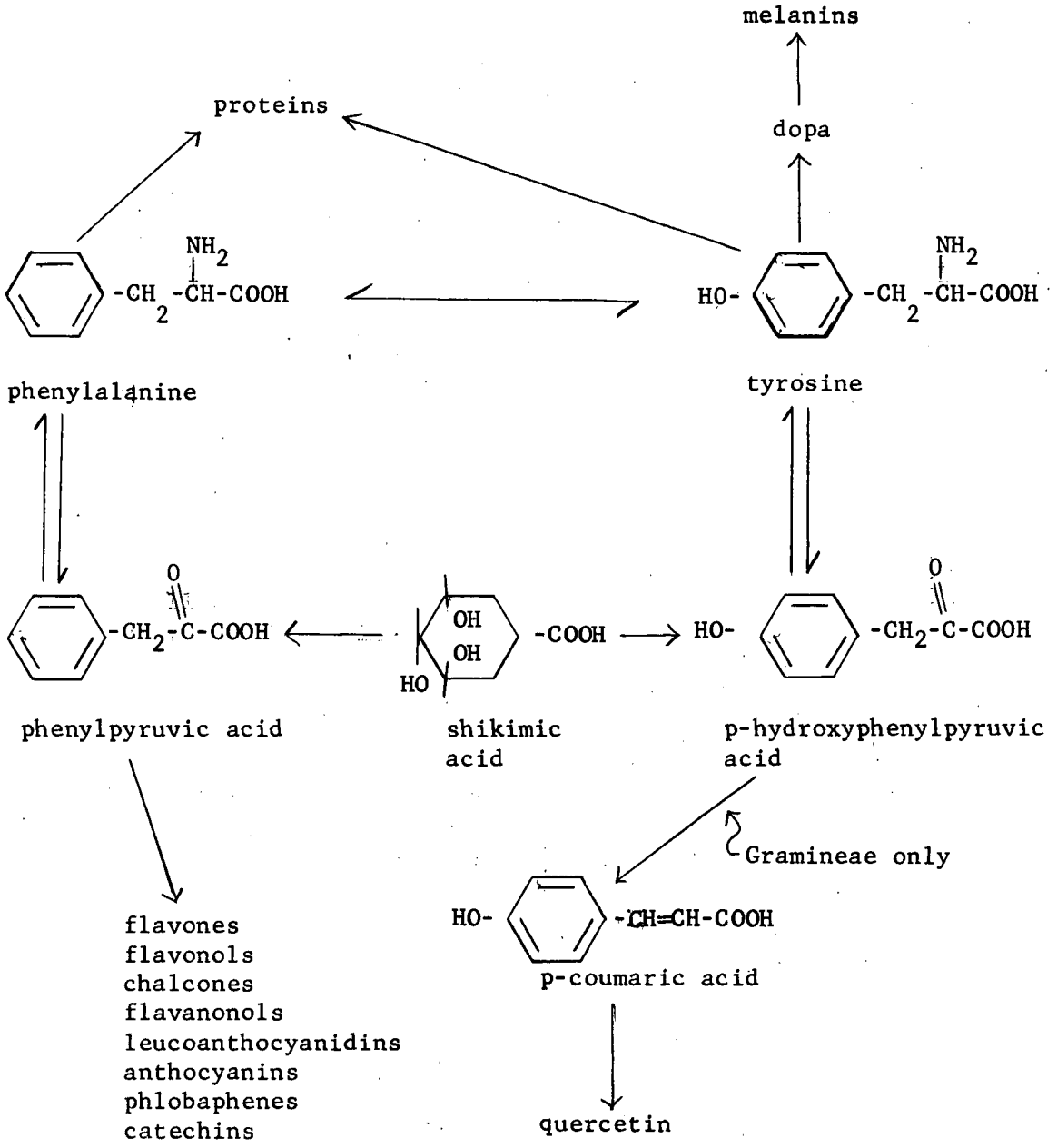


Fig. 4.2.
Pathways showing possible biogenetic unity of melanins and flavonoids in barley.

numbers of plants may be limited. The fact that slightly different environmental conditions prevailing at the time of tiller initiation may lead to pigmented or non-pigmented tillers on the same plant may be of value in the design of physiological experiments (see Fig. 4.3).

Fig.4.3.
Variations in pigmentation of barley tillers initiated at different states under a changing environment. The plant (71-Pr Pr-10 x 71-pr pr-10) planted in the greenhouse Aug. 4, 1961, first headed ca. Nov. 10. Until mid January 1962 tillers lacked color; tillers heading after mid February were increasingly pigmented.

4.5. SUMMARY

As a preface to biochemico-genetical studies, an intimate knowledge of the nature and duration of pigments, in different tissues, during plant development is necessary. Observations were made on the development

of flavonoidal, anthocyanic and melanic pigments in each pigment-competent tissue, particularly the pericarp, spermoderm and aleurone tissues, of several color variants by direct examination. Ridgway's color designations were used for determining aleurone colors (Appendix: Tables 27.I to 27.XX), however, simple color designations were used for all other tissues.

In general, all tissues (except pericarp), which are green initially become pale green. Then depending upon the genetic competence, they develop either yellow and eventually dull yellow colors, or anthocyanic and eventually dull brown and dull yellow colors, or anthocyanic and melanic colors, or only melanic colors. There is a pigmentation cycle in all plant tissues. Whereas melanins may develop in a tissue following the appearance of yellow and/or anthocyanic colors, the anthocyanins never develop following the appearance of melanic colors.

Although, the pigment dynamism in each tissue is consistent, environment greatly influences the ontogeny of pigments. For example the tillers from the genetically purple stocks, which headed between November to January in greenhouses lacked anthocyanins, and those which headed after mid-January showed increasing anthocyanins in the grain and spike tissues.

Anthocyanins develop in the node in regions not covered by the sheath. Removal of the sheath causes anthocyanin production. Thus, although, the genes for coloring are present, light filtered by green sheaths determines the genetic expression.

A large number of the variants, whether they do or do not develop anthocyanins in other parts of the spike, do develop anthocyanins, at least ephemerally, in the awn tips as soon as the tips are exserted. Marked pigment changes occur in the awn remainders. The pigmentation cycle in the glume tip and glume remainder, within a single stock, is identical to

that of the awn tip and awn remainder. Anthocyanins in hull veins and interveins are determined likely by independent sets of genes. Sterile florets of the purple variants are readily recognized because they develop anthocyanins ahead of the fertile florets. The rachis develops anthocyanins profusely in the purple variants. The pericarp is a colorless tissue in the white, near-white, gray and blue variants; it develops melanic pigments (late dough stages) in the black variants, and anthocyanins, if at all, near the tips (opposite the embryo) of the purple variants. The spermoderm is chlorophyllous until early dough and is a principal anthocyanin-containing tissue of the purple variants. It develops anthocyanins ephemerally at the slit bounded by lemma and palea in the black and several other variants. The pericarp tissue, however, does not develop anthocyanins at the slit.

The developing aleurone tissues of several variants, which are visually anthocyanin-free, develop anthocyanins on exposure to acid due to the occurrence, in nature, of anthocyanin pseudo bases, and not due to leucoanthocyanins. The occurrence of anthocyanin pseudo bases in nature was also recorded for the hood veins of the black variety, Gatami. Thus surficial examination of at least some tissues is insufficient for the detection of anthocyanins. The point deserves attention of breeders and taxonomists.

The detailed observations on a) the ontogeny and duration of flavonoidal, anthocyanic and melanic pigments, b) the polymerization of anthocyanins to brown and jet black colors (Sec. 19.3.3.), and c) the known hybridization data and the records of spontaneous mutations suggest that melanic, flavonoidal and anthocyanic systems are related in biogenesis probably irrespective of whether melanins arise from the tyrosine-

tyrosinose system or polyphenolic polymerisation.

The observations will aid delineation of appropriate stages of plant development for studying sequential gene action with the inter-tissue complementation technique and for other biogenetic studies.

BIOCHEMICAL DIFFERENTIATION OF ANTHOCYANIN PHENOTYPES

(Sections 5 - 8)

The inheritance of color in barley has been extensively studied by geneticists and breeders and has been reviewed on a number of occasions (Smith, 1951; Nilan, 1964; Robertson, Weibe, Shands, and Hagberg, 1965). The chemistry and biochemistry of the pigments of barley have, however, been given very little attention although a substantial literature exists on the pigments of many other plants some of which are other graminaceous crop plants such as maize (Zarudanaya, 1950; Coe, 1955a, 1955b, and 1957; Straus, 1959, 160; Reddy and Coe, 1962; Reddy, 1963, and 1964). Until recently, the chemical study of plant pigments relied on a relatively few and often laborious techniques which generally gave very incomplete patterns. More recently techniques such as chromatography, electrophoresis and spectral analysis have been employed very effectively. Having first examined ocularly the color patterns of a number of varieties, lines and tissues of barley, they were next scrutinized chromatographically. The general results of this scrutiny are reported in the next sections. It might in fact be said that having examined the ocular phenotype, the chromatographic or biochemical phenotypes were examined then.

BIOCHEMICAL DIFFERENTIATION OF ANTHOCYANIN PHENOTYPES

5. ANTHOCYANINS OF THE SEPARATE MATERNAL TISSUES OF THE GRAIN

It is highly desirable to determine if there are differences in the pigments of the separate tissues of the grain for, it is well known, there are fundamental genetical differences (and, ipso facto, biochemical differences) between them. In the early studies of grain pigments, for lack of better techniques, the chromatography had to be conducted on composite tissues, i.e., pearlings of the outer coverings and aleurone tissues. The development of the peeling technique, described in section 3, made a refined chromatographic study possible. An investigation to delineate the biochemical phenotypes of the color in different maternal, and maternal-paternal tissues was undertaken in the summer of 1958. It was shown in Sections 3 and 4, that in purple varieties, the major development of anthocyanins occurs in the spermoderm layer; the pericarp proper possibly develops if at all, only minor concentrations of anthocyanins near the apical end (see Sec. 8). The anthocyanins present in the pericarp proper

and spermoderm layers were investigated separately. The results were partly compiled (Mullick, 1959). Although several unusual features of these pigments were noted, no satisfactory explanations were forthcoming at that time. It was not until 1961-62, when the identification studies on the anthocyanin pigments were undertaken (Sec. 19) that the inherent complexity of barley anthocyanins was appreciated. The results of the 1958 summer studies were re-evaluated and after re-evaluation are presented in this section.

5.1. MATERIALS AND METHODS

5.1.1. Collection of Materials

Two purple varieties, Gopal and Black Hulless, which develop anthocyanins in grain tissues profusely were chosen for this study. The materials were planted at the University Farm on May 15, 1958. The following grain tissues, namely awn tips, lemma, pericarp, spermoderm and aleurone, were collected for analysis in early August, 1958.

The plant material was not graded on the basis of color intensity, as was done in 1959 (Sec. 8). Although the collection of materials was at random, in general, only those parts of tissues showing color (probably, equivalent to stage III of 1959: Sec. 8.1.1) were collected using stainless steel scissors. The caryopsis layers were collected by manual peeling (Sec. 3). The layers cannot be peeled after late hard dough stage, the stage around which fortunately they develop maximum pigment. Only in the case of Gopal was an additional collection of all the grain tissues made at the stage when anthocyanins had just started to develop (equivalent of stage I of the 1959 studies).

In addition to the developing plant material mentioned above, the pearled dust obtained from the mature barley kernels of Gopal (Sec. 6.3.1.c) was also used later in 1962 for this study.

5.1.2. Extraction

The maternal tissues were extracted with ethanol containing 1% conc. HCl. The awn tips, and lemmas (hulls) were extracted at the rate of 1 gm tissue per 15 ml and 20 ml solvent, respectively.

The collection of pericarp and spermoderm tissues is time-consuming. In order, therefore, to minimize autolysis, these tissues were extracted directly in the solvent following peeling; weighing was not undertaken. The tissues were usually extracted overnight on a shaker, decanted, centrifuged and concentrated *in vacuo* at 30°C to obtain a highly concentrated anthocyanin extract. At least two high speed centrifugations (8000 rpm), one in the process of, and the other at the end of, volume reduction *in vacuo*, were essential to obtain a clear anthocyanin extract for chromatography. In general, the extraction, concentration and spotting for chromatography were completed within two days.

The pearled dust of Gopal was extracted in 1962 with 0.1% methanolic HCl at the rate of 20 ml per gram dust by shaking for 6 hours at room temperature. The extract was centrifuged at high speed, concentrated *in vacuo* at 30°C and banded immediately on Whatman paper No. 3. The chromatography was carried out in the BAW solvent by ascent as described elsewhere (Sec. 10). Thus the extraction and chromatography was completed on the same day within about 10 hours.

5.1.3. Hydrolysis

The anthocyanin extracts were hydrolysed by the following method. To 0.5 ml of concentrated alcoholic extract was added 1 ml of 1% aqueous HCl, and the mixture heated gently for about two minutes to evaporate most of the alcohol. Then 1 ml conc. HCl was added, and the mixture was hydrolysed for 10 minutes. The hydrolysate was centrifuged at 3000 rpm for five minutes and the aglycones were displaced into a few drops of isoamyl alcohol to obtain a highly concentrated solution for chromatography.

5.1.4. Paper Chromatography

The chromatography of anthocyanins and anthocyanidins was carried out on Whatman Paper No. 1, at 30°C, by ascent. The anthocyanin extracts were spotted immediately after processing on at least three sheets using different amounts to determine the optimum amount of the extract for good resolution. The anthocyanins were resolved in BAW solvent consisting of 1-butanol-acetic acid-water (4:1:5; v/v; upper phase) and anthocyanidins in Forestal solvent consisting of acetic acid conc. HCl-water (30:3:10; v/v). The R_f value measurements were made to the leading edge of the spot.

The chromatograms were examined under visible and ultraviolet light. The ferric chloride test was carefully applied to anthocyanin spots; concentration was important and was varied from 0.05 to 1 percent to suit the circumstances. The least concentration was used in the case of weak anthocyanin spots. Spots to be tested were cut out and slipped slowly sideways into the ferric chloride solution, placed in a flat enamel tray.

5.2. OBSERVATIONS AND RESULTS

5.2.1. Anthocyanins, General

The chromatographic characteristics of the anthocyanins of awn tip, lemma, pericarp* and spermoderm tissues of Black Hulless and Gopal are shown in Fig. 5.1 and 5.3., respectively. The R_f values, colors (visible and ultraviolet), and ferric chloride reactions of the spermoderm anthocyanins of Black Hulless are shown in Table 5.I; the data for the first two anthocyanin spots (R_f 0.23, 0.31) were taken from Gopal spermoderm III (Fig. 5.3). The colors and ferric chloride reactions of other tissues are similar to those of the spermoderm tissues.

Because of the instability of barley anthocyanins, the identification work posed several problems (see Secs 11, 12, 13 and 15), and the initial efforts proved fruitless. Accordingly, any attempt to relate the identity of one spot with the other on physical parameters, such as, colors, R_f values etc., alone appears hazardous. Although, chromatography by ascent affords excellent resolution (Acheson, Harper and McNaughton, 1956) the R_f values are found to be highly variable. (The anthocyanin spots in the figures, therefore, have not been designated in order to eliminate any implication of their correspondence to one another). However, for ease of reference, the spots will be numbered with arabic numerals in the text, the numbering beginning from the spot closest to the

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*It became apparent subsequently that the pigments from pericarp obtained in this study must have originated from the lacerated spermadermal tissues at the time of manual peeling (cf. Secs. 3, 4 and 8).

starting line. Some idea of the chromatographic relationship of one spot to another may be gained by reference to the position of crescent-shaped fluorescent spots shown in the chromato-diagrams. The fluorescent spot, probably, arises from the paper.

TABLE 5.I.
R_f VALUES (ASCENDING), COLORS AND FERRIC CHLORIDE, REACTIONS OF THE ANTHOCYANINS FROM THE SPERMODERM LAYER OF BLACK HULLESS.

R_f x 100 (BAW)	Colors		FeC_{13} 0.05%
	V	UV	
23	Reddish[1]	Dull bluish purple	Blue
31	Reddish[1]	Dull bluish mauve	Blue
46	Rose Red	Dull purple-magenta	Blue
50	Rose Red	Dull purple-magenta	Blue[2]
55	Rose Red	Dull mauve	Blue[2]
61[3]	Reddish (wk)	Dull (wk)	Blue
70[3]	Reddish (wk)	Dull (wk)	Blue
95[3]	Rose Red	Dull bluish purple	Blue
99[3]	Rose Red	Dull blue	Blue

1. Color is red during chromatography, it turns bluish red when chromatograms are dried. The red color reappears after 3-4 days.

2. Although most of the anthocyanin color at this spot turns blue and disappears upon reacting with 0.05% ferric chloride; a small portion of the red color does persist.

3. Note that the 'fast-moving' anthocyanins do not show the fluorescence characteristic of anthocyanidins.

The photograph of anthocyanins in different tissues of Black Hulless (Fig. 5.1) demonstrates several striking features. First, the presence of at least two 'fast-moving' (R_f above 0.5 in BAW) anthocyanins may be noted. These anthocyanins show ultraviolet absorption and give

ferric chloride and ammonia reactions characteristic of anthocyanins (Table 5.I.). The fast-moving anthocyanins, surprisingly, were not

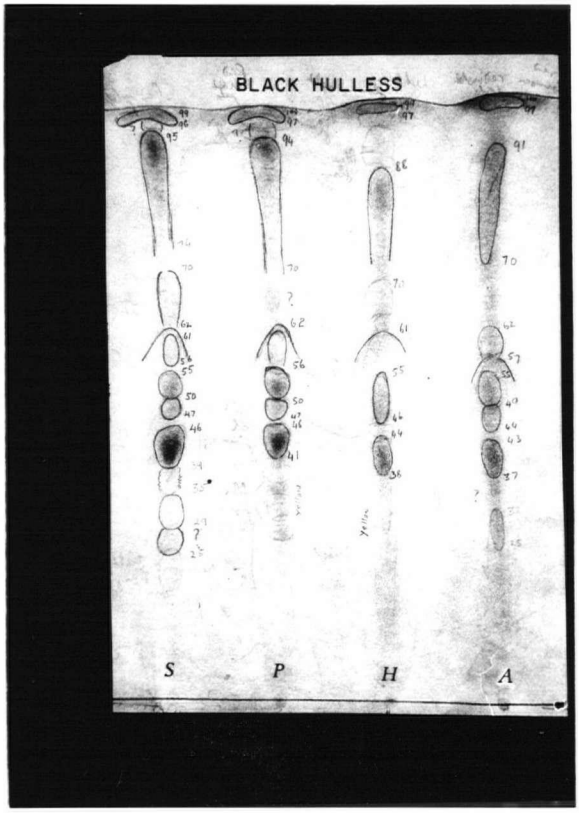

Fig. 5.1.
Chromatograms of acidified ethanolic extracts from the spermoderm, S, pericarp, P, hull, H, and awn tips, A, of immature Black Hulless barley grain showing novel anthocyanins with high R_f values in BAW solvent.

recovered from the combined tissues of mature caryopsis reported previously (Mullick et al., 1958: Fig. 1, pp. 449). Additional observations on the fast-moving anthocyanins are given later in this section and in sections 7 and 8.

The patterns of the major anthocyanin spots in the 2n grain tissues of Black Hulless are not uniform (Fig. 5.1). The lemma and spermoderm tissues show, additionally, weak spots of 'slow moving' (R_f below 0.5 in BAW) anthocyanins not present in pericarp and awn tips. The patterns of anthocyanin distribution of lemma and spermoderm, if the fast

moving anthocyanins are excluded, compares well with those of the mature caryopsis of Black Hulless (Mullick, et al., 1958). A few observations may be added: First, the chromatography of anthocyanins in Figs. 5.1 and 5.2 was done by ascent and that in the early work by descent; the R_f value differences may, therefore, be more apparent than real. Second, the most prominent spot in the tissues of Fig. 5.1 (R_f values ranging from 0.43 to 0.46) is, probably, equivalent to the Black Hulless spot C in the early work. Third, although the two spots D and E of the early work may appear to be equivalents of the two spots above the prominent spot (R_f 0.43-0.46) in Fig. 5.1, they possess different colors and give different ferric chloride reactions; upon treatment with ferric chloride, the two spots of Fig 5.1 turned blue and left very weak reddish colors (Table 5.I); the spots D and E of early work did not change.

The results using Gopal tissues, stage I and stage III are diagrammed in Fig. 5.2. All tissues show the presence of the fast-moving anthocyanins. The patterns of anthocyanin distribution in all tissues at the same stage of development are similar: the stage I collections, however, do not show the slow-moving anthocyanins. The anthocyanin distribution, at stage III, in all Gopal tissues is similar to the Black Hulless spermoderm tissues. Further, spot No. 3 (in all tissues representing stage III) appears to be similar to spot No. 1 of tissues at stage I. The concentration of this spot is at a maximum at stage III and at a minimum in stage I. The spots No. 2 and 3 of stage I are well developed when compared to the corresponding spots No. 4 and 5 of stage III; the nature of the two spots of stage I, most probably, is different from that of stage III. It was recorded in the 1958 study (Mullick, et al.,) that the spot B (Fig. 5.2) gave bluish red (i.e. purple) color in Black Hulless,

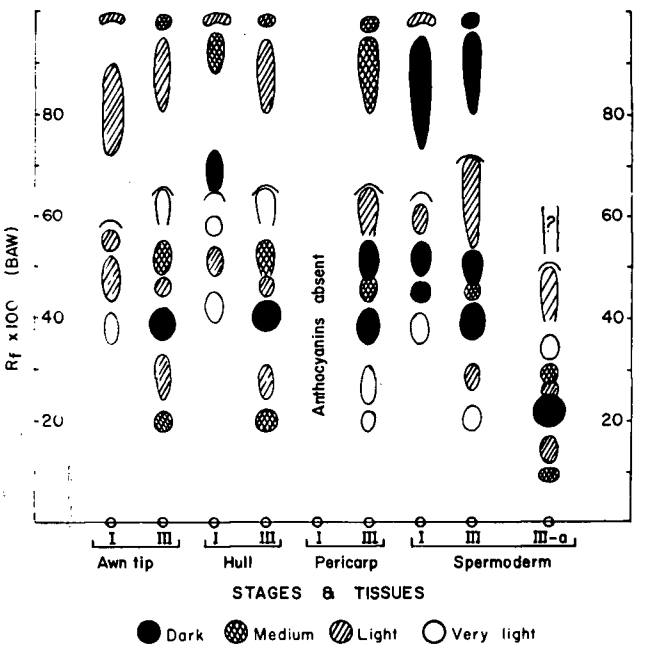

Fig. 5.2.
Chromatograms of acidified ethanolic extracts from the spermoderm, pericarp, hulls, and awn tips, of developing (stages I and III) Gopal barley grain showing novel anthocyanins with high R_f values in BAW solvent. Anthocyanins do not occur in pericarp stage I. Spermoderm anthocyanins stage III extract, after one year's storage at 5°C is also shown (S-III-a). Solvent (B:A:W, 4:1:5), S, spermoderm.

and brownish red color in Gopal visually, and also gave blue black and blue colors with ferric chloride, respectively. It was observed that none of the spots in Fig. 5.1, and Fig. 5.3, showed the purple color visually or gave blue black color with ferric chloride (Table 5.I).

5.2.2. Anthocyanins, 'Fast-Moving'

The anthocyanin extracts described in the preceding section were processed, prior to chromatography, by the procedure described by Mullick, et al., (1958): the acidified alcoholic extracts of anthocyanins were purified by transferring to an acidified aqueous system to facilitate the

removal of contaminants and related non-anthocyanin flavonoids by ethyl acetate, petroleum ether, and benzene scrubbing. Elaborated, the procedure consisted of evaporating the alcoholic anthocyanin extracts: (a), to near dryness, in vacuo, and then dissolving the anthocyanins in water containing 1% conc. HCl, (b), the volume reduction in vacuo was continued until most of the methanol had evaporated; the aqueous extract was concentrated by washing with 3 to 4 times the quantity of ethyl acetate. The ethyl acetate, apart from extracting related flavonoids, also extracts water and thus concentrates the aqueous extract of anthocyanins. The chromatograms of the anthocyanins extracts (a), and (b) are shown in Fig. 5.3. It will be noted that when the anthocyanin

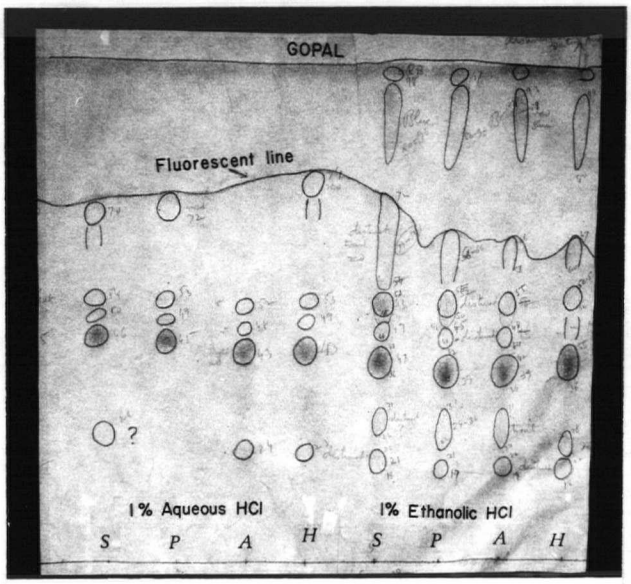

Fig. 5.3.
Anthocyanins from awn tips, A, hulls, H, pericarp, P, and spermoderm, S, of Gopal. I, extracted with and chromatographed from 1% conc. hydrochloric acid in ethanol. II, extracted with 1% conc. hydrochloric acid in ethanol and chromatographed from 1% conc. hydrochloric acid in water. (Solvent B:A:W, 4:1:5).

extracts of awn tips, hulls, pericarp and spermoderm were chromatographed directly from 1% ethanolic HCl, they showed fast moving anthocyanins above the wavy fluoresent line (Fig. 5.3). Upon transfer of the acidified ethanolic extracts to the corresponding acidified aqueous system, the fast-moving anthocyanins disappeared (Fig. 5.3). In the course of

repeated chromatography of the acidified ethanolic and aqueous extracts, it was observed that fast-moving anthocyanin streaks were present above the fluorescent wavy line in the aqueous system for the first five hours of chromatographic development; the streaks gradually vanished, when the chromatograms were allowed the usual time of development (ca. 20 hours). The fast moving anthocyanins, thus, are extremely labile. The factors affecting their lability are understood poorly.

It was observed that whenever volume reduction in vacuo followed the transfer of anthocyanins from the alcoholic to the aqueous system, apart from the general precipitation of alcohol soluble - water insoluble material, a small fraction of anthocyanins, floated on the surface of the aqueous system. The 'floating' material appeared during the evaporation of the alcohol in vacuo. It was collected by centrifugation, redissolved in a few drops of the acidified ethanol, and chromatographed. The chromatogram showed a strong, very well resolved spot, which by co-chromatography was found to be identical to spot No. 3 (Fig. 5.3). The spot No. 3, in turn, is identical to the band No. 5, which is a cyanidin derivative (Table 19.VIII.5). In addition, it gave a spot equivalent in R_f value to spot No. 1 (Fig. 5.3), which, in turn, is identical to band No. 1, also is a cyanidin derivative (Sec. 19.3.7.3), and a brownish red weak trail extending, both above and below the well resolved major spot.

Another observation on the fast-moving anthocyanins came from the 1958 spermoderm extracts (Fig. 5.2, Spermoderm stage III), which were chromatographed after storage in a refrigerator (-5°C) for a year. The results were shown at S-III-a in Fig. 5.2. Upon extended storage, the fast moving anthocyanins, which were present in the original extract,

completely disappear; the intensity of slow-moving anthocyanins increases, and spot No. 3 emerges as a major spot. The differences between the R_f values of the two adjacent spermoderm extracts (S-III, and S-III-a) are, probably, due to temperature variation during chromatography. The crescent shaped fluorescent spot, used as a reference mark, also shows a corresponding R_f change.

5.2.3. Anthocyanins, Flinty Stage Grain

The chromatographic results from the anthocyanins of the pearled dust obtained from the flinty grains of Gopal, which was extracted and processed within a day under mild conditions (cf., Sec. 23) are shown in Fig. 5.4. It is clear that no fast moving anthocyanins are present in the mature barley grain. The fast moving anthocyanins were also absent from the mature kernels of Black Hulless under identical conditions (cf., Sec. 19).

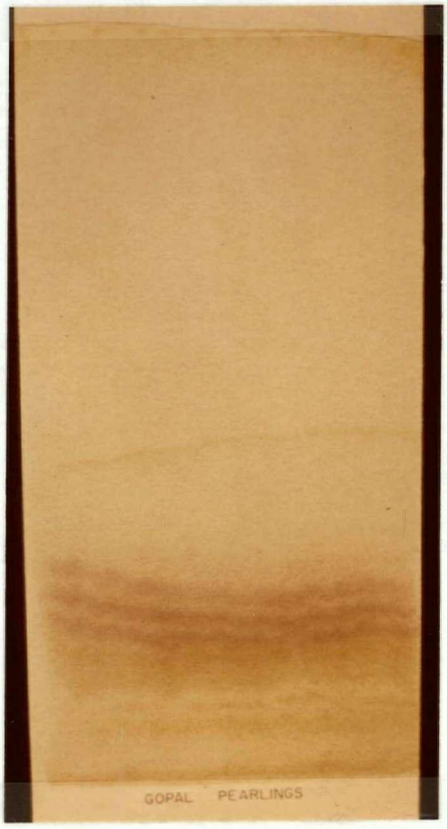

Fig. 5.4.
A chromatogram of anthocyanins from the pearlings obtained from the flinty Gopal grains. The pearlings were extracted with 0.1% methanolic HCl, and chromatographed in the BAW solvent on the same day. Note, the chromatogram does not show the fast-moving anthocyanins.

5.2.4. Anthocyanidins

The anthocyanin extracts from the tissues, listed in Figs. 5.1 and 5.2, were hydrolysed separately and chromatographed in Forestal solvent. Only two anthocyanidin spots, a major and a minor, were recovered from all extracts. The identity of the major spot was established as cyanidin by co-chromatography with a synthetic sample, by comparison of visible and ultraviolet colors and by the ferric chloride test. The minor spot always appeared on chromatograms as a weak trail beginning from the cyanidin spot and approaching the R_f values of synthetic pelargonidin. Despite repeated chromatography, the trailing could not be eliminated. Since the color of the trail was weak, its true visible, ultraviolet and ferric chloride characteristics could not be ascertained with certainty. Therefore, no definite identity could be assigned to the trail. The color appears to resemble that of peonidin rather than pelargonidin. It was appreciated subsequently that peonidin and pelargonidin cannot be resolved dependably in Forestal solvent (Sec. 17). This point was not realized in the earlier studies when the weak anthocyanidin trail was considered to be due to pelargonidin (Mullick, 1959). It now appears that the weak trail could either be due to peonidin or a mixture of peonidin and pelargonidin. The same observation may apply to the pelargonidin spot found in the mature ceryopsis tissues of the purple varieties (Mullick, et al., 1958). It is significant that delphinidin, which was present in the combined pearlings of ceryopsis (Mullick, et al., ibid.) was not recovered from the peelings of the spermoderm and pericarp and other maternal tissues.

5.3. DISCUSSION

The peeling technique, although simple, has been the means by which the biochemical phenotypes in the separate maternal tissues of the grain are differentiated. The results of anthocyanin hydrolysis establish that cyanidin derivatives are the major, and peonidin and (or) pelargonidin derivatives are the minor anthocyanins of the maternal grain tissues of Gopal and Black Hulless. Significantly, neither delphinidin, nor its derivatives, which were present in the composited pearlings of pericarp, spermoderm, and aleurone of caryopsis as examined by Mullick et al., 1958; Mullick, 1959, were recovered from the separate tissues of pericarp and spermoderm. Delphinidin derivatives, thus by deduction, originate from aleurone layer. Consequently, the maternal (reddish purple) and maternal-paternal (blue) tissues have not only phenotypic differences but also distinct biochemical differences. These observations lend added interest to the biochemico-genetical studies, inasmuch as the color development in the maternal and aleurone tissues is determined by independent sets of genes located on different chromosomes (review: Nilan, 1964).

On the basis of current literature (Nilan, 1964; Robertson, Wiebe, Shands and Hagberg, 1965), it appears that the determination of purple color in lemma and pericarp* (+spermoderm) in some genetic stocks is conditioned by a single gene, while in others by the action of two complementary genes. The same genes are believed to determine the purple

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*The pigment competent tissue is largely spermoderm and not pericarp (cf., Secs. 3, 4 and 8).

color in the three maternal tissues, namely, lemma, and pericarp ($^+$ spermoderm). The purple vs. non-purple color development in the three tissues is determined by the genes Re_2 re_2 located in chromosome No. 2, and (or) Re re associated with chromosome No. 5. That the same genes may control the color development in the three tissues is supported by the chromatographic findings, inasmuch as the biochemical phenotypes of color of all the maternal tissues of Gopal (Fg. 5.2, stage III) are almost identical.

The color development in lemma is under genetic control, and, it has been shown that three dominant gene pairs (P_c -1; P_e -?; P_f -?) control the color development in lemma veins (Buckley, 1930). In the case of the two purple varieties, Gopal and Black Hulless, the anthocyanins first develop in the interveinal region of lemma when the veins are still green; the color in the veins develops a few days later (Sec. 4). Thus it is likely that the color in veins and interveinal region of lemma may be determined by an independent set of genes. This possibility was not appreciated at the time these studies were undertaken. No attention to these aspects has been given by earlier workers. It was noted in the case of all varieties included in Table 2.I. that whenever lemma interveins are colored the veins are also colored; the reverse, however is not true. For instance 71-Pr Pr-10 or 71-pr pr-10 has color in veins but no color in interveins. Further work appears highly desirable to discern if differences between the biochemical phenotypes of lemma interveins and lemma veins, similar to that of the maternal and aleurone tissues, are involved. If the complementary factor hypothesis is accepted then, theoretically, the genotype of the varieties investigated would be Re Re_2 P_c P_e P_f for lemma, and Re Re_2 for spermoderm.

It is likely that the genetic constitution of the stocks may still be further confounded. Although, the current studies indicate that the same genes may control color development in the three maternal tissues, it was observed that in the purple varieties the genes controlling color development in the tissues do not become active at the same time: the color first appears in lemma, then in spermoderm, and finally perhaps in pericarp. The ontogeny of anthocyanins in the pericarp proper is the hardest to discern towards the later stages of kernel maturation. It is clear from the studies described elsewhere (Sec. 3, 4 and 8, and see also Appendix: Sec. 27) that pericarp tissue may develop anthocyanins at hard dough stage, at best, only near the apical end. Although the pericarp tissues in Figs. 5.1 and 5.3 show patterns of anthocyanins identical to those of spermoderm, most of the pigments, if not all, would have arisen from the lacerated spermodermal tissues (Secs., 3, 4, and 8). Nonetheless, the point has been made that the development of pigments in the three maternal tissues is not related temporally. Thus it remains to be demonstrated that if the color development in the three maternal tissues is governed by the same gene or set of genes, viz. Re Re_2 Pc Pe P_f, then what governs the temporal aspects of gene activation? The studies on the time gene relations appear to be too involved to be handled by the genetical techniques alone. Continuing biochemical studies on pigments, it appears, will provide fruitful foundations for further genetical work.

The chromatographic results included in Figs. 5.1 and 5.2 demonstrate that the maternal tissues of Black Hulless and Gopal do not show the presence of the purple anthocyanin spot B of earlier work (Mullick *et al.*, 1958) which gave the blue black color with ferric chloride. The purple spot which is a delphinidin derivative (Sec. 19.3.7.3), was present only

in Black Hulless, the blue aleurone variety, and not in Gopal, the non-blue aleurone variety (Mullick et al., 1958). The fact that delphinidin did not occur in the spermoderm or pericarp of Gopal or Black Hulless is supported by that fact that its glycoside (equivalent of the spot B) did not occur in these tissues either.

The fast-moving anthocyanins show novel chromatographic characteristics, inasmuch as the R_f values of the anthocyanins, both simple and acylated, reported in the literature thus far do not exceed ca. 0.5 in the BAW solvent. In this solvent, polyglycosylated anthocyanins give low R_f values and monoglycosylated high R_f values (Harborne, 1959a). Several acylated anthocyanins are generally highly glycosylated and give R_f values as high as their corresponding monoglycosides; the higher R_f values are due to the greater solubility of the acyl components in the organic phase of the solvent. A noteworthy observation may be cited in this connection. A row of semilunar fluorescent spots (Figs. 5.1, 5.2), or a wavy fluorescent line (Fig. 5.3); sometimes forms on the chromatograms. It was observed that when wet chromatograms, after development by ascent, were hung to dry, the region above the fluorescent row dried faster than the region below. The observation suggests that most of the aqueous phase of the BAW solvent is partitioned and held by cellulose below the wavy line, and that the region above the line contains predominantly the organic phase. The concentration of the aqueous phase of the solvent, particularly in ascending chromatography, is a maximum near the starting line and a minimum at the solvent front. The concentration of the organic phase is a minimum near the starting line and a maximum at the solvent front. Because of the preponderance of the organic phase near the solvent front, anthocyanidins and related planar compounds, which are sparingly

soluble in water, give high R_f values in BAW solvent; the highly glycosylated anthocyanins, which possess greater solubility in aqueous phase as compared to the monoglycosides, give lower R_f values. The R_f values of the acylated anthocyanins (which are highly glycosylated), as high as those of their corresponding monoglycosides, are doubtlessly attributable to the greater solubility of the acyl function (in contrast to the glycosidic function) in the organic phase, the concentration of which increases in the direction of the solvent front.

The extraordinarily high R_f values of the fast moving anthocyanins suggest that they have a novel type of organic residue, readily soluble in the organic phase of BAW. These anthocyanins are not only found to be unstable when stored for a year (cf., S-III and S-III-a, Fig. 5.2; also refer Secs. 7, and 8), but also when stored for a few hours (Sec. 7).

The 'floating' fraction of anthocyanins, obtained upon the transfer of the alcoholic extracts to the aqueous media, require, as do the fast-moving anthocyanins, further scrutiny. Certain features of the floating fraction such as lability, the presence of a brownish-red trail above the major spot, and other chromatographic characteristics suggest that the floating material could be the fast moving anthocyanins, and acyl groups may be responsible for the behaviour in aqueous media.

After a year's storage of the spermoderm extracts of 1958 (Fig. 5.2: cf., S-III, and S-III-a), the fast-moving anthocyanins disappear, the slow-moving ones increase in intensity with spot No. 3 emerging as a major anthocyanin. The slowest moving spot No. 1, and also spot No. 3, on the basis of identification data described elsewhere (Sec. 19), are the glycosides of cyanidin. Inasmuch as these spots intensify upon the disappearance of the fast-moving anthocyanins, and inasmuch as

the major anthocyanidin derived from the hydrolysis of the extracts containing the fast-moving anthocyanins is cyanidin, it appears very likely that the fast-moving anthocyanins are cyanidin derivatives. Further observations on the nature of fast-moving anthocyanins will be found in Secs. 7, 8, 19.4.4, and 22.

The fast-moving anthocyanins were recovered only from the developing grain tissues; they were absent in the composited pearlings from flinty caryopsis of earlier work (Mullick et al., 1958). The studies on the grain tissues were repeated in 1959 and 1960 (Sec. 8). When the results of these and subsequent studies were compiled, it became clear that the fast moving anthocyanins were highly labile during handling and storage. This suggested that the combined tissues of mature caryopsis could have contained the fast-moving anthocyanins, and that the absence of these pigments in the 1958 analysis (Mullick et al.,) was only due to handling and processing. Accordingly, the work was repeated in 1962 (Sec. 23) using a 'mild' treatment; the extraction, processing and chromatography was completed the same day, but again the fast-moving anthocyanins were not recovered. Inasmuch as the fast-moving anthocyanins, as indicated by their chromatographic properties, may be acylated, the total observations indicate that the grain tissue anthocyanins are probably deacylated towards the final stages of kernel maturation.

5.4. SUMMARY

The maternal tissues from the developing spike and grain, namely awn tips, hulls and spermoderm, of the purple varieties, Gopal and Black Hulless, show, by standard methods, eight identical anthocyanins.

Three of the eight anthocyanins show novel chromatographic characteristics (R_f 0.5 to 0.99 in BAW). Such exceedingly fast-moving anthocyanins were not recovered from the flinty grain tissue (see Mullick, Faris, Brink and Acheson, 1958). It is postulated on the basis of chromatographic properties that the fast-moving anthocyanins are acylated with novel type(s) of organic residue(s).

The identity of the pigment patterns in the maternal tissues supports the previously reported genetics of anthocyanins, inasmuch as the same genes are known to goven anthocyanin production both in the lemma and pericarp (The pigment-competent tissue, in fact, is largely spermoderm (see Sec. 3 and 4), and not pericarp). However, the anthocyanin genes in the maternal tissues do not become active simultaneously. Thus, the temporal aspects of anthocyanin development are likely controlled by the 'time' genes.

BIOCHEMICAL DIFFERENTIATION OF ANTHOCYANIN PHENOTYPES

6. THE BLUE ANTHOCYANIN PIGMENTS OF THE ALEURONE LAYER

6.1. INTRODUCTION

As a result of double fertilization the aleurone tissue is composed of cells with triploid nuclei in which 2/3 of the chromatin comes from the maternal parent and 1/3 from the paternal parent. In barley a xenia effect may be observed in aleurone pigmentation when pollen carrying genes for 'blue' produces blue aleurone after pollinating a 'homozygous colorless' maternal style.

Breeders and taxonomists have used aleurone color in classifying barley varieties. In Canada, a rough distinction has been maintained between feed varieties and malting varieties in that the former have generally white aleurone and the latter blue aleurone. More recently the

brewing and malting industries have displayed interest in the aleurone pigments, because of their relationship to the chill haze and flavor (see review by Pollock, 1963).

Color inheritance for the aleurone tissue has been reviewed by Smith (1951) and Nilan (1964). The chemistry of aleurone pigmentation has received scant attention. The fact that the pigmented tissues, peri pheral to the aleurone, can be peeled away (see Section 3) made a definitive chemical study possible.

In the spermoderm and pericarp, which are maternal and diploid, anthocyanins when present are red; in the aleurone, which is triploid, anthocyanins, when they occur, are blue (Section 4). Although it is well known that the red color of anthocyanins is due to oxonium salt formation, how the blue and violet color transformation is brought about is not known. "Despite all efforts of many investigators," states Hayashi (1962), "this intriguing phenomenon still remains enigmatic."

The pH, the co-pigmentation and the adsorption theories, as put forward to explain flower color variations, are well known. New light shed on the matter has been competently reviewed by Hayashi (1962). Only a few comments are therefore given here.

Karrer and his co-workers (1927), on the basis of qualitative and quantitative analysis of the ash content of flowers, found that the ash of red flowers imparted an acidic reaction, and that of blue flowers an alkaline reaction to their cell saps. The results obtained by several other workers, however, showed that it was the complexing of metals (present in ash) such as, aluminum, magnesium, calcium, potassium, iron, etc., with anthocyanins that played an important role in the formation of bluer flower colors. Shibata, Hayashi and Isaka (1949), and Shibata

and Hayashi (1949a and 1949b) claimed that no simple relationship exists between ash content and flower color. According to them, the blue color of the flowers is due to an alcohol insoluble, organo-metallic-anthocyanin complex containing magnesium and calcium.

Recently, the properties of certain blue anthocyanins, which have been isolated from natural sources in a highly pure form, indicate that the blue colors may, indeed, be due to organo-metallic-anthocyanin complexes. Hayashi, Abe, and Mitsui (1958) have isolated a blue anthocyanin "commelinin" as crystalline brilliant-blue prismatic needles from the flowers of Commelina communis. Commelinin is soluble in water but is insoluble, or does not retain its color, in common organic solvents. It is non-dialyzable, is neither an acid nor a base, and retains its blue color even in the presence of 1% HCl solution. Tentatively, commelinin consists of 4 moles of delphinidin-3, 5-diglucoside plus 1 mole of p-coumaric acid, held together by 1 atom of magnesium. In addition, two atoms of potassium, probably as the phenolic salts of the hydroxyl groups in the anthocyanin moiety, and a faintly yellow flavonoid substance, which could be the cause of the stability of the blue color, are also present (Hayashi, 1962). Similarly, a blue anthocyanin, "protolupinin" isolated from Lupinus polyphema by Bayer (1959) is a non-dialyzable high molecular weight substance in which delphinidin glycoside is complexed with aluminum and iron. Bayer (1958, 1960) has, also, isolated from cornflowers, a blue anthocyanin "protocyanin," which is complexed with aluminum (0.27%), iron (0.54%) and an unknown nitrogenous substance of colloidal nature.

6.2. MATERIALS

The following barley varieties, Black Hulless (purple), Kwan, Montcalm and Trebi (blues), and Gatami (black) were chosen for this investigation. The varieties Gopal (purple), Lion (black) and Golden Pheasant (white) were not used because they either do not develop, or, develop as traces, blue anthocyanins in the aleurone layer (see section 4 and Appendix I). All of the aforementioned varieties were used in an earlier study by Mullick, Faris, Brink and Acheson (1958).

6.3. METHODS

6.3.1. Extraction from the Aleurone of Flinty Grains

(a) The aleurone of Black Hulless obtained from mature (flinty) grains, by peeling after softening (Sec. 3), were left at room environment for three months in methanol containing 1% conc. HCl, in 1% conc. HCl (aqueous), and in methanol.

(b) About 3 to 5 grams of mature kernels of each variety were left in 25 ml 1% conc. HCl in methanol for several days. The extraction was hastened by shaking for about 12 hours at a time. The extraction by shaking, in the case of Black Hulless was repeated several times using fresh solvent each time.

(c) The barley grains were first dehulled by pearling. Liberal quantities of the dehulled grains were pearled to obtain dust (pearlings) from the outer coverings and aleurone layer with as little endosperm material as possible. The dust was thoroughly scanned with a magnet to remove metallic bristles. About 3 to 5 grams of the powder was placed in an erlenmeyer flask containing 50 ml 1% conc.* HCl in ethanol, extracted on a shaker for about 12 hours, and centrifuged. The pellet was re-extracted several times using fresh solvent. The pearled dust, later, was also extracted in 1% conc. HCl in methanol, in the manner described above.

(d) To 8 grams of the dust, was added 1% conc. HCl in methanol at the rate of 10 ml per gram of dust. The mixture was extracted in a 10 KC Raytheon sonic oscillator for 30 minutes, withdrawn after extraction, and centrifuged at 14,000 rpm for 5 to 7 minutes. The pellet was re-extracted by sonication, in several cases up to 10 times, using fresh solvent at the rate of 5 ml per gram of dust.

In general, the dust was first extracted for a few times in acidified methanol by sonication, until most of the readily extractable pigments had been extracted. The pellet, obtained after centrifugation of the last extract, was dissolved in 1% conc. HCl aqueous (5 ml per gram dust), extracted in the sonic oscillator and centrifuged. The pellet was reextracted as above using 1% conc. HCl in methanol. Alternate extractions with the acidified aqueous and methanolic solvents were

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*The extraction solvent used previously (Mullick et al, 1958; Mullick, 1959) also contained 1% conc. HCl, and not 1% HCl. The error is regretted.

repeated as often as necessary. The aqueous supernatants, which contained some colloidal material were combined; the colloidal matter was removed as described in Section 6.3.4. The combined alcoholic supernatants were evaporated to near dryness in vacuo at 30°C, and the aqueous supernatant freed of colloidal matter was added. The combined extracts were reduced in volume in vacuo to about 10 ml, centrifuged, washed with ethyl acetate and benzene as usual (Mullick et al., 1958; see Sec. 5), and further concentrated in vacuo, and recentrifuged to obtain a highly concentrated extract for chromatography.

6.3.2. Detection of Proteins

The presence of proteins was detected by the standard A.O.A.C., qualitative tests, viz., biuret test and xanthoproteic test.

6.3.3. Examination of Aleurone Colors

The aleurone colors were always observed by peeling the outer coverings of the caryopsis (see Sec. 3).

6.3.4. Removal of Colloidal Material

The aqueous extracts of anthocyanins, obtained from the dust, contained colloidal materials which overloaded the chromatography paper. Several methods, such as precipitation with 4-10% trichloroacetic acid, butanol and i-pentanol extractions were tried to obtain colloid-free anthocyanin extract. The colloids, however, were removed in large

quantities by simple freezing and thawing. The aqueous extract was frozen at -15°C for, at least, 48 hours in a beaker. On undisturbed thawing, the colloids coalesced as a thin disc, and were removed by centrifugation.

6.3.5. Extraction from the Aleurone of Developing Grains

(a) Three hundred and fifty Black Hulless grains at hard dough stage from which the pericarp and spermoderm had been peeled but on which the aleurone envelope (cf., Sec. 3) and aleurone layer remained intact, were deposited, one by one, in 15-20 ml 1% conc. HCl in methanol, over a period of 2 to 3 days, in amber bottles as described elsewhere (Sec. 8.1.2). The extract was discarded. Five of the peeled grains were left for several months in the acidified methanol, in darkness, both at room environment and in a refrigerator (~5°C) for further observations. Attempts were also made to extract anthocyanins from the peeled grains by homogenization. A few of the extracted aleurones were homegenized in 1% conc. HCl aqueous. The homogenate was centrifuged, the supernatant was treated with 4%, and, also, 10% trichloroacetic acid, and centrifuged. The supernatant, as well as, the pellet after rehomogenization, was shaken separately with butanol and i-pentanol. The remaining peeled grains were pearled and the dust extracted as described in Sec. 6.3.1.d.

(b) Five hundred and seventy peeled grains with intact aleurones from the cross between 'Black Hulless' (♀), and '33-b1b1-13' (♂) were deposited, one by one, in an amber bottle, containing about 40 ml 0.1% HCl in methanol over a period of 10 days in the summer of 1962. The amber bottles were kept cold as described elsewhere (Sec. 8.1.2). On the basis of

certain observations, to be referred to later, made on this extract, the concentration of acid in the methanolic solvent was adjusted to one per cent hydrochloric acid.

The extract was filtered through glass wool, concentrated in vacuo at 30°C, to 8-10 ml, centrifuged, passed through celite column and eluted with 0.01% HCl in methanol. The eluate was again concentrated in vacuo, centrifuged and chromatographed. It is to be noted that the extract was only concentrated but never dried at any stage prior to chromatography.

(c) The dehulled kernels of Black Hulless around hard dough stage (representing Field Collections No. 272, 274, and 278: Section 19) were extracted in 1% conc. HCl in methanol on a large scale in an erlenmeyer flask. The erlenmeyer was placed in a freezer (-15°C) for about two months when the extract was decanted for identification work. Fresh solvent was added and the erlenmeyer remained in the freezer for another few months when the second extract was removed. A third extract was obtained after storage for another few months.

In another case, Black Hulless seeds at hard dough stage were extracted twice in August, 1959, as above. The acidified solvent was added to the extracted kernels which were left for storage in the freezer until May, 1965, when further observations were made.

6.3.6. The hydrolysis was carried out by the method described elsewhere (Sec. 8.1.4). The chromatography of anthocyanins was carried out in the BAW solvent, and of anthocyanidins in Forestal solvent on Whatman paper No.3 by banding (see Sec. 10.2.2).

6.4. OBSERVATIONS AND RESULTS

6.4.1. Observations on the Aleurone of Flinty Grains

(a) When the intact blue aleurones of 'Black Hulless,' obtained by peeling softened mature kernels (6.3.1.a), were placed in the acidified methanol, the blue color gradually became violet purple and, finally, bright rose-red within half an hour. Although, small amounts of anthocyanins diffused into the solvent, the bulk of the anthocyanin remained *in situ*, unextracted, over a period of three months. The *in situ* anthocyanins could not be extracted completely even by repeated extractions with fresh solvent. The pigment extract degraded in the course of volume reduction *in vacuo*.

When the blue aleurones were placed in acidified water, the color change from blue to red took a slightly longer period of time. The behaviour of the aleurone was, otherwise, similar to that in the acidified alcohol, except that the aqueous extract, as expected, showed turbidity owing to the diffusion of water soluble proteins from the aleurone (intact with endosperm). The aleurones on standing in the aqueous medium became soggy, but in the methanolic medium, they remained firm. An observation, which needs more substantiation, indicated that the extractable anthocyanins were more stable in the acidified aqueous than in the alcoholic medium.

When placed in methanol, the peeled grains with intact aleurone retained their original blue color for several weeks; the blue color faded gradually about three months.

(b) When the intact mature kernels of 'Black Hulless' are placed in acidified methanol (6.3.1.b), the anthocyanins are extracted slowly from the spermoderm and pericarp of the grain (cf., 6.4.2.c). However, when the kernels are extracted by shaking over a period of several days with frequent changes of solvent, the anthocyanins from the spermoderm and pericarp are, virtually, extracted completely.

A few further observations appear in order. The extraction from the intact kernels requires both large quantities of the acidic solvent and extended periods of laboratory processing. Both of these requirements are undesirable from the stability of anthocyanins (Sec. 23), hence, this method of extraction from the intact mature kernels (6.3.1.b) was discontinued.

When the Black Hulless kernels were added to the acidic solvent, the color of the pericarp and spermoderm was such a dense deep red, that the blue color of the aleurone could not be observed in a majority of the kernels, except by peeling or scraping the outer coverings. However, in a few kernels, the blue color of the aleurone was discernible, indicating thereby that color expression in the pericarp and spermoderm was not uniform. The behaviour of the blue color and its extraction characteristics, when the outer coverings are intact, will be presented later (6.4.2.c). When the intact mature kernels of the blue and black varieties are placed in the solvent, anthocyanins are not extracted. It will be recalled that small amounts of anthocyanins, however, are extracted from the peeled aleurones (6.4.1.a).

(c) Following early studies by Mullick et al., in 1958, it was ascertained (Sec. 4) that the non-purple varieties used in this study, do

not contain anthocyanins in the spermoderm and pericarp at maturity. Thus, the pearled dust (6.3.1.c), obtained from the mature grains of the three blue, and one black varieties, namely, Montcalm, Kwan, Trebi, and Gatami, contains blue anthocyanins from the aleurone only. It was observed, that despite repeated extractions of the pearlings from the blue and the black varieties with acidified ethanol or methanol by shaking (6.3.1.c), only a small quantity of extractable anthocyanin was obtained; the bulk of the anthocyanins remained in the pellet. The improvement in extraction with the acidified methanol was small.

The pearled dust, in contrast to the intact kernels, of 'Black Hulless' gave a far heavier yield of anthocyanins both in the acidified ethanol and acidified methanol. The extraction with the latter was somewhat better. It was noted that most of the color from the dust could be extracted by 4 to 5 repetitions. The pellet obtained after the extractions was, invariably, red. Repeated extractions removed only a small quantity of the color from the pellet. The extractability of the 'Black Hulless' pellet, is about the same as that of the pellets obtained from the blue and the black varieties. Since the color from the pericarp and spermoderm is extracted completely, it is clear that what goes in the extract are largely the anthocyanins of the pericarp and spermoderm and what remains in the pellet are the anthocyanins of the aleurone. The differential extractability of anthocyanins from the different caryopins tissues, and the localization of anthocyanins in the blue and black varieties exclusively in the aleurone were appreciated only after the development of peeling technique (Sec. 3).

(d) The poor extractability of anthocyanins from the non-purple varieties, and the long time taken for pigment extraction by shaking necessitated the extraction of the pearled dust by sonication (Mullick et al., 1958). Several interesting feature of extraction by sonication, especially, of the Black Hulless dust, which were briefly reported earlier (Mullick, 1959), will be elaborated in the light of total experience. The sonication improved the rate of extraction immensely; the number of days taken previously for extraction by shaking were reduced to about the same number of hours by sonication.

It was observed that most of the color from Black Hulless pearlings could be readily extracted in the first few extractions in acidified alcohol by sonication (6.3.1.d). The pellet obtained by centrifugation, when most of the extractable color had been removed, was always red. The reddish color from the pellet could not be extracted completely even after about twenty extractions by sonication using acidified methanol each time. The extractability of the dust of the non-purple varieties was greatly improved by sonication, but as with Black Hulless, the color could not be extracted completely from the pellet.

The reddish pellets of all varieties obtained from the acidified methanol were found to be rich in proteinaceous matter, because they were positive to biuret and xanthoproteic tests. The extraction of the reddish pellet with 1% HCl aqueous by sonication gave a colloidal supernatant containing weak anthocyanin color, and a reddish pellet. A relatively good yield of anthocyanins was obtained when the reddish pellet was re-extracted by sonication with acidified methanol (Mullick, 1959). Alternate extractions with the acidified aqueous and alcoholic solvents extracted the hard-to-extract anthocyanins from the pellet in good yield.

Nevertheless, a color-free pellet was neither obtained from the purple nor from the non-purple varieties despite extensive repetitions. The reddish pellet was still positive to the protein tests. When the colloidal supernatants, obtained from the aqueous extractions, were combined, they also gave positive biuret and xanthoproteic tests. Although several methods such as trichloroacetic acid precipitation, extraction with i-pentanol (6.3.4), to obtain protein-free anthocyanin extracts, were tried, the freezing and thawing of the aqueous extracts only was truly successful. When the combined aqueous and alcoholic extract, after volume reduction (6.3.1.d), was shaken with ethyl acetate, a slightly reddish coagulum was observed at the interface. This was removed by centrifugation, and the extract was further concentrated in vacuo, as described in Sec. 6.3.1.d, for chromatography. It is worth noting that only 4-5 applications of this extract caused overloading of the chromatography paper, probably, due to the presence of soluble proteinaceous matter. Satisfactory chromatographic resolution of anthocyanins could not be obtained. Since, the aqueous extractions were largely responsible for the proteinaceous matter in the extract, they were discontinued.

6.4.2. Observations on the Aleurone of Developing Grain

(a) In the few preliminary observations on the extraction of anthocyanin from aleurone described elsewhere (Mullick, 1959), it was noted that anthocyanins, from both the mature and developing aleurone, could not be extracted. Later on, in the course of color classification of the developing aleurone (Sec. 4), it was noted that anthocyanin color did diffuse from the young aleurones when they were placed in 1% HCl in

methanol. Accordingly, when the 350 peeled aleurones of Black Hulless were deposited in the solvent (6.3.5.a), one by one, the extraction of anthocyanins in the initial period of collection was observed. After the completion of peeling, which took a period of about a week, the amber bottle was stored in the freezer. A few days later, when the bottle was removed for processing, it was noted that the extract had decolorised. The aleurone, however, was still colored and contained anthocyanins in situ. On the basis of previous experience (Mullick, 1959), the decolorisation of the extract was relegated to the instability. The decolorised extract was discarded and attention was devoted to the extraction of anthocyanins still present in the aleurone.

A few of the extracted grains with intact aleurones, when placed in the acidified methanol, at room temperature and the refrigerator temperatures allowed only small amounts of the color to be extracted even after months of standing. The extraction by homogenization (6.3.5.a) was also unsuccessful. It may, however, be noted that when the centrifuged homogenates were shaken with i-pentanol, a slightly reddish coagulum was observed at interface. Owing to the limited availability of peeled aleurones, further studies on the interface coagulum were not undertaken. Instead, the remaining peeled grains were pearled. The pearling of very small quantity of materials is difficult, and therefore, only a small quantity of the pearled dust could be obtained. Although, some color was extracted from this dust by the alternate extractions with the aqueous and the alcoholic solvents on the sonic oscillator (Sec. 6.3.1.d); the pellet, as usual, could not be freed of the red color completely. The quantity of anthocyanin in the extract was too small and suffered breakdown upon volume reduction. Because of the difficulties of extraction from the

aleurone, no further attention was devoted to this tissue, until by chance, towards the final stages of this research program in 1962, a simple but revealing observation was made as described in the following section.

(b) It became necessary to reduce the concentration of HCl in the extraction solvent to 0.1% in subsequent studies (Sec. 23). The spermoderm and pericarp tissues of 570 seeds (6.3.5.b) of the hybrid Black Hulless x 33-b1b1-13 (studies on the hybrid stocks are not reported in the thesis), after peeling, were extracted in 0.1% methanolic HCl and the peeled aleurones were also deposited in about 40 ml of this solvent for the detection of _in situ_ anthocyanins for the purposes of genetic classification of aleurone color. (Details of this work could not be reported in the thesis). The aleurone extract certainly showed a good red color in the course of peeling which took a period of 10 days. Like the previous observations of 1959 (see 6.4.2.a), the extract, again, became colorless when examined after several days. Since the aleurone layer is believed to be alkaline and since the concentration of acid in the solvent was low, the aleurone could have neutralised the solvent. Indeed, upon acidification brilliant red anthocyanins reappeared. From then on the concentration of acid in the extraction solvent for the developing aleurones was increased to 1% HCl in methanol. The extract was concentrated _in vacuo_ and chromatographed. The results are shown in Figure 6.1; the R_f values (by ascent), relative concentration ratios, and visible colors of anthocyanins are given in Table 6.I. It was established from subsequent studies that aleurone anthocyanins are derived largely from delphinidin and petunidin (Sec. 22).

TABLE 6.I.
R_F VALUES, RELATIVE CONCENTRATION RATIOS[1], AND VISIBLE COLORS OF ALEURONE ANTHOCYANINS FROM A HYBRID BETWEEN BLACK HULLESS AND 33-b1b1-13 (GREENHOUSE COLLECTION 448)

Band No.	R_f x 100	Rel. Conc.	Visible Color
?	17	wk	Weak
1	20	15	Reddish purple to carmine
2	22	1	Weak
3	24	5	Bluish red
4	27	1	Bluish red
5	29	3	Bluish red
6	34	3	Bluish red

[1]Estimated values.

(c) Like the mature grain tissues, the anthocyanins from the spermoderm and pericarp of the developing Black Hulless intact grains (Collections No. 217, 270, 272, 274 and 278; Sec. 19) were extracted almost completely by the second extraction (6.3.5.c); however, the aleurone of the majority of kernels remained, distinctly, blue (note: blue in the acidic solvent). In another case, Black Hulless kernels, which after two extractions had been left in storage for six years showed colorless spermoderm and pericarp, but the majority of aleurones were still distinctly blue in the acidic solvent. However, in kernels, which showed mutilated embryos, the solvent had seeped through the coverings, apparently, in trace amounts, because the blue aleurone color had become reddish-violet or purplish but not red. Several aleurone layers were, also red, largely because of chipping or other injuries.

The chromatography of the first and the third extracts (6.3.5.c) from unpeeled Black Hulless caryopses (Collection 272) is shown in Fig. 6.2, chromatograms 272S-I and 272S-III, respectively. The first

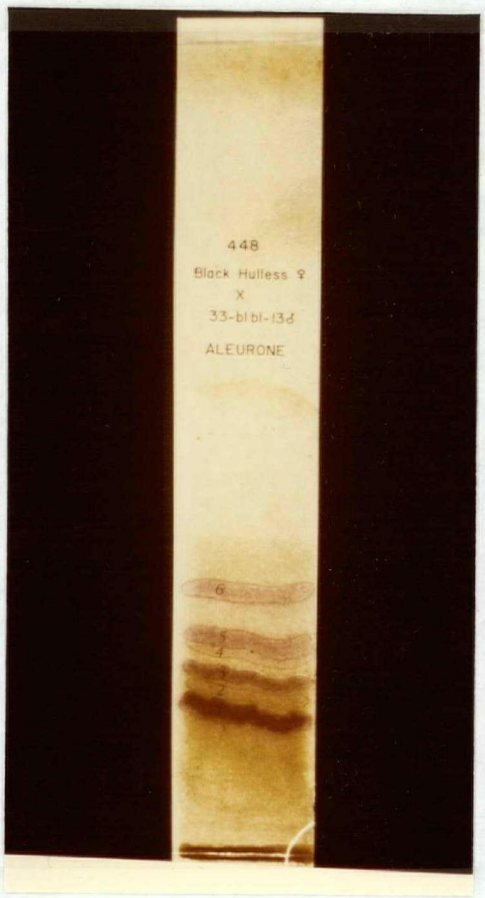


Fig. 6.1. Chromatogram of anthocyanins obtained from young aleurone tissues of the hybrid Black Hulless x 33-blbl-13. The tissues were peeled manually and extracted in 0.1% methanolic HCl. The extract, which became colorless on standing, was reacidified with 1% methanolic HCl prior to chromatography.

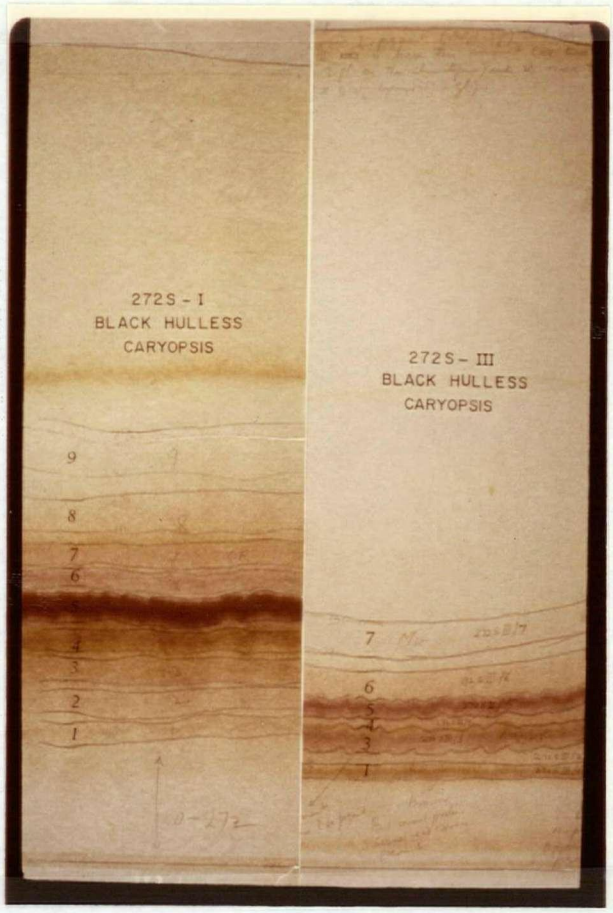


Fig. 6.2. Chromatography of the first and the third extracts from intact Black Hulless caryopses (field Collection 272). The pigments obtained from the first extract are largely of spermodermal origin, and those from the third extract are largely derived from chipped or damaged aleurone. Note the differences in R_f values of the two extracts.

extract contains at least nine anthocyanins most of which are highly complex as revealed by their identification characteristics described elsewhere in Section 19.3.7.3. The third extract shows fewer anthocyanins

than the first extract. Moreover, the R_f values of the anthocyanins are notably quite different from those of the anthocyanins of first extract. The R_f values, in fact, correspond to those of the aleurone anthocyanins shown both in Fig. 6.1 and elsewhere in Fig. 22.1.

It has been established on the basis of detailed characterization of each anthocyanin that the first extract of 1960 caryopses collections 217, 270, 272, 274 and 278 consists of cyanidin, peonidin, pelargonidin and delphinidin derivatives (Sec. 19). It will be recalled that delphinidin derivatives were not recovered from the 1958 maternal grain tissues, particularly, spermoderm (Sec. 5). Delphinidin was also absent from the 1959 maternal grain tissues, including spermoderm (Sec. 8).

A detailed characterization of the anthocyanins obtained from the third extract of Collection 272 was not undertaken. However, on the basis of direct hydrolysis on paper by the techniques described in Section 20, it was established that the anthocyanin band No. 3 (Fig. 6.2, chromatogram 272S-III) is a delphinidin derivative (Fig. 19.6). The band No. 3 of the chromatogram 272S-I (Fig. 6.2) is also a delphinidin derivative (Sec. 19.3.7.3). The relative concentration ratio of the delphinidin derivative is strikingly very weak on Chromatogram 272S-I as compared to Chromatogram 272S-III.

The results obtained from the hydrolysates of the first and the third extracts of collections 272 and 278, in Forestal solvent, are shown in Fig. 6.3, Chromatograms 272S-I, 272S-III, 278S-I, and 278S-III. On the basis of their R_f values in Forestal, the first two bands from the starting line have been identified as delphinidin and cyanidin. In the first extracts (Chromatograms 272S-I and 278S-I), cyanidin is present as a very strong band, and delphinidin a barely detectable band. In other words,

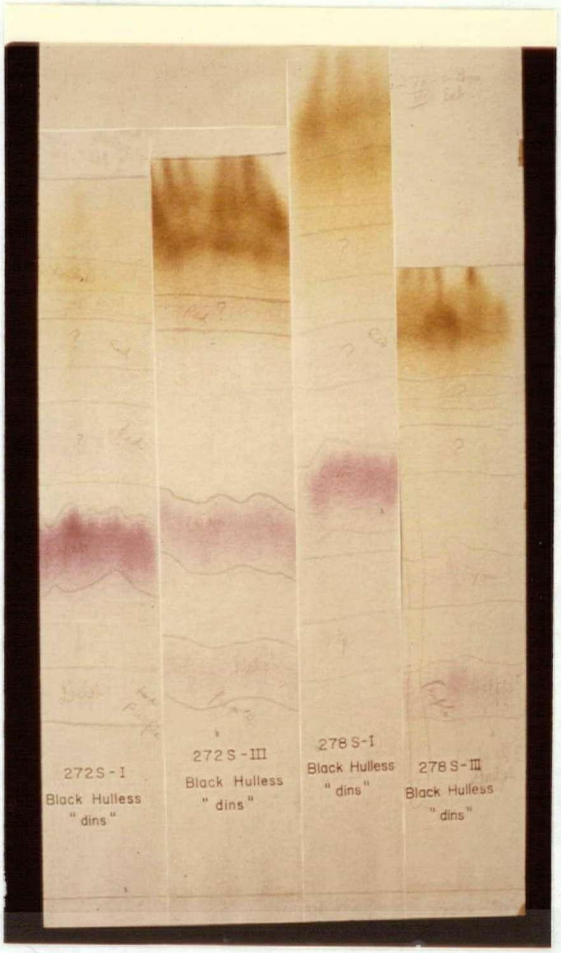


Fig. 6.3. Chromatograms obtained from the hydrolysates of the first and the third extracts of Black Hulless caryopses collections 272 and 278. Note, delphinidin is present in very small amounts in the first extracts (chromatograms 272S-I and 278S-I), and large amounts in the third extracts (chromatograms 272S-III and 278S-III).

the ratio of delphinidin to cyanidin is very low. In the third extracts (Chromatograms 272S-III and 278S-III), however, the ratio of delphinidin to cyanidin is very high; in fact, the delphinidin band is stronger than the cyanidin* band in chromatogram 278S-III. The results obtained from

*The second band from the hydrolysates of third extracts, chromatograms 272S-III and 278S-III probably is a mixture of cyanidin and petunidin (see Section 22).

hydrolysates of the first and the third extracts of Black Hulless caryopses Collections No. 270 and 274 were identical to those shown in Fig. 6.3.

The differences in anthocyanin patterns obtained from the first, and the third extracts also bring out the difficulties of working with the grain tissues.

6.5. DISCUSSION

6.5.1. The preparation of aleurone tissues by manual peeling is a laborious undertaking. The observations on several occasions had to be restricted to small samples. Moreover, it was difficult to make detailed and repeat observations in every case, because the developing aleurone tissues are available for observations only once a year for a short period. Nonetheless, it is clear from this study that the aleurone layer of barley contains two types of anthocyanins, (a) those that are soluble in the acidified water or methanol, and (b) those that are virtually unextractable, and are tissue bound. The soluble anthocyanins from the peeled aleurone of developing grains, in contrast to the mature grains, are readily extracted. The tissue bound anthocyanins of the developing, as well as, mature aleurones cannot be extracted by the usual means. That the aleurone possesses two types of anthocyanins is also supported by the studies on the pearlings of the blue and the black varieties which contain anthocyanins only in the aleurone. The pearlings, like the peeled aleurones, also, showed the presence of the two types of anthocyanins, inasmuch as, the pellet could not be freed of anthocyanins by extensive treatments ranging from sonic waves to protein precipitants. The behaviour of the pearlings of the purple variety, Black Hulless, following

the initial extractions to remove anthocyanins of spermoderm and pericarp is similar to the pearlings of varieties without anthocyanins in spermoderm and pericarp.

6.5.2. The only method by which the hard-to-extract anthocyanins could be extracted from the pellet was that of the alternate extraction with the aqueous and alcoholic solvents accompanied by sonication. Surprisingly, a good yield of anthocyanins was obtained when the reddish pellet, extracted with the aqueous solvent, was reextracted with the alcoholic solvent by sonication (6.4.1), but only a poor yield was obtained if the reddish pellets were not extracted first with the aqueous solvent. This behaviour of the pellet is intriguing and, undoubtedly, requires further attention. It is, however, clear that the aqueous solvent does remove some proteinaceous coagulum, and, also, some anthocyanins, and once the materials soluble in the aqueous solvent are removed, anthocyanins from the pellet are again extractable. Since the pellet, after the series of alternate extractions, still retains proteins and anthocyanins, it was postulated (Mullick, 1959) that it is the protein which is holding the anthocyanins in the pellet.

It is well known that phenolic hydroxyl groups of flavonoid compounds, such as, tannins, and leucoanthocyanins also, known as anthocyanogens) cause precipitation of gelatin and other proteins (see review by Freudenberg et al., 1962). It appears that the attachment of tannins to proteins, in vitro, occurs by adsorption. However, it is not clear, whether the anthocyanogen bound hordein fractions of barley (Pollock, Kirsop and Pool, 1959; Kloos, 1961; McFarlane and Sword 1962), are held to proteins, in vivo, by a chemical bond or merely by adsorptive forces.

Studies reported elsewhere indicate that a chemical linkage may be involved (Mullick, 1964a; 1964b). The facts that anthocyanins abound in phenolic hydroxyls, that the blue anthocyanins isolated from other plants, usually contain high molecular weight nitrogenous constituents (Bayer loc. cit.; Hayashi, loc cit.), and that the color of the aleurone anthocyanins is invariably blue, further support the postulate that the hard-to-extract anthocyanin fraction of the aleurone may, in fact, be protein bound or linked. Several other observations, which will be discussed subsequently, also support this view.

6.5.3. The extraction of the soluble anthocyanins from the mature aleurone was extremely slow, and that from the pearlings relatively fast. The differential rate of extraction of the soluble anthocyanins is, probably, influenced by two factors. First, the aleurone envelope (Sec. 3) may act as a barrier to the movement of solvents, for on exposure to the acidic solvents, the blue anthocyanins of aleurone change to red color faster in pearlings than in mature grains from which pericarp and spermoderm have been removed. The latter may generally take as long as half an hour for the gradual transformation from blue to the red state. Second, the slower extractability from the intact mature aleurone, as compared to the pearled dust, may, also, be due to the stereospecific arrangement of the extractable pigment molecules within the aleurone cells at maturity. It is to be reckoned that the aleurone layer is believed to be rich in lipoidal, glutinous and other proteinaceous matter (Collins, 1918; Mann and Harlan, 1915) which is laid down in aleurone cells towards maturity. It is, therefore, possible that rich deposits of the colloidal materials in the aleurone may prevent faster diffusion of anthocyanins towards the

later stages of maturity. It was earlier stated (6.5.2) that the superior yield of anthocyanins from the pellet previously extracted with the acidified aqueous solvent is poorly understood. However, it suggests that the release of anthocyanins from the pellet is blocked by proteins, which are soluble in acidified water but insoluble in acidified methanol; as soon as the acidified water-soluble proteins are removed, the anthocyanins can be extracted in the alcoholic solution. This observation, though circumstantial, supports the view that the extraction is influenced by stero-specific arrangements (also see below).

Other observations, yet, indicate that the extractability may not merely be influenced by physical barriers. For instance, the soluble anthocyanins from the young intact aleurones are readily extracted in high yield, but from the pearlings of mature aleurones (when most of the physical barriers are eliminated by pearling) they are extracted in low yield, even with sonication. This differential extractability with maturity, apparently, is due to fundamental physiological changes. During early stages of kernel maturation, the major part of anthocyanin complement in aleurone may exist in a 'free' state (soluble form), and only a minor part in a conjugated state. However, at maturity, it appears that most of the anthocyanins are conjugated with proteins. It is tempting to speculate that the conjugation may be a mechanism of inactivating some enzymes at maturity of the grain.

6.5.4. It is known that some of the outer coverings of barley caryopsis act as a semi-permeable membrane and allow only a limited exchange of water. Brown (1907; 1909) felt that the semipermeability involved the nucellar epidermis in a primary way (see Sec. 3), whereas Collins (1918)

maintained that the tegmen was primarily involved. Collins (ibid.) also established that little exchange of water occurred from the outer coverings, and that absorption of water mainly occurred through the sheaf of chalazal cells, which also acted as a semipermeable membrane. Brown (1907), however, made an excellent empirical observation which has not been given much attention. He noted that when undamaged grains of blue barley are steeped in a dilute acidic solution, although the contents of the grain become soft and swollen, the aleurone cells retain their original blue color at least for a week. However, the seeds, on extended steeping in a N sulphuric acid, or N hydrochloric acid become red within a period ranging from 7 to 24 days (Brown, 1909). Thus, the semi-permeability either of the coverings or of the absorption region (chalaza) is impaired after a week when the acid, along with water, seeps through. Our observations that the aleurones of the unpeeled developing kernels remained blue in methanol containing 1% conc. HCl, even after six years' storage, apart from supporting the earlier observations of Brown (loc. cit.) also indicates that the semi-permeability of the undamaged seeds in the acidified methanolic solutions is retained indefinitely. The observations on the differential extractability and the semi-permeability were later exploited in the development of a method for the color classification and biochemical studies of aleurone pigments (Sec. 21). It is because of the semi-permeability that in the early stages of this research program (Faris, 1956; Mullick, 1959), the anthocyanins from the intact kernels of varieties, other than the purples, could not be extracted, and pearling and eventually sonication had to be resorted to.

The observations in Sec. 6.4.1 and 6.4.2 also establish that since the aleurone of the intact seeds maintains its blue color in-

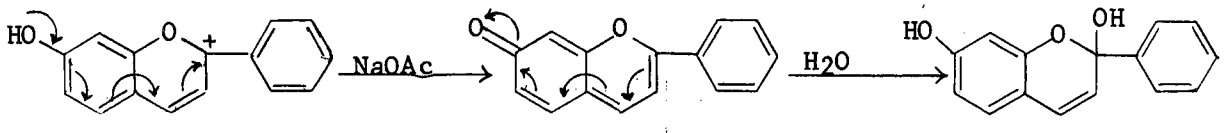
definitely under our conditions, the soluble pigments of aleurone cannot be extracted from undamaged kernels both during development and at maturity. It was, however, mentioned (6.4.2.c) that the young aleurone of the damaged seeds (unpeeled) of several collections, for instance, Nos. 272, 274, and 278, showed red to reddish-violet colors depending upon extent of the damage. The chromatography of the first extract of the collection No. 272 of the young unpeeled kernels showed only a small amount of the delphinidin derivative (Fig. 6.3, chromatograms 272S-I, and 278SI), however, the third extract, (Fig. 6.3, chromatogram 272S-III, and 278SIII), which did not contain the pigments of pericarp and spermoderm (6.4.1.c, and 6.4.2.c) showed only the preponderance of delphinidin. The concentration of the delphinidin derivative, as ascertained by the direct hydrolysis, was several fold (ca. 10x) greater in the third extract than the first extract. These observations clearly indicate the influence of the duration of extraction on the extractability of soluble anthocyanins, both qualitatively and quantitatively, from the grain tissues. Future work must take into account this differential extractability.

Since no delphinidin derivatives were recovered from the pericarp and spermoderm tissues of 'Black Hulless' (Sec. 5) at any stage of development (Sec. 8), the delphinidin must originate with the aleurone in the third extract of the collection No. 272 and 274. Although, the origin of the delphinidin derivatives could have been established conclusively by the direct hydrolysis of the aleurone extract of the cross between Black Hulless and 33-blbl-13 (Sec. 7.4.2.b), this could not be done owing to the small quantity of the extract. However, the colors of the lowermost band (Fig. 6.1) are similar to the delphinidin band No. 3 of the third extract (Fig. 6.2), an observation, which further supports the fact that the

delphinidin derivative of the third extract must have originated from the aleurone. Since the soluble anthocyanins cannot be extracted from undamaged seeds, it is concluded that the delphinidin derivative, and also other soluble anthocyanins of aleurone layer (Fig. 6.1), in the third extract must have diffused into the extract through the chipped and damaged developing seeds.

On the basis of the foregoing observations and those described elsewhere (Sec. 21), a method for extraction of pericarp-spermoderm and aleurone pigments separately without the intervention of peeling technique has been worked out; details of the method will be presented in Section 22.

6.5.5. The decolorization of the anthocyanins in acid extracts from developing aleurones, on standing in darkness (6.4.2.a), is a rather unusual phenomenon. The regeneration of the red color upon acidification indicates that the decolorization must have been brought about by the alkaline exudates from the aleurone layer. This is because, anthocyanins (I) under mildly alkaline conditions lose a proton, and give rise to colored anhydro bases (II) which upon standing, or dilution, form colorless



I II III

pseudo bases. The pseudo base formed in aqueous medium is called a carbinol (III), and in methanolic medium, used in this investigation, is called a methyl ether of carbinol or more conveniently, a methyl ether base. The colorless methyl ether bases upon acidification give rise to

the flavylium salt form (I) of anthocyanins (Wawzonek, 1951; Jurd, 1963). It was the appreciation of this fact, which finally laid the basis for extraction of soluble anthocyanins from the aleurone layer. Accordingly the anthocyanins from the aleurone were extracted with 1% HCl in methanol as against 0.1% HCl in methanol used for all other tissues in later studies (Sec. 23). It may be emphasized that the aleurone extracts were only concentrated *in vacuo* (6.3.5.b), but never *dried*. The drying of extracts, although recommended by others (Harborne, 1958) was not undertaken because barley anthocyanin extracts in 1% HCl in methanol suffered a great deal of degradation (Sec. 23). It is evident from the studies described in Sections 23, and 15 that barley anthocyanins are extremely labile in acid; standard extraction procedures are not satisfactory and the extraction solvent for aleurone anthocyanins, in view of the observations will have to be modified.

6.5.6. The chromatography of the aleurone extracts of the cross between Black Hulless, and 33-blbl-13 (Fig. 6.1) shows that aleurone possesses several anthocyanins which are different from those of the maternal tissues. Thus, the biochemical differences support the genetical findings that the anthocyanin development in aleurone, as compared to maternal tissues of the grain, are determined by different genes. The presence of anthocyanins in aleurone is determined by one gene pair, in certain cases by two gene pairs (see review by Nilan, 1964) and in others by the complementary interaction of two-gene pairs (see review by Smith, 1951). Undoubtedly, more biochemical work will have to be undertaken on the aleurone pigments of a large number of barley color variants to set the stage for combined biochemico-genetical studies on the mechanism of gene

action.

6.5.7. The differential extractability of anthocyanins from the spermoderm-pericarp tissues on one hand and the aleurone on the other, and the localization of anthocyanins in the blue, and the black varieties, exclusively, in the aleurone were not well recognized until after the development of peeling technique (Sec. 3). Thus, the two anthocyanins of the varieties, Montcalm, Kwan, Trebi and Gatami, which were previously considered to have been derived from pericarp and aleurone tissues (Mullick, Faris, Brink and Acheson, 1958), in fact, represent the blue anthocyanins of aleurone layer. It was also shown that of the two anthocyanins, the major anthocyanin was the delphinidin derivative and the minor a cyanidin derivative. These anthocyanins were obtained by aqueous and alcoholic extractions by sonication; the proteinaceous coagulum from the extracts was removed by freezing and thawing (Mullick et al., ibid; see also Sec. 6.3.4). Although the significance of these observations was not realized at first, only a few applications of the extracts overloaded the chromatography paper. It was the presence of proteinaceous matter in the extracts, as found in the latter studies (6.4.1.d), that must have been responsible for the overloading. This, at best, is circumstantial evidence that the proteins were present in the extract but it does not indicate that the proteins were bound to the anthocyanins. On the basis of their chromatographic behaviour, it appears, that the two anthocyanins are not the protein-bound but the soluble anthocyanins of aleurone. The soluble anthocyanins of the aleurone, most probably, exist free of proteinaceous matter. The future investigations must establish this point conclusively.

6.5.8. Although, the difficulties in extracting the blue anthocyanins from aleurone have been believed for some time to be due to binding or conjugation with tissue proteins, the establishment of the fact has been brought nearer by the observations included in Sec. 6.5.2 and 6.5.3, and by the recent isolation of blue anthocyanins bound with high molecular weight nitrogenous substances (Bayer, 1958, 1959, 1960; Hayashi, 1962). No effort has been made to isolate the protein bound anthocyanins; the fact that they are anthocyanins is supported by qualitative tests. The red aleurone tissues, from which extractable anthocyanins have been removed, on exposure to ammonia fumes turn from red to greenish blue to greenish yellow. In dilute sodium acetate and dilute sodium hydroxide solution, the red colors gradually become decolorised and yellowish colors appear in the aleurone. In all cases, however, when the kernels after the alkaline treatments are exposed to acid solutions, the red color is re-generated. These observations confirm that the unextractable red color that remains in the aleurone is due to anthocyanin pigments.

A discussion on the nature of the protein bound anthocyanins appears to be connected with the protein bound anthocyanogens (leuco-anthocyanins) of barley kernels. It is well known that both malt and barley contain leucoanthocyanins (Harris, 1956; Mullick et al., 1958). The leucoanthocyanins may exist in a free state as the delphinigens and cyanigen of Reynold, Atterton, Kirsop and Pool (1961), may exist as ethyl acetate soluble and insoluble anthocyanogens, may exist as hordein bound anthocyanogen (Pollock, Kirsop and Pool, 1959; Pollock and Pool, 1959; Kloos, 1961; McFarlane and Sword, 1962), may exist as albumin bound anth-ocyanogens (Meredith, 1963), and may exist as bound to true proteins, low molecular weight proteins, and to several other fractions which are

soluble and insoluble in several concentrations of ammonium sulfate (Mullick, 1964b). Thus, barley contains several kinds of anthocyanogens and these assume importance in this discussion because they are localized in aleurone layer (Sec. 9) as, indeed, are also the blue anthocyanins.

The question arises that following the repeated extraction of the soluble anthocyanins, is the unextractable red color that remains in the aleurone due to anthocyanins per se, or due to the transformation of leucoanthocyanins to the corresponding flavylium salt state. Since the leucoanthocyanins are transformed to the red flavylium state gradually even in weakly acidified alcoholic solutions (Robinson and Robinson, 1933), it is probable that in the course of extraction of the soluble anthocyanins, the leucoanthocyanins of aleurone could have turned red. Thus, the hard-to-extract red color, from this point of view, may not be due to anthocyanin bound proteins but leucoanthocyanin bound proteins.

It was shown in Section 4 and Appendix (Sec. 27) that grains of several varieties, with aleurone exposed by peeling, do not develop blue anthocyanins. However, when these aleurone tissues were placed in 1% conc. HCl in methanol, or in 1% conc. HCl aqueous, some showed gradual intensification of red color. A similar observation was made in the hood veins of the black variety, Kitchen (Appendix: Table 27.XX) during early stages of development, when the colorless veins on exposure to acidified alcohol developed red anthocyanin coloration (Sec. 4). However, the color intensification in the veins was faster than the aleurone. The slow development of red color both in the colorless and blue aleurones is likely to be influenced, as already discussed, by the barriers of the aleurone envelope, or the stereospecific arrangement of the pigment molecules within the aleurone cells. Once these barriers are ruptured, as in the pearled dust,

the red color in the dust may develop almost as fast as in the hood veins. The rapid transformation of colorless to the colored state, in weakly acidified alcoholic solutions, as is well established (Robinson and Robinson, loc. cit.), is not due to leucoanthocyanins but pseudo base transformations. Thus, it is clear that the rapid development of color, at least, in the hood veins of Kitchen is most likely to be due to the transformation of anthocyanin pseudo bases and certainly not due to leucoanthocyanins. The pseudo bases of anthocyanins, contrary to the existing belief, are very stable (Sec. 24; also see below). This, then, appears to be the first demonstration of the occurrence of anthocyanin pseudo base modification in nature. The significance of this observation in biogenetic and phytochrome studies is self evident.

On the bases of studies reported elsewhere (Mullick, in press), a few additions to the observations of the Robinsons (loc. cit.) on the rapid versus gradual transformations of the pseudo bases and leucoanthocyanins to their corresponding flavylium salts, upon acidification, may be made. It was found that although the pseudo bases of anthocyanins upon acidification transform to the flavylium state rapidly even upon extended standing for several days, that of the aglycones are transformed rapidly only if acidified within a few hours. Under appropriate conditions, it was further shown (Mullick, in press) that the transformation of the pseudo base of cyanidin occurs only gradually. The gradual regeneration of the tinctorial intensity of cyanidin from its pseudo base lends credence to the view that of the several kinds of leucoanthocyanins present in the aleurone, possibly some may be due to the pseudo base modifications. This possibility is further supported by the recent demonstration of the gradual transformation of chalcone-pseudo base

modifications of several flavylium salts lacking 3-substituent, to their corresponding flavylium state by Jurd and Geissman (1963). Although, the pseudo bases of anthocyanidins *in vitro* are unstable, it is possible that they may be stabilized *in vivo* by colloidal 'binding' or adsorption (presumably, due to their phenolic hydroxyls). Doubtlessly, this appears to be a desirable area for further investigations.

It is necessary to look into the nature of the blue aleurone color. Because the blue aleurone becomes red upon acidification, the blue color may be due to the anhydro base form directly due to the pH effect. However, the pH of the cell sap, as being the determinative factor for flower color variation, of late has lost its support, because of the extensive studies of Shibata, Hayashi and Isaka (1949), Bayer (1958), and others (see review by Hayashi, 1962). These studies indicate that the pH variation of 0.5 to 1.0 units from the average pH range 4-6 is too small to account for the color variation of anthocyanins *in vivo*. It is, however, the pH range 4-6, contrary to the earlier beliefs, that appears to be most important for the color transformations of anthocyanins (Jurd and Geissman, 1963). pH and time is importantly involved in such transformations. The only factor that may negate the proposition that the blue color of the aleurone is due to anhydro base transformations is the demonstrated instability of the anhydro bases (*cf.*, Jurd and Geissman, *loc. cit.*). Although, it will not be possible to record at this point, the writer's studies on the factors affecting the stability of anhydro bases (which, in part, were prompted in reference to the blue colors of aleurone), it may, nonetheless, be definitely stated that the instability of anhydro bases is due to artificial conditions in the *in vitro* environment: the writer has been able to keep anhydro bases stable for

weeks. In support of the widely held belief (review: Weibe and Reid, 1961) that aleurone is an alkaline tissue, the observation on the decolorization of anthocyanin extracts of aleurone on standing (Sec. 6.5.5) do indicate that the pH of the aleurone exudates is dufficiently high even to neutralise the acidic solvent and to cause pseudo base modifications of the flavylium state of anthocyanins. Thus, above all other reasons, the alkaline nature of the aleurone tissue reinforces the belief that the blue color of the aleurone may be only due to anydro base modifications.

On the basis of their extractability from the young and inextractability from the mature aleurones, it was suggested in Sec. 6.5.3 that the anthocyanin may become protein bound towards maturity. Thus, the anhydro base state of anthocyanins could be stabilized *in vivo* by colloidal adsorption as originally proposed by Robinson and Robinson (1931). The situation of the blue anthocyanins of aleurone towards maturity may be similar to the blue anthocyanins bound with nitrogenous materials isolated by Bayer (*loc. cit.*) and Hayashi et al. (*loc. cit.*). The nature of these anthocyanins, however, still remains sketchy.

It was noted from observations made elsewhere (Mullick, 1963; 1964a) that when the pearled dust of the blue varieties is extracted in neutral solvents such as methanol or acetone, the blue color of the aleurone is hardly visible in the dust. The color is much less likely to be seen in barley grist as commonly used in the isolation of protein bound anthocyanogens by the trade. It is also likely that the blue anthocyanins in the grist would have undergone pseudo base transformation in the presence of unacidified solvents generally used for the isolation. Thus, at least, part of the colorless anthocyanogens of barley, as described in the brewing and malting literature, may not be in the original sense

anthocyanogens but may be due to anthocyanin bound proteins, and also other pseudo base modifications as already discussed. Undoubtedly, the above discussion stresses the need for detailed investigations of the aleurone pigments.

6.6. SUMMARY

Detailed studies on the aleurone tissues are difficult to make because of the limitations of manual peeling of caryopses past hard dough stage, and because of the availability of developing aleurones only once a year for a short period of time.

The studies indicate that blue anthocyanins of aleurone are of two kinds: a) those that are soluble, and b) those that are tissue bound and most likely protein bound. During dough stage, a major part of the anthocyanins exists in the soluble or 'free' state, and only a minor part in the bound or conjugated state. The situation, however, is reversed at flint state maturity, when the bulk of the anthocyanins exist in the conjugated state. The blue anthocyanins are, therefore, physiologically important, and it is probable that their conjugation may be a mechanism for inactivation of some enzymes towards maturity.

Anthocyanins extracted from the aleurone tissues with 0.1% methanolic HCl become colorless on standing due to pseudo base modification. The modification confirms that the aleurone is an alkaline tissue and that the blue color of aleurone is due to the anhydro base state.

The chromatographic patterns of anthocyanins obtained from the maternal-paternal aleurone differ markedly from those of the maternal tissues, such as, spermoderm, awns, and hulls. The chromatographic

anthocyanin patterns from the intact seeds of Black Hulless, which contains anthocyanins both in the spermoderm and aleurone, differ markedly from those of the third extract from the same seeds. The observation indicates the difficulties of working with the grain tissues, and also suggests that the first extract contains anthocyanins largely from the outer investments of the caryopsis and the third extract largely from the aleurone tissues of chipped and unhealthy seeds. It is demonstrated that anthocyanins from the aleurone of healthy seeds (unpeeled) cannot be extracted even when the seeds remained in the acidified methanol for six years. These observations were exploited latterly in the development of a method for extraction from the separate layers of the caryopses without manual peeling (Sec. 22).

The studies indicate that the two anthocyanins of the varieties Montcalm, Kwan, Trebi and Gatami, which were obtained from the composited pearlings of the pericarp and aleurone tissues (Mullick et al., 1958), in reality, are the blue anthocyanins of aleurone.

The importance of the above observations in reference to beer chill haze is discussed briefly.

BIOCHEMICAL DIFFERENTIATION OF ANTHOCYANIN PHENOTYPES

7. THE ANTHOCYANINS OF THE BASAL LEAF SHEATHS, NODES AND AURICLES

Although the genetics of grain tissue anthocyanins has been extensively investigated, only cursory attention has been given the genetics of plant tissues (Smith, 1951; Nilan 1964). The lack of attention, presumably, is due to variability in color expression attributable to environmental influences (Aberg and Weibe, 1948). The anthocyanin chemistry of plant tissues, has not been investigated previously. The studies reported in this section were undertaken (a) to delineate qualitatively the anthocyanin patterns of different varieties and lines of barley, and (b) to compare at the biochemical level, patterns in plant and grain (excluding aleurone) tissues. The patterns obtained from the grain tissues of several color variants were reported previously (Mullick, Faris, Brink and Acheson, 1958).

The studies will also extend our understanding of the inter-relationship of anthocyanin production and of the molecular basis of anthocyanin potentiation by phytochrome, inasmuch as barley leaf sheaths have been used in the pioneering investigations of the phytochrome pigments.

7.1. MATERIALS AND METHODS

The barley stocks listed elsewhere (Table 2.I) were planted on May 15, 1958 at the University Farm. The stocks were not harvested in the Fall. Late in October, 1958, it was noticed that several varieties developed brilliant anthocyanins in plant tissues, particularly in the basal leaf sheaths. The following stocks namely, Lion (black)*, C-54-55 (a near white), Hanna and Golden Pheasant (whites), Montcalm, Kwan and Trebi (blues), and Black Hulless and Gopal (purples) were chosen for their red colors in the basal leaf sheaths. These two purple varieties were earlier employed for anthocyanin studies of the maternal grain tissues in August and September, 1958 (Sec. 5). The basal leaf sheaths of all the nine varieties, the auricles of Lion and C-54-55, and the nodes of C-54-55 only were used for the pigment analyses. It was shown in Section 4 that the nodes of C-54-55 develop anthocyanins in the region not covered by the sheath, and that upon removal of the sheath, anthocyanins develop in the region previously covered by the sheath. In this study, however, only the anthocyanin containing portions of the node were used. The basal leaf

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*Varieties are characterized by grain and head color, not by plant color.

sheaths, after harvesting, were washed with water and dried between paper towels. The washing, however, was discontinued because anthocyanins leached out during washing. Instead, the leaf sheaths, after harvesting, were cleaned of soil particles with a hard hair brush. The anthocyanin containing portions of the tissues were snipped off with stainless steel scissors and extracted in ethanol containing 1% conc. HCl at the rate of 1 gm tissue per 20 ml solvent. The tissues were extracted for 3-4 hours on a shaker. The extraction was repeated once and occasionally twice.

The procedures for processing the anthocyanin extracts, hydrolysis and chromatography have been described previously (Sec. 5.1). The optimum quantity of extract that showed the maximum number of anthocyanins, without interfering with the resolution, was determined empirically by depositing a series of concentrations of each extract. The analysis of the tissues reported in this section was commenced on October 31, 1958, and concluded on December 11, 1958.

7.2. RESULTS

7.2.1. First Chromatography of Plant Extracts

The distribution pattern of anthocyanins in basal leaf sheaths of the nine barley varieties, in auricles of Lion and C-54-55, and in nodes of C-54-55 is chromato-diagrammed in Fig. 7.1. Whenever necessary, the anthocyanins are numbered beginning with the spot nearest the starting line. The color of all anthocyanins was red with a slight bluish hue, except a few, marked with an asterisk, were either orange red or else their color was not clearcut. When treated with ferric chloride, anthocyanins,

in general, turned blue; those marked with an asterisk gave doubtful reactions.

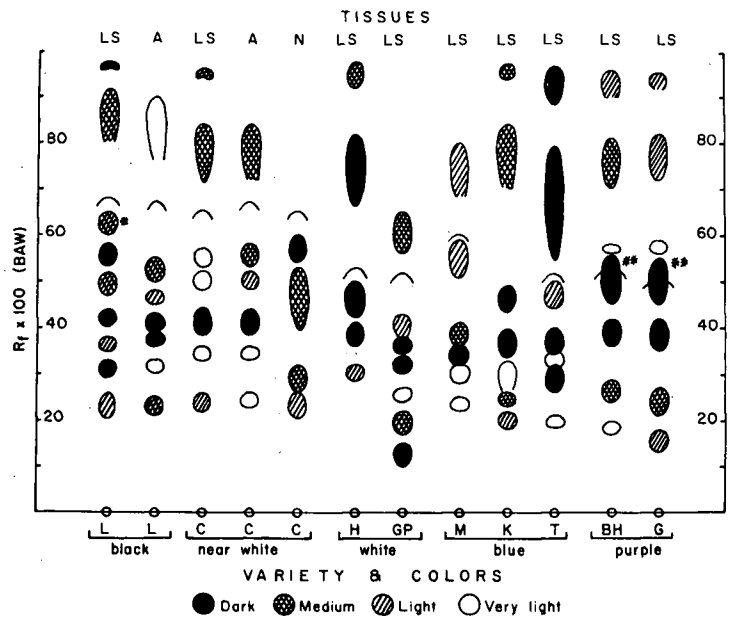


Fig. 7.1.
Anthocyanin patterns from leaf sheaths (LS), auricles (A) and nodes (N) of the black variety, Lion (L), the near-white variety C-54-55 (C), the white varieties Hanna (H) and Golden Pheasant (GP), the blue varieties, Montcalm (M), Kwan (K) and Trebi (T) and the purple varieties, Black Hulless (BH) and Gopal (G). Note the pattern similarity for BH and G; note the markedly different pattern of GP. Notable also are the anthocyanins with high R_f values, the "fast-moving" anthocyanins.

It was shown in Section 5 that grain tissues contain novel types of fast-moving anthocyanins, which are likely acylated, and that these acylated anthocyanins were detected only in the developing grain tissues and not in the pearlings obtained from mature grains. It will be noted from Fig. 7.1, that similar fast-moving acylated anthocyanins are present in all tissues and varieties with the notable exception of C-54-55 nodes and Golden Pheasant sheaths. The colors of the acylated anthocyanins are identical to those recorded in Table 5.I (Sec. 5). They are generally bluish red in the

course of chromatographic development, and turn blue within a few hours of removal from the chromatography tank; the reddish color, however, is restored, either automatically over a period of a few days, or immediately upon exposure to hydrochloric acid fumes. The anthocyanin spot No. 1, and in some cases No. 2, notably from Lion, C-54-55, Kwan, Gopal, and most probably, from all stocks and tissues, also undergo the characteristic transformations of color from red to blue to red upon standing, as do the acylated anthocyanins.

No identification work was undertaken in this pilot study. However, an attempt was made to relate the similarities and differences in the pattern from one variety or tissue to another. Each extract of the basal leaf sheaths was chromatogrammed separately, and also in mixture with each extract from each of the other varieties. Although several anthocyanin spots when run from mixtures, resolved at the same chromatographic loci, their chromatographic correspondence could not be concluded because the extracts showed considerable degradation upon repeat chromatography following a brief period of storage (see Sec. 7.2.2.). It was, however, established that the prominent spot No. 1 of Golden Pheasant, which characteristically turns dull blue upon standing, is different from all anthocyanins included in Fig. 7.1. Since R_f values by ascent were found to be variable some idea of the correspondence of one spot with another may be obtained in reference to the semilunar fluorescent spot (cf., Sec. 5.2). It may, however, be noted, that data in the chromatodiagrams of Fig. 7.1 were drawn from four separate chromatograms. The data of the white and the blue varieties were obtained from a single chromatogram, the data of the purple varieties from another chromatogram, that of Lion and C-54-55 leaf sheaths, and C-54-55 auricles from another chromatogram, and the data of

Lion auricles and C-54-55 nodes from still another chromatogram. The extracts, on the four chromatograms, were spotted as soon as they were processed.

The auricles and basal leaf sheaths of Lion and C-54-55 show similar anthocyanin distribution, except that Lion sheaths have a few additional spots. The anthocyanin distribution in nodes is noticeably different. The distribution in the white varieties is highly variable, but in the blues somewhat similar; Trebi, however, showed extremely strong acylated anthocyanin spots. The pattern of anthocyanin distribution in Gopal and Black Hulless, remarkably, is almost identical.

The hydrolyses of the anthocyanin extracts chromatogrammed in Fig. 7.1 yielded a major spot and a minor spot. The concentration ratio of the major and minor spot, as determined visually, was about 20:1 for almost all varieties. The major spot was identified as cyanidin; it R_f values on co-chromatography with synthetic cyanidin, both in mixture and separately on the same chromatogram were identical. The second spot did not resolve properly; it trailed above cyanidin to the R_f value of synthetic pelargonidin, which was run on the same sheet. The identity of this trail could not be concluded. The trail appears to be similar to that obtained from the grain tissues (cf., Sec. 5); it, probably, is a mixture consisting largely of peonidin and traces of pelargonidin. The suggestion is based on the observation that only Lion showed a distinct orange red spot marked with an asterisk (*) in Fig. 7.1; the presence of orange red anthocyanin in other extracts could not be established beyond doubt. It is significant that delphinidin was not present in any extract. Thus, the pattern of anthocyanidin distribution is very similar to that observed for the maternal tissues of developing grain (Sec. 5).

7.2.2. Changes in Anthocyanin Extracts in Storage

It was indicated earlier that when the chromatography to relate the similarities and differences in the biochemical phenotypes of different varieties (Fig. 7.1) was undertaken, the extracts showed considerable modifications. Primarily, because of the modifications, no valid chromatographic identities of anthocyanin spots between the varieties could be established. The results shown in Fig. 7.1 represented those of the chromatograms, which were spotted immediately after the extraction and processing had been completed. Before describing the results obtained when chromatography of the extracts, shown in Fig. 7.1, was repeated (Fig. 7.2), it is necessary to state the approximate time taken from extraction and processing to spotting of the anthocyanin extracts (Fig. 7.1). The information is included in Table 7.I. In column 4 of the table (First Chromatography) are included the dates on which the tissue extracts of Fig. 7.1 were chromatographed immediately after processing was completed. In column 5 (Subsequent Chromatography) is recorded the period of time, the appropriate extracts remained in storage in a dark cold room ($0^{o}C$) until rechromatography was undertaken (Fig. 7.2).

It is clear from Table 7.I that anthocyanin extraction, processing and chromatography shown in Fig. 7.1 was completed within two days at the maximum. The typical results of rechromatography of only a few of these extracts, after a period of storage specified in Table 7.I, are shown in Fig. 7.2. The Black Hulless basal leaf sheath extract, the processing of which was completed on November 12, 1958 (Table 7.I: Column 4) was chromatographed after storage of 0 day (Row I), 1 day (Row II), and 15 days (Row III) as shown in Fig. 7.2. It will be noted

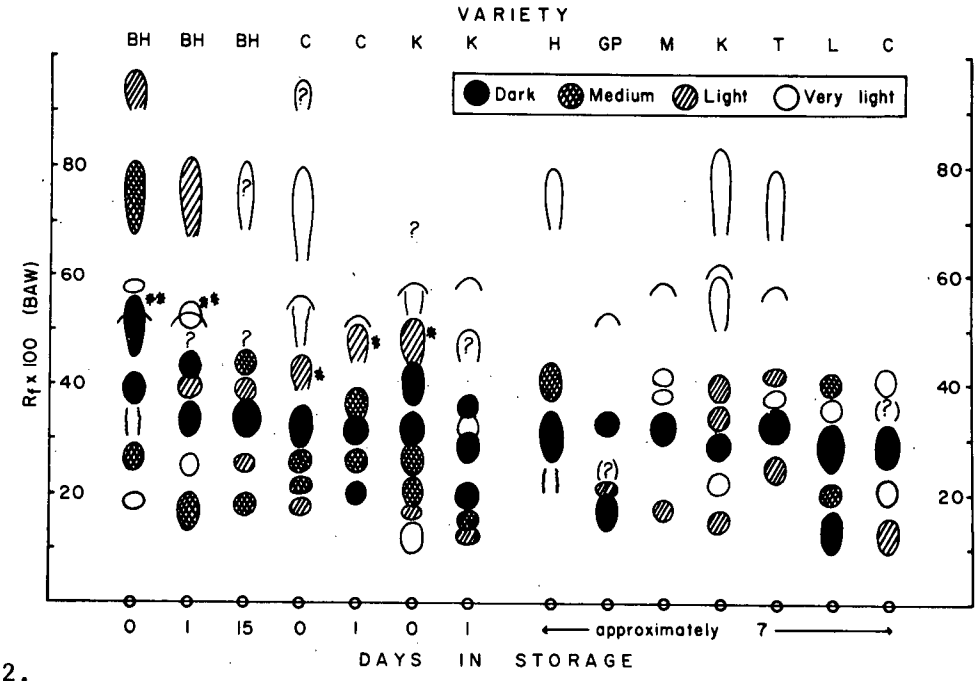


Fig. 7.2.
Anthocyanin behaviour in storage. Black Hulless (BH) I to III showing disappearance of fast-moving anthocyanins over a 15 day storage period. C-54-55(C) and Kwan (K) anthocyanin patterns (IV to VII) over a 24 hour storage period showing somewhat similar trends. Chromatopatterns VIII to XIV were obtained after 7 days storage of extracts whose day one chromatopatterns are shown in Fig. 7.1 where Hanna (H), Golden Pheasant (GP), Montcalm (M), Kwan (K), Trebi (T), Lion (L) and C-54-55(C) are the varieties used. Again the disappearance of certain anthocyanins in storage is to be noted.

that within one day of storage, the fastest moving anthocyanin (R_f 0.97) disappears, the major spot No. 4 (R_f 0.56) suffers a considerable degradation, and the relative concentration of the spot at R_f 0.81 decreases. Further, as these spots degrade, the concentration of the spot Nos. 1, 3, and 5 (Row II) increases greatly. Upon standing for another 15 days, the fast-moving anthocyanin (R_f 0.81) is scarcely detectable (Row III). It was observed from other chromatograms that spot No. 6 (Row II) disappears within three days of storage. The intensity, and number of anthocyanins below the fluorescent line, in Row III are almost similar to those of

TABLE 7.I
TIME TAKEN FOR EXTRACTION, PROCESSING, AND SPOTTING OF THE ANTHOCYANIN EXTRACTS PRIOR TO FIRST CHROMATOGRAPHY (FIG. 7.1) AND TIME THE EXTRACTS REMAINED IN STORAGE PRIOR TO SECOND CHROMATOGRAPHY (FIG. 7.2)

Variety	Tissue	Date of Extraction	First Chromatography	Subsequent Chromatography
Lion	Sheath*	21.10.1958	2.11.1958	After several days
Lion	Auricle	6.11.1958	7.11.1958	-
C-54-55	Sheath*	31.10.1958	2.11.1958	After several days
C-54-55	Auricle	31.10.1958	2.11.1958	-
C-54-55	Node	6.11.1958	7.11.1958	-
Hanna	Sheath*	18.11.1958	19.11.1958	
Golden Pheasant	Sheath*	18.11.158	19.11.1958	Dependable records
Montcalm	Sheath*	18.11.1958	19.11.1958	not available;
Kwan	Sheath*	18.11.1958	19.11.1958	chromatography
Trebi	Sheath*	18.11.1958	19.11.1958	repeated before end of Nov. 1958.
Black Hulless	Sheath*	10.11.1958	12.11.1958	After 1 day & after 15 days.
Gopal	Sheath*	10.11.1958	12.11.1958	After 1 day & after 3 days.
C-54-55	Sheath*	7.12.1958	8.12.1958	After 1 day.
Kwan	Sheath*	7.12.1958	8.12.1958	After 1 day.

*Basal leaf sheath.

Row II. It was observed in Fig. 7.1 that the anthocyanin patterns of the purple varieties, Black Hulless and Gopal are remarkably similar. The anthocyanin patterns of Black Hulless leaf sheaths shown in Row III (Fig. 7.2) is also similar to that of Gopal leaf sheaths obtained in 1960 (see Fig. 10.3). It will be noted from Table 7.I that Gopal basal leaf sheaths were extracted, processed and chromatographed at the same time as those of Black Hulless. The chromatography of the one- and three-day old Gopal extracts was similar to that of the similar Black Hulless

extracts and, therefore, has not been shown here. It is, however, notable that the chromatograms of Gopal, run after three days of storage, showed anthocyanin patterns parallel to those of Black Hulless run after 15 days of storage.

The results of the basal leaf sheath anthocyanin extracts of the white (Hanna, and Golden Pheasant), and the blue (Montcalm, Kwan and Trebi) color variants when chromatographed immediately after processing (November 19) are shown in Fig. 7.1. Repeat chromatography of these extracts after a few days' storage (see Table 7.I) gave the results shown in Fig. 7.2, Rows VIII, IX, X, XI and XII. On comparison of the results shown in Figs. 7.1 and 7.2, it will be noted that the fast-moving anthocyanins of Hanna disappear upon standing (Row VIII) and, seemingly, do not give rise to any new anthocyanin. Golden Pheasant (Row IX) shows considerable degradative modifications. From Montcalm (Row X) and Kwan (Row XI), the fast moving anthocyanins disappear, the spot around R_f 0.33 emerges as a major spot, and the relative concentration of spot No. 1 in both cases increases slightly. In Trebi, the fast-moving anthocyanins, which were present in large quantity when the extract was first chromatographed (Fig. 7.1) almost disappear following the storage (Row XII: Fig 7.2); the concentration of the spot around R_f 0.33 increases appreciably, and, apparently, no other change in the slow-moving anthocyanins is noticeable.

The results of Lion (black), and C-54-55 (near white) basal leaf sheaths upon first chromatography are also shown in Fig. 7.1. The results of subsequent chromatography after several days (Table 7.I) are shown in Fig. 7.2, Rows XIII and XIV. On comparison, it will be noted that the fast-moving anthocyanins disappear from both the extracts. In Lion the

number of anthocyanin spots present below the fluorescent line (i.e., the number of slow-moving anthocyanins) undergo appreciable modifications during storage; the spots No. 1 and 3 finally emerge as major spots. In C-54-55, spot No. 3 emerges as the major spot (Row XIII).

The foregoing results clearly indicate that barley anthocyanins are highly labile upon storage. The results obtained from extracts of Black Hulless and Gopal show that the lability is a maximum in the first 24 hours of storage, and, presumably, also during extraction and processing. Because of the lability, the work was repeated on November 26, 1958 for basal leaf sheaths of Golden Pheasant, Black Hulless and Lion, and on November 29, 1958 for the basal leaf sheaths of all the nine varieties used in this study. The extraction, processing and chromatography of the extracts of November 26, 1958 was completed within a day, and that of the November 29, 1958 within two days. The chromatograms of most varieties either did not show, or else showed a weak concentration of the fast-moving anthocyanins. Since the results did not correspond with those of Fig. 7.1, the discrepancies were attributed to the breakdown of anthocyanins during processing and handling of the extracts.

The work was repeated on basal leaf sheaths of several varieties on December 7, 1958; the processing, and chromatography was completed within a day (Table 7.I). The results of only two varieties, C-54-55, Rows IV and V and Kwan, Rows VI and VII are shown in Fig. 7.2. The pattern and relative concentration of anthocyanins obtained in C-54-55 sheath extracts (Row IV) are notably different from those found in the November 2, 1958 chromatography (Table 7.1), which is shown in Fig.7.1. Upon repeat chromato-

graphy of the December 8 extract (Table 7.I) 24 hours later, the fast-moving anthocyanins disappear (Row V). The results of the December 8 extract of Kwan on the first chromatography (see Table 7.1) are shown in Fig. 7.2, Row VI. On comparison with Fig. 7.1, it is clear that the fast-moving anthocyanins disappear and several new slow-moving anthocyanins appear. Upon rechromatography of extract after 24 hours, the spots No. 6, 7, and 8 (Row VI), disappear and only five spots remain (Row VII).

7.2.3. Later Chromatography of Anthocyanins from Basal Leaf Sheaths

It became exceedingly difficult to ascribe the absence of the fast-moving anthocyanins in the November 26, 29 and December 7 extracts only to the lability, because the techniques of extraction and processing were comparable. Accordingly, the work was repeated on December 11, 1958 taking as little time for extraction and processing as possible. The extraction, processing, and chromatography was handled in semi-darkness and completed in a continuous operation lasting 20 hours; the results of Lion, Hanna, Golden Pheasant, and Trebi basal leaf sheaths, and C-54-55 auricles are chromato-diagrammed in Fig. 7.3. The figure also shows the results of Kwan transposed from Fig. 7.2. Additionally, the results of the second leaf sheath (from the base) of C-54-55, which showed fairly dead leaf blades and which was extracted and processed on December 7 and chromatographed within a day are also included in Fig. 7.3. The purple varieties, Gopal and Black Hulless, and the blue, Montcalm, were not analysed because they did not show sufficient pigment.

The colors of all anthocyanins shown in Fig. 7.3 were bluish red,

except those marked with an asterisk, which are not known with certainty. All spots, except those (*), turned blue with 0.05% ferric chloride; (*) spots gave doubtful reactions, except the spot No. 4 of Hanna, No. 7 of Kwan and No. 3 of Trebi. A portion of the anthocyanin of the latter spots remained red after the ferric chloride reaction. The lower most anthocyanin spots of all varieties showed the usual color transformations

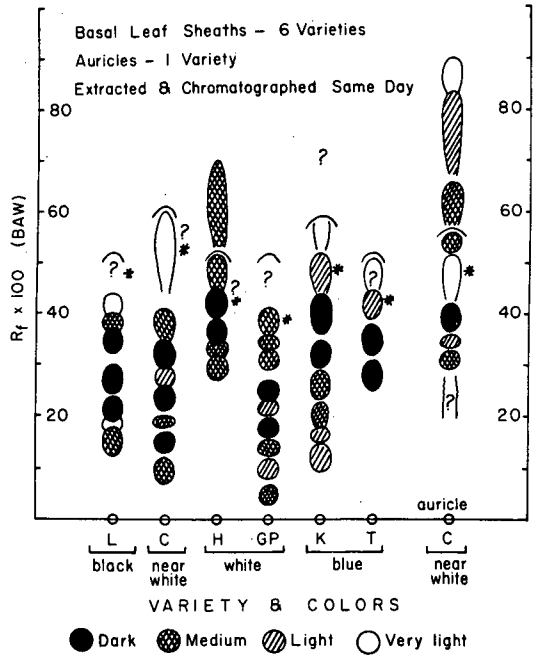


Fig. 7.3.
Patterns of anthocyanins extracted, purified and chromatographed in parallel as quickly as possible (20 hr. period). Fast-moving anthocyanins are obtained only from Hanna (H) leaf sheaths and from C-54-55 (C) auricles. The number of anthocyanins obtained from the aforementioned varieties and from Golden Pheasant (GP), Kwan (K), and Trebi (T) is greater than the number obtained from the corresponding varieties included in Fig. 7.1.

on chromatograms from red to blue to red upon standing without any treatment.

Several important differences are noticeable on comparison of the results of Figs. 7.1 and 7.3. In general, the fast-moving anthocyanins are absent (exception: C-54-55 auricles) from the December 11, 1958 collection. Since no identification work was undertaken, no extended effort to read between the differences shown by a particular variety from one stage of development (Fig. 7.1) to another stage of development (Fig. 7.2) has been made. Nonetheless, a general scrutiny of the two chromato-diagrams reveals several distinctive differences. For instance, the number of spots below the semilunar fluorescent line increases; Golden Pheasant, Kwan, and Hanna show a relatively larger number of spots thereby indicating the presence of additional anthocyanins. Also, the differences in the intensity of anthocyanin spots of the same extract below the fluorescent line as shown in Figs. 7.1 and 7.3 are notable. In general, when the fast-moving anthocyanin spots are present, the number, and intensity of the spots below the fluorescent line is less, but when they are not present, it is, significantly, more. The appearance of more anthocyanins in the same extract, both, in quantity and quality, upon the disappearance of the fast-moving anthocyanins must be due to modifications rather than the degradation of fast-moving anthocyanins (see discussion).

The anthocyanin extracts of Fig. 7.3 were left in the freezer (-5^{o}) until September 1960 by oversight. The extracts however, did not show any visual degradation and were hydrolysed. The period of hydrolysis was extended to 30 minutes. Lion and Kwan basal leaf sheaths and C-54-55 auricles, gave the same two spots, cyanidin, and peonidin or palargonidin trail, as recorded for the hydrolysates of Fig. 7.1. Golden Pheasant gave two distinct spots; the R_f values were identical to those of cyanidin and peonidin. The results of C-54-55, Hanna and Trebi basal leaf sheaths were

distinctly different; they gave only one major spot, a portion of which was purple (mauve under ultraviolet) with R_f values of petunidin, and another portion of which was magenta with R_f values of cyanidin. The visual concentration ratio of the petunidin and the superimposed cyanidin spot was approximately 1:1. Inasmuch as, the extracts were hydrolysed after an extended period of storage, and because the work was not repeated, the above results of hydrolysis are only tentative.

7.3. DISCUSSION

It was observed in the course of studies described in Section 4 that anthocyanin colors of basal leaf sheaths of barley color variants such as purples, blues, whites, near-whites and blacks are invariably reddish but they cannot be differentiated visually. From the comparative investigation on the qualitative distribution of leaf sheath anthocyanins in the color variants chromatodiagrammed in Fig. 7.1, it is clear nevertheless, that the colors of the variants are given by biochemically discrete pigments. Moreover some variants possess pigments which are missing in other variants. For example, Lion (black) shows a maximum number of slow-moving anthocyanins (R_f value below ca. 0.5 in BAW) and Hanna (white), a minimum number. Golden Pheasant (white), has pigments which characterize it alone. That the color possesses biochemically discrete differences is fully supported by the results chromatodiagrammed in Fig. 7.3. Thus, whereas the visual phenotypes of the color variants are undifferentiable, the biochemical phenotypes are readily differentiable.

The patterns from the two purple varieties, Gopal and Black Hulless, however, are identical, and ipso facto, the two variants should

have identical genetic background. That the patterns of the two purple varieties are similar does not imply that the patterns of all purple varieties are similar. For instance, the patterns obtained from the purple isoline, 71-Pr Pr-10, and the white isoline, 71-pr pr-10, in 1961 and 1962 (not reported in this thesis), although similar, differed in several respects from those of Gopal and Black Hulless.

Although, the variants studied show differences in patterns, all possess several anthocyanins in common. Only Golden Pheasant appears to possess a few anthocyanins, equivalents of which were not encountered in the other lines. The fact that some of the variants possess very similar anthocyanin chemistry and some do not, offers some intriguing possibilities for biogentic studies of anthocyanins. Extended to a chromatographic survey of world barleys such studies might be of value in delineating geographic and evolutionary regions of species, and sub-species and races within the genus.

It will be noted that the anthocyanin patterns from auricles and basal leaf sheaths of Lion are essentially similar, and those from C-54-55 almost identical (Fig. 7.1). It was shown in a previous investigation that the patterns from several maternal grain tissues were also chromatographically identical (Fig. 5.1). Although the patterns from Black Hulless grain tissues (Fig. 5.2) did show some differences, the differences are more apparent than real probably because of the developmental dynamism of the pigments (Sec. 8). The patterns of basal leaf sheath anthocyanins of Gopal and Black Hulless are not only similar, but they compare well with those of the corresponding grain tissues. It is well established that independent genes control the development of anthocyanins in different tissues of barley plant (reviews: Nilan, 1964;

Smith, 1951). The broad chromatographic identities of anthocyanins in different tissues imply that anthocyanin genes in all tissues are identical and they act in the same fashion, except that they act at different stages of plant development (because the expression of color in different tissues does not occur simultaneously). Thus, the temporal expression of anthocyanins appears to be controlled by 'time genes'.

In order to place the genetical overtones of this investigation on a sound footing, it is necessary that a detailed characterization of anthocyanins must be undertaken. To meet these objectives, it was intended, as a first step, to compare chromatographically the correspondences of anthocyanins of different color variants by cochromatography of their extracts in suitable mixtures. These objectives, as is clear from the results presented in Fig. 7.2, could not be attained because extensive anthocyanin modifications occurred in the extracts immediately following a brief period of storage.

It is clear from the results presented in Fig. 7.2 that extensive changes in chromatographic patterns of anthocyanins ('nins) occur immediately following the preparation of extracts. Following brief storage, the fast-moving anthocyanins (R_f 0.5 to 0.98 in all cases disappear. On the basis of other observations that were made in subsequent studies (not reported in this thesis) it may be added that the fast-moving 'nins in several instances were observed to disappear in the course of preparative chromatography in the BAW solvent. The observations in the course of preparative development were facilitated because the chromatography was carried out by banding rather than spotting. Frequently, less than a quarter length of the fast-moving 'nin band showed anthocyanin coloration, while the rest of the band was colorless. Precise reasons

for their disappearance in the course of chromatographic development are not known. Inasmuch as the nature of the fast-moving anthocyanins has not been investigated so far except the partial investigation of anthocyanins K and L of the greenhouse collections 407 and 408 (Sec. 19.3.7.2), a few comments on their nature appear appropriate.

It was noted from the rechromatography of the stored extracts (Fig. 7.2), that as the fast-moving 'nins disappear, conspicuous changes occur in the number and concentration of the slow-moving 'nins. In particular, the concentration of the spot around R_f 0.30 to 0.35 increases invariably. The increase in the concentration suggests that at least some of the fast-moving 'nins do not degrade but are modified to the slow-moving 'nin spot, R_f 0.30 to 0.35. The spot is equivalent to the anthocyanin DE-400s, and has been identified as a complex mixture of cyanidin derivatives in studies described elsewhere in Section 19.3.7.2. The identification of the spot strongly supports the earlier suggestion (Sec. 5.3) that some of the fast-moving anthocyanins are cyanidin derivatives.

It was observed in a previous study (Sec. 5) that as the fast-moving anthocyanins of Black Hulless and Gopal grain tissues disappear, apart from the spot, R_f 0.30 to 0.35, the concentration of the lowermost spot, also, increases. The results of Black Hulless leaf sheath anthocyanins shown in Fig. 7.2 (Rows I and II) and also those of Gopal leaf sheaths support the above observation. However, the results of Hanna and Trebi (Fig. 7.2: Rows VIII and XII) suggest that upon disappearance, the fast-moving anthocyanins do not give rise to the lower-most spot, but only the spot around R_f 0.30-0.35. The lowermost spot in Black Hulless (e.g., Fig. 7.2: Rows I and II) and, also in Gopal leaf sheaths, therefore, could have arisen from the transformation of the anthocyanin spot at R_f 0.56.

Doubtlessly, the transformation process is more complex (see Sec. 19), than is revealed by the above deductions and a detailed study is warranted.

The fast-moving 'nins in the BAW solvent resolve at the anthocyanidin region. It was, because of their greater mobility in the organic phase, postulated that the fast-moving 'nins are acylated with a novel type of organic residue(s), which promote their solubility in the mobile organic phase of the solvent (see Sec. 5). The chromatographic characteristics of the fast-moving anthocyanins presented in Fig. 7.1 further support the postulate.

The results in Fig. 7.2 show that during storage of the concentrated extracts in darkness, the fast-moving anthocyanins are far more labile than the slow-moving anthocyanins. The differential lability, therefore, is not induced by light. Since the concentrated extracts, other factors being equal, contain greater concentrations of acid (cf., Secs. 15 and 23), than the non-concentrated extracts, the lability appears to be induced by acid. The results presented elsewhere (Sec. 23) strengthen the view because a marked increase in acid concentration occurs following flash evaporation. It is interesting that the lability to acid supports acylation, and the acylation supports the lability to acid. Since the ester linkage of acyl components are labile to acids, it is likely that novel kind of organic residues of the fast moving components are directly esterified to the anthocyanin molecule. The esterification of the barley anthocyanins does not appear to be through the sugar molecule, as has been postulated by Harborne (1960) for the acylated anthocyanins of potato. This is because, Harborne's postulate (that the acylation was through the sugar molecule) is based on the observation that the acyl linkage of potato anthocyanins is stable to acid.

It is clear from the results presented and from other chromatographic observations made in the course of this tudy that several changes in the number and concentration of the slow moving anthocyanins also occur within a brief period of storage: for instance, compare the results of Black Hulless sheaths Rows I, II, and III (Fig. 7.2). However, following the brief period of storage, the anthocyanin patterns, for example, those obtained in Row III (Fig. 7.2), undergo no apparent modification and are therefore reproducible on repeat chromatography. This reproducibility in patterns of the slow-moving anthocyanins was observed throughout the studies (see Secs 8, 10 and 19). This is, however, not to imply that degradation of anthocyanin extracts does not occur in storage; degradation does occur for the intensity of several spots is reduced and the anthocyanin spot around R_f 0.30-0.35 emerges as a major spot (see also Sec. 19.3.7.2). However, upon extended storage, some spots were found to disappear completely but not the major spot, R_f 0.33. Thus, the anthocyanins of barley have differential stability, some degrade quickly, others slowly, while still others remain stable for years if stored in a freezer (-15°C), and in darkness. The differential stability of barley anthocyanins will be further elaborated upon in Sections 11, and 12.

It is also clear from the chromatographic comparison of Black Hulless leaf sheath extracts shown in Rows I, II and III (Fig. 7.2) and also from those of Gopal leaf sheath extracts that, of the fast-moving anthocyanins, the spot at R_f 0.97, and 0.56 are highly labile. The disappearance of the fastest-moving 'nin spot, R_f 0.97, following a period of brief storage, is also observed consistently in the hulls, awns, and spermoderm extracts (Sec. 5). However, the major fast-moving 'nin spot (R_f values 0.8 to 0.9; Fig. 7.1) is stable for several days under the

conditions employed. It is, primarily, on the observation that fast-moving 'nins are detectable only over a period of brief storage that the anthocyanin extraction, processing and chromatography of the December 11 extracts shown in Fig. 7.3 was carried out in a 20-hour continuous operation. Thus, the presence of the fast-moving 'nins in the November 2 extracts (Fig. 7.1) and their absence from the December 11 extracts (Fig. 7.3) seems to be due to the differences in the physiological state of the plants, rather than to the lability. The observations (a) that the patterns obtained following storage (Fig. 7.2) are quite different from those obtained without storage (Fig. 7.3), both, in the number and relative concentration of anthocyanins, and, (b) that the patterns of fast-moving 'nins of C-54-55 auricles obtained from the December 11 chromatography (Fig. 7.3) are different from those observed in the November 2 chromatography (Fig. 7.1) support the view that the differences observed in Fig. 7.1 and Fig. 7.3 are due to physiological differences. The results of hydrolysis tend to strengthen the view further because petunidin was recovered only from the December 11, 1958 extracts and not from the November 2, 1958 extracts. It is notable that Bockian et al.,(1958), Nutsubidze et al., (1959), Albach et al., (1959) have shown the existence of a developmental sequence of anthocyanins and anthocyanidins in grape and also the occurrence of considerable qualitative and quantitative variations of anthocyanins from year to year and from location to location.

The studies on the grain tissues in Section 5 (hulls, spermoderm and awns) indicate that fast-moving anthocyanins appear during development and disappear at maturity. The foregoing results and discussion indicate that a similar dynamism of pigment appearance and disappearance exists for the plant tissues.

It is clear that, biochemically, the anthocyanins vary both within and between genotypes. The variability makes desirable the delineation of distinct biochemical phenotypes. The developmental dynamism complicates the delineation of a distinct phenotype. It will be shown in subsequent investigations that the pelargonidin derivative(s), G-130, G-138 (Sec. 13), which was recovered from the field grown leaf sheaths was absent from the greenhouse grown sheaths (Sec. 19). Thus environments influence the expressivity of genes for anthocyanin. The tentative identification of petunidin from the December 11, 1958 extracts indicates that the occurrence of petunidin may be due to winter conditions in the field. Thus, both differences in environment and development may complicate the delineation of a distinct biochemical phenotype. How the petunidin or pelargonidin 'genes' could remain dormant specifically, while the rest of the anthocyanin gene mileau remained penetrant is a challenging area for future work.

The conclusions from this investigation are, namely (a) that the leaf sheath colors of the several lines are not differentiable visually but they are differentiable biochemically (b) that leaf sheath color cannot be treated as a monofactorial mendelian entity (c) that the biochemical mutants in nature can be differentiated by chromatographic survey. It may be assumed the number of 'anthocyanin genes' in different maternal tissues of the one individual must be identical; however, the anthocyanins are active in metabolism and deaclyation may occur towards later stages of development. Therefore, color, although on the surface is static, is developmentally dynamic. Although, the conclusions appear to be sound, it appears highly desirable that the results should be re-appraised by the milder techniques to be described in subsequent sections of

the thesis.

7.4. SUMMARY

The basal leaf sheaths, when grown under winter field conditions, showed deep anthocyanin coloration. The leaf sheath, node, and auricle anthocyanins from black, near-white, white, blue and black variants, which were extracted on November 2 and December 11, were analysed chromatographically by standard methods. The purple variants, Gopal and Black Hulless, show similar chromatographic patterns. The patterns, however, differ between the other variants. The basal leaf sheath color of the variants is so homogeneous that it cannot be differentiated visually. Thus, whereas the visual phenotypes of the color variants are undifferentiable, the biochemical phenotypes are differentiable. The anthocyanin color in barley is therefore a complex genetic character.

Although several variants show differences in anthocyanin patterns, they seem to possess several anthocyanins in common. A world survey of the differences in anthocyanin chemistry of the color variants offer intriguing possibilities in biogenetic and evolutionary studies.

The similarities of chromatographic patterns obtained from basal leaf sheaths, auricles and other maternal tissues of the same plant indicate a priori that the anthocyanin genes in different maternal tissues are similar and that they act in an identical fashion. However, the differences in the temporal expressivity of anthocyanins in different tissues indicate that the genes act at different stages of plant development. Thus, the temporal expresivity of anthocyanins appears to be

controlled by 'time' genes.

The November 2 sheaths from all variants showed the novel fast-moving anthocyanins in patterns identical to those recorded earlier for the grain tissues (see Sec. 5). It is shown that the fast-moving anthocyanins are highly unstable in storage. Accordingly, the December 11 sheaths were processed and chromatographed within 20 hours, but the fast-moving anthocyanins were not recovered. Thus, either the developmental dynamism, or the instability or both complicate the delineation of a distinct biochemical phenotype.

BIOCHEMICAL DIFFERENTIATION OF ANTHOCYANIN PHENOTYPES

8. PRELIMINARY STUDIES OF THE DYNAMICS OF ANTHOCYANINS IN AWNS, HULLS, PERICARP AND SPERMODERM

This investigation was undertaken in the summer of 1959 to confirm and extend the field observations on the dynamics of pigment development (Sec. 4), and to determine if there were, indeed, 'ontogenetic' development of anthocyanins in the grain tissues, namely, awn tip, hull, pericarp, and spermoderm, the indications for which were first obtained from the 1958 study (Sec. 5).

At the time these studies were initiated, little was known of the stepwise development of anthocyanins. Subsequently, several papers have appeared on the developmental sequence of anthocyanins in grape (Bockian et al., 1955; Albach et al., 1959; Nutsubidze et al, 1959; and Colagrande et al., 1960).

8.1. MATERIALS AND METHODS

The two purple varieties, Black Hulless and Gopal, and a blue isogenic line 36-B1 B1-21, planted on the University Farm April 24, 1959, were used in this study. The analysis was conducted on awn tip, lemma and palea (hulls), pericarp, and spermoderm.

8.1.1 Grading Plant Material by Stages of Development

Progression in anthocyanin development was delineated as follows:

Stage I: when anthocyanins just make their appearance in a tissue
Stage II: when anthocyanins show further intensification
Stage III: when anthocyanins show maximum development, and
Stage IV: when anthocyanins show gradual disappearance.

The different layers of caryopsis (Fig. 4.1) were separated by manual peeling (Sec. 3). The 'ontogeny' of anthocyanins in the different layers of the caryopsis does not occur simultaneously (cf., Sec. 4). Since pericarp pigmentation is absent or weak, and spermoderm pigmentation, comparably strong and, since peeling of these tissues is laborious, collection stage was determined by the degree of pigmentation in the spermoderm. Since spermoderm is the first caryopsis tissue to develop anthocyanins, when spermoderm stage I is collected, pericarp stage I is colorless.

Whenever the color gradation could be easily seen in hull or caryopsis, material was collected at a large number of stages. In order to maintain the designation of the main stages of collection, the intergrades or sub-collections were numbered IIIa, IIIb, etc., or IVa, IVb, etc., as the case may be.

The awn tip collections were made by reference to the development of pigment in hulls. However, since the color in awn tips develops first,

the earliest stage of collection of awn tips was already late in terms of development and well past the maximum stage of coloring for this organ.

8.1.2. Extraction

The tissues were extracted in methanol containing 1% conc. HCl not in the acidified ethenol as used in studies under Sections 5 and 7. The extraction was carried out in amber bottles, kept cold in beakers filled with ice during the period of tissue collection by manual peeling. Whenever the peeling operation was suspended or completed, the amber bottles were transferred to a refrigerator at -15°C for extraction and storage. The deep freezing apparently breaks the cell walls, thus aiding the extraction. The collection of different tissues took a period of about three weeks. The amber bottles containing the extracts remained in the refrigerator ready for use.

In general, slightly larger quantities of tissues at stage III than of tissues at other stages were collected for purification and identification work. In a few specified cases, anthocyanins were also extracted with methanol-conc. HCl (97:3) (Harborne, 1958) for comparative purposes.

8.1.3. Processing Prior to Chromatography

The processing of the acidified methanolic extracts of the grain tissues had to be modified for practical reasons (see under discussion). No special extraction techniques were needed, because extraction proceeded in storage. The extracts, following filtration through glass wool, were concentrated to one-third volume *in vacuo* at 30°C in semi-dark conditions, and centrifuged at 10,000 rpm for 30 minutes. The supernatant was placed in the freezer. In storage, chlorophyll along with other

non-anthocyanin contaminants precipitated, particularly from the extracts derived from tissues where anthocyanins were beginning to develop; from tissue extracts, where anthocyanins were past maximum development, large quantities of yellow and brownish pigments also precipitated. The extracts were purified by celite filtration and high speed centrifugation. Further volume reduction *in vacuo*, with intermittent exposure to cold, and high speed centrifugation were undertaken as often as necessary. This eliminated most of the non-anthocyanin contaminants and gave a highly concentrated anthocyanin extract. Although the quantification of these almost contaminant-free anthocyanin extracts would have been useful, it was not undertaken because of unequal losses of anthocyanins during extraction and processing, and because the qualitative objectives of this study, viz., the determination of total number of anthocyanins present at each stage of development could be realized by manipulation of chromatography procedure (Sec. 8.1.5).

8.1.4. Hydrolysis

Aliquots of the extracts were hydrolyzed for about 30 minutes as described in Section 5.1.

8.1.5. Chromatography

The chromatography was carried out by ascent at about 25°C (*cf.*, Sec. 5). Although the bulk of non-anthocyanin contaminants were eliminated in processing (Sec. 8.1.3), extracts representing earlier 'anabolic' stages, or later 'catabolic' stages in which the anthocyanins were relatively weak had to be applied in larger quantities on the chromatography paper. Generally, in such cases, trial runs were performed

to determine the limits set by overloading (of chromatography paper), and by the minimal amount required for detection of anthocyanins on paper. Once experience with respect to the maximum quantity of extracts that can be applied without sacrificing resolution is gained, chromatography becomes straight forward. Although this procedure is not quantitative, in practice dependable 'ontogenetic' comparisons were obtained.

8.1.6. Elution and Purification of Anthocyanins

A few tissues of the purple varieities were extracted on a fairly large scale for purification and identification of their anthocyanins. The extracts, after obtaining R_f values, by an aforementioned procedure (Sec. 8.1.5) were banded on Whatman paper No. 3, and chromatographed by descent in the BAW solvent, using "RSCO" chromatocabinets. The solvent fronts were run off as far as possible to obtain clear separations. The bands were cut out, eluted with water-methanol-acetic acid (25:70:5;v/v)(Harborne, 1958), and the extracts were concentrated *in vacuo* at 30°C, and rechromatographed in the BAW solvent; the solvent front was again run off the sheet for maximum resolution. The bands were again eluted, and eluates concentrated, as described above, for repurification in an aqueous solvent. The eluates, whenever it became necessary (for chromatographic considerations) following volume evaporation *in vacuo*, were diluted with, or dissolved in methanol containing 1% conc. HCl.

8.1.7 Chromatography, Elution and Purification of Anthocyanidins

The anthocyanin extracts were hydrolyzed and chromatographed in Forestal solvent as described elsewhere (Sec. 5). The elution and purification was attempted by the procedure of Harborne (1958). The hydrolysates

were banded on Whatman paper No. 3, developed in Forestal, eluted with the anthocyanin eluent and concentrated *in vacuo* for rechromatography as described in Sec. 8.1.6.

8.1.8. Spot Test

Ferric chloride spot test was applied as described in Sec. 5.1.4.

8.2. RESULTS

8.2.1. Anthocyanins of the Purple Varieties

Some of the data on anthocyanin development in various grain tissues of Black Hulless and Gopal are given in Figures 8.1 and 8.2. All spots up to R_f 0.44 turn blue with ferric chloride. The spots above R_f 0.44 do not give clear cut reactions; most of the red color disappears without turning blue. The extracts marked with an asterisk were extracted with Harborne's solvent (*loc. cit.*).

Lemma and Palea (Hulls)

The results of four successive stages of pigment 'ontogeny' in Black Hulless hulls are shown in Fig. 8.1. The stage I collection was made when the lemma showed a slight anthocyanin coloring near the awn base; it shows only two anthocyanins and a distinct pale yellow unresolved streak. It is apparent from the intensity of spots that the stage I anthocyanins could not be concentrated sufficiently in the extract owing to the presence of non-anthocyanin contaminants. An additional anthocyanin appears at stage II, and the pale yellow streak persists. By the time color is at a maximum (stage III), several additional anthocyanins appear;

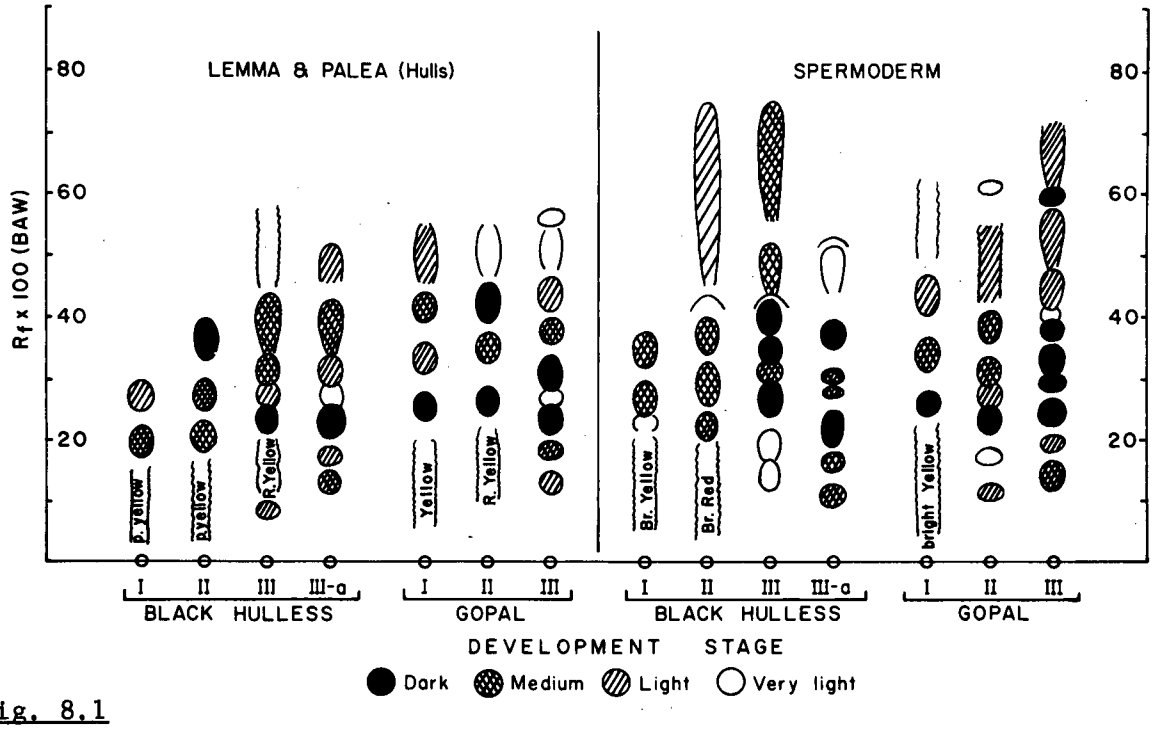


Fig. 8.1
Chromatograms of anthocyanins at different stages of development of lemma and palea and of spermoderm. Varieties used were Black Hulless and Gopal. Note the low intensity and paucity of anthocyanins in the early stages; note also the occurrence of yellow and red materials of low R_f values during stages I and II.

the pale yellow streak around spot No. 1 disappears and becomes reddish yellow in the region of spot No. 2. A few days later when the color was still near the maximum stage, another collection marked stage IIIa* (Fig. 8.1) was made using Harborne's solvent. In the region of the reddish yellow trail (stage III), a distinct anthocyanin spot is detectable; the distribution pattern does not change much except that spots No. 4 and 5 became weak in relation to the intensity of spot No. 3.

The developmental patterns of Gopal hulls, stage I, II and III (Fig. 8.1), are broadly similar to the patterns for Black Hulless hulls.

The presence of a distinctly yellowish streak at Stage I in the region of spot No. 1 and 2 is notable.

Spermoderm

The results of four successive stages of anthocyanin development in Black Hulless spermoderm are shown in Fig. 8.1. The spermoderm is chlorophyllous until soft dough stage (Secs. 3,4 and Appendix). Later, as the chlorophyll disappears, slight development of anthocyanin and yellowish pigments occur in restricted areas (stage I). At this stage only two, or possibly three anthocyanins, and a light brownish streak is present. At stage II, distinctive changes occur; the brownish streak (stage I) becomes reddish brown, and an unresolved fast-moving-anthocyanin streak above the fluorescent line appears. At the maximum stage of color development (stage III), the brownish streak increases in intensity and two weak anthocyanins are clearly discernible. Apart from the appearance of an additional anthocyanin spot below the fluorescent line, the fast-moving component (stage II) splits into two well resolved fast-moving anthocyanins. Another collection (stage IIIa*) was made a few days later using Harborne's solvent (97 parts methanol: 3 parts conc. HCl). This collection does not show the fast-moving anthocyanin; the changes in the relative intensity of several anthocyanins are notable. Spermoderm cannot be readily peeled past hard dough stage and hence the chromatography of later stages was not attempted.

The developmental patterns of the spermoderm of the purple variety Gopal at stages I, II and III (Fig. 8.1) are similar to the spermoderm patterns of the purple variety, Black Hulless. The anthocyanins of stage III show some differences but even these are probably simply

explained. For example, the trailing spot of Gopal near the solvent front, probably, is an equivalent of the fast-moving anthocyanin spot of Black Hulless stage II and III. However, spot No. 10 (Fig. 8.1: R_f 0.61) of spermoderm of Gopal at stage II and III does not appear to develop in the spermoderm of Black Hulless at any stage of development. Earlier studies on the two purple varieties, also, confirm the additional presence of the spot No. 10 in Gopal (Mullick et al., 1958; Fig. 1, pp. 449).

Pericarp

Temporally, the pigments in the different tissues of the caryopsis develop independently. In Black Hulless, at stage I of the color development in spermoderm (corresponding to soft dough stage), the pericarp is a colorless tissue (Sec. 4). Chromatography with strong concentrations of the extract also does not show anthocyanins at stage I. The second collection of the pericarp was made at stage II of the color development in spermoderm (corresponding to about mid dough stage). Although the pericarp visually was colorless, it did show a weak antho - cyanin spot (Fig. 8.2). The presence of anthocyanin, presumably, is due to diffusion: in the peeling process, fingers and thumbs become purplish due to diffusion of anthocyanins from the spermoderm. This purplish color is transferred to the pericarp during peeling. At stage III, pericarp can be peeled apart easily in a colorless state from about two-third of the kernel; the peeling near the apical end, however, becomes difficult and the peelings from this region show reddish color. The reddish color, yet to be confirmed by histological study is, probably, due to partially lacerated spermoderm adhering to the pericarp peelings. The pericarp at stage III shows three spots on chromatography (Fig. 8.2)

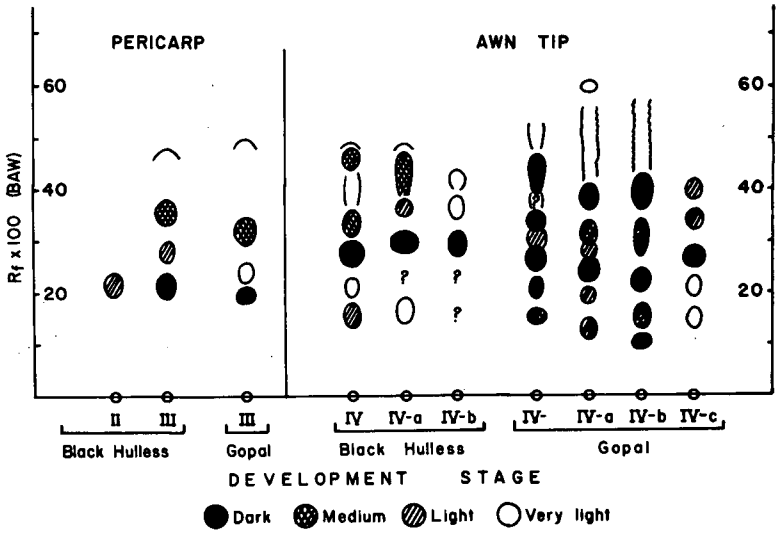


Fig. 8.2.
Chromatograms of anthocyanins at several stages of development of pericarp and awn tips. Varieties used were Black Hulless and Gopal. Note the decreasing number and lowered intensity of anthocyanins as the tissues progress towards maturity. The pigments shown as derived from pericarp, possibly are derived from lacerated spermoderm.

which are similar to those found in spermoderm.

Awn Tips

The awn tips of both the purple varieties develop anthocyanins as soon as they are exserted. The coloring following exsertion is so rapid that it attains maximum expression within a day or so. The awn tips could not be collected during this early period of color development. However, collections were made corresponding to the progressive stages of pigment development in lemma. Thus, the first awn tip collection was made at stage I of lemma. Since color development in awn tips is past the maximum by this time, and fading has set in, the first collection of awn tips might be said, in an ontogenetic sense, to be at stage IV of

anthocyanin development. The results of both the purple varieties are shown in Fig. 8.2. The varieties do not show fast-moving anthocyanins. The slowest moving anthocyanins are present only in traces.

8.2.2. Anthocyanidins of the Purple Varieties

Aliquots of the anthocyanin extracts shown in Fig. 8.1 and 8.2 were hydrolysed; the results are summarized in Table 8.I. The chromatography of the hydrolysates either gave cyanidin alone, or cyanidin and the usual trail above the cyanidin spot (cf., Sec. 5 and 7). In order to resolve the nature of this trail, the hydrolysates of different stages of the same tissue were mixed, chromatographed and eluted. The eluates, in the course of volume reduction in vacuo turned yellow. The nature of the trail above cyanidin, therefore, could not be resolved. It appears that the trail is either due to peonidin or a mixture of peonidin and pelargonidin (cf., Sec. 5 and 7).

The relative concentrations of the two anthocyanidin spots, estimated ocularly (Table 8.I), obtained from Black Hulless and Gopal, are comparable at the same stage of development. For instance, lemma of Black Hulless at stages I and II show the presence of cyanidin only. The second anthocyanin spot develops only at stage III. The results of Gopal are broadly similar. Although the determination of relative concentration ratio, at best, is only crude, nonetheless it does indicate probable trends; the concentration of the second spot increases towards maturity.

The spermoderm hydrolysates of both the varieties show the two anthocyanidin spots at all stages. The conspicuous shifts in concentration ratios with maturity are notable.

The hydrolysis of the anthocyanin-free extracts of pericarp

TABLE 8.I
THE RELATIVE DISTRIBUTION OF ANTHOCYANIDINS IN DIFFERENT TISSUES OF GOPAL AND BLACK HULLESS AT DIFFERENT STAGES

Tissue	Stage	Anthocyanidin Concentration*			
		Black Hulless		Gopal	
		Cya.	Peo. &/or Pelar.	Cya.	Peo. &/or Pelar.
Hulls	I	p		p	
	II	p	?	p	t
	III	1.0	0.1	1.0	0.2
	IIIa*	1.0	0.3		
Spermoderm	I	p	t	p	t
	II	1.0	0.1	1.0	0.4
	III	1.0	0.5	1.0	0.7
Pericarp	I	No leuco-anthocyanin present			
	III	1.0	0.1	1.0	0.4
Awn tips	IV	1.0	0.05	1.0	0.1
	IVa	1.0	0.05	1.0	0.1
	IVb	1.0	t	1.0	0.05

*estimated values.

p = present

t = traces

Cya. = cyanidin

Peo. = Peonidin

Pelar. = Pelargonidin

(stage I) of both the varieties did not reveal the presence of leuco-anthocyanins. The direct hydrolysis of the colorless pericarp tissues in 3N HCl (Bate-Smith, 1954), also did not reveal the presence of anthocyanins. The stage III collection, however, shows two anthocyanidin spots in both varieties.

The awn tips show the same two anthocyanidins. The relative concentration of the second spot gradually decreases with maturity.

8.2.3. Anthocyanins and Anthocyanidins of 36-B1 B1-21

Hulls

This line typical of so called blue varieties develops anthocyanins only in the veins of lemma; the interveinal region develops yellowish flavonoids (cf., Sec. 4 and Appendix I). The development of anthocyanins proceeds only after early soft dough stage. Only two collections were made: one for stage I, and the other for stage III of pigment development. Both the anthocyanin extracts contained a relative preponderance of non-anthocyanin contaminants which caused overloading of chromatography paper. The extracts were chromatographed in sufficient quantity on Whatman paper No. 3. They showed a major spot (R_f 0.26) and an extremely weak spot at R_f 0.40. The hydrolysis of the extracts gave cyanidin only. The direct hydrolysis of the lemmas, prior to anthocyanin development, did not yield evidence of leucoanthocyanins.

Pericarp

Pericarp does not develop anthocyanins at any stage of development and furthermore, direct hydrolysis did not yield evidence of leucoanthocyanins.

Spermoderm

The anthocyanins in spermoderm of this line develop only at the lateral slit not covered by lemma and palea; they are, notably, absent in pericarp even at the slit. The spermoderm anthocyanin development at the slit is ephemeral; anthocyanins appear at the end of soft dough stage and disappear during the hard dough stage. Only two antho-

cyanins were recovered in chromatograms during their ephemeral occurrence. These anthocyanins were qualitatively and quantitatively similar to those of the hull veins. Their hydrolysates contained cyanidin only.

Awn Tips

To the eye anthocyanin development in awn tips is similar to that described for the purple varieties. The maximum stage of pigment development could not be collected. Nonetheless, three collections were made: (a) two from the awn tips, the lemmas of which were used for the two collections of anthocyanin in the lemma veins, and (b) one from the awn tips from which anthocyanins disappeared at later stages of development. The chromatography of the two anthocyanin extracts showed the presence of only the major anthocyanin (R_f 0.26), and that of the hydrolysates only cyanidin. Hydrolysis of the anthocyanin-free extract did not show leucoanthocyanins.

8.2.4. Elution and Purification of Anthocyanins

The elution and purification of anthocyanins of the stage III extracts of the tissues included in Fig. 8.1, and also of Gopal basal leaf sheaths anthocyanins, posed similar problems when purification was attempted. Typical results will be illustrated with spermoderm stage III extracts of Gopal anthocyanins.

Although in this study, attention has solely been devoted to anthocyanins, it appears desirable to indicate that spermoderm extracts of different varieties, whether purple, blue, or yellow, show at least two well resolved, and a few unresolved brownish pigments below the the anthocyanin spot No. 4 (Fig. 8.1: Gopal spermoderm, stage III). A

photograph of these spots is shown elsewhere (Fig. 19.3). As noted by Mullick, Faris, Brink, and Acheson (1958), the brownish pigments considerably influence the resolution and also, the unambiguous detection of the anthocyanin bands present in that region. The resolution and R_f values particularly, of the anthocyanin spots No. 1 and 2 of the whole seed and spermoderm extracts are, therefore, not precise. With this background, the results of the spermoderm stage III extracts of Gopal will be described.

The R_f values of the anthocyanins of this extract are shown in Fig. 8.1. For the purposes of purification, the extract was banded on Whatman paper No. 3. The solvent front was run off to achieve greater separation of the bands. The fast-moving unresolved anthocyanin component (Fig. 8.1) was not recovered. Banding was found to be extremely superior to spotting inasmuch as it lent a greater measure of confidence in the detection of even weak anthocyanins. In the region of anthocyanin spot No. 1 (Fig. 8.1), in fact, two anthocyanin bands superimposed by the brownish pigments were detected. The brownish red eluates turned blackish upon volume evaporation to dryness in vacuo. The blackish material was quickly redissolved in the acidified methanol, it did turn slightly reddish, but upon chromatography, no anthocyanins were recovered. All the remaining anthocyanin eluates were chromatographed, simultaneously, by banding on Whatman paper No. 3 in the BAW solvent. The solvent fronts had to be run off in the interest of resolution. The relative resolution, or the relative distances travelled by the anthocyanin spots obtained from each eluate are shown in Fig. 8.3.

The visible colors are noted on the left and ultraviolet on the right of each spot (Fig. 8.3). Color differentiation is subjective;

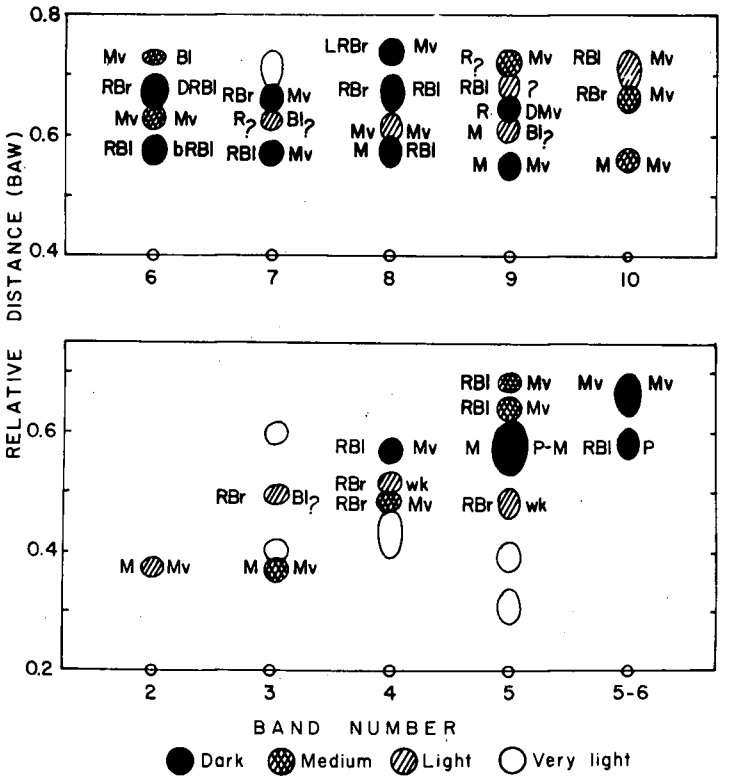


Fig. 8.3.
Standard chromatographic techniques yielded many anthocyanins from few on rechromatography of the spermoderm eluates. The *in vivo* complexity of barley anthocyanins, and their tendency to split *in vitro*, is indicated. The upper case letters associated with the chromatospots are color designations.

the color specification of each anthocyanin spot is, therefore, trying. Difficulties in color judgment arise due to quantitative variation of anthocyanins, and to superimposition of the yellowish copigments on the paper. Wide variations in hues and, occassionally, in basic color designations occur even in the Ridgway's, the Royal Horticultural Society's, and the Printers' color charts. All anthocyanin spots, basically, were red, but they showed distinctive variations in hue; the red were 'shaded' with blue, brown or orange. The hue of the basic red color of the anthocyanins has been denoted by additional words, e.g., RBl means that the spot is basically red but shows definite blueness and so on. Although this system of color classification may lack precision, it nonetheless

shows the differences between red spots in familiar terms.

It will be noted from Fig. 8.3 that several anthocyanin spots obtained from the rechromatography of each eluate differ from one another on the basis of color. The anthocyanin eluate of the spot No. 2 (Fig. 8.1) turned reddish yellow brown in the course of evaporation in vacuo, and gave a weak anthocyanin band; several yellowish brown bands were also present on the paper. The rechromatography of the other eluates was relatively straightforward, except that of eluates No. 9 and 10, which also showed a great deal of degradation in the course of their concentration in vacuo. The eluate marked '5-6' was obtained from the region between band No. 5 and 6; it was not detectable in chromatograms run by spotting (Fig. 8.1), and denotes the superiority of banding over spotting. It should be noted from Fig. 8.3 that some eluates split into two major components (see eluate No. 5-6 and, also No. 3, the latter, however, does show the presence of two anthocyanins in traces), while others split into several components. The split components resolve both above and below the major component. For instance, the spot No. 4 of eluate No. 5 (Fig. 8.3) is the equivalent of spot No. 5 of Fig. 8.1, and it shows two anthocyanins above and three below it in the same chromatographic solvent. It will be seen that a total of 37 anthocyanins were recovered from 10 eluates. Some spots must be identical to one another inasmuch as this was only the first purification and, therefore, does not indicate an equivalent diversity in vivo. Extracts were not available in sufficient quantity to undertake further purification and identification.

8.3. DISCUSSION

8.3.1. Preparation and Gradation of Developing Plant Material

Variations in the times of tiller production and variability in pigment expression attributable to climatic changes makes the determination of physiological age difficult.

The criterion used in this study for approximating the successive stages of pigment development is arbitrary but is quite dependable in a relative way. Thus if a large quantity of plant material is harvested at a time, the tissues showing different stages of color development can be assorted easily; an overlap between the two successive stages when the different stages of development are collected at one time is thus minimized. This procedure, however, required a large number of persons for manual peeling so that the harvested material could be processed quickly and autolysis minimized.

8.3.2. Extraction

In the course of investigations on the grain tissues (Mullick et al., 1958; Mullick, 1959), it was observed that methanol, being more polar, gave better extraction of anthocyanins than ethanol. It was also observed (Sec. 5 and 7) that anthocyanins showed variations in solubility in the aqueous, and the ethanolic solvents. Although the reasons for this variability are poorly understood, it was decided to use the solvent most commonly used by acknowledged authorities in the field, for instance Harborne (1958). Accordingly, ethanol was replaced by methanol, the latter was then consistently used throughout the course of investigations reported herein.

The collection of caryopsis tissues by peeling in quantities suitable for accurate weighing is a highly time consuming process. To minimize artefact production due to autolysis, the tissues following peeling were, therefore, immediately extracted in the acidified methanol. Thus, weighing was not undertaken. Inevitably, a compromise had to be struck between quantification on one hand and artefact production on the other.

8.3.3. Processing Prior to Chromatography

The chromatography of anthocyanin extracts representing different stages of anthocyanin ontogeny posed practical problems which necessitated a re-examination of the procedure for processing anthocyanin extracts prior to chromatography. The tissues in which anthocyanins are beginning to develop (stage I), as compared to the same tissue at maximum color development (stage III), abound in methanol soluble non-anthocyanin contaminants. Differences in the quantity of the non-anthocyanin contaminants in anthocyanin extracts at different stages of development created chromatographic problems; relatively fewer applications on the paper of the extract (standardized on the basis of tissue dry weight following preliminary extraction) of stage I as compared to stage III caused overloading of chromatography paper. Consequently, equal quantities of anthocyanins could not be deposited on the paper. In reality, quantification was not necessary for this pilot inquiry which basically sought to determine if, indeed, there were any developmental sequences of anthocyanins. What was important in this study was the chromatographic resolution of the pigments, and the determination of the number of anthocyanins present at each stage of development. Inasmuch

as the chromatographic resolution is influenced greatly by the purity of the compounds, the method of processing the anthocyanin extracts described in section 8.1.3 was undertaken to reduce contamination without loss of anthocyanin pigment.

8.3.4. Anthocyanins of the Purple Varieties

Although most of the concomitants were removed during processing, the stage I extracts could not be freed of all the contaminants. Although stage I tissues had to be extracted in larger quantities, as compared to the stage III tissues, the extracts obtained in no case gave as intense extracts. Further concentration and removal of contaminants from the stage I extracts was not attempted because the anthocyanins were observed to fade in the process of volume reduction and handling. Perhaps, the contaminants would have been minimized, had only the tissue regions containing anthocyanins been used for extraction, as was done for the tissues used in section 5. The spotting of stage I extracts, therefore, had to be undertaken very carefully. Several chromatograms with different quantities of stage I extracts had to be run to make sure of the number of anthocyanin spots present.

In all the tissues, excluding awn tips, the number of anthocyanin spots increases with the progressive stages of pigment ontogeny implying step-wise biogenesis of anthocyanins. At stage I_4 only two or at most three anthocyanin spots were present. The slow-moving anthocyanins were conspicuously absent; in the chromatogram region where on might expect them to be present, was found a clear-cut pale yellow streak, which did not show anthocyanin colors even in traces. In hulls, the pale yellow streak almost disappeared when the slow-moving anthocyanins

appear at stage III. The disappearance of the yellow streak, is suggestive but is not a strong argument in favour of the step-wise ontogeny, because there remains a possibility that most of the yellow components of the streak could have been eliminated in the processing of the extracts (cf., Sec. 8.1.3). However, it is unlikely that this could have happened in all cases.

The instability of the anthocyanin extracts in storage (fully appreciated only in the final stages of this research), may likewise cast doubts on the involvement of step-wise biogenesis. However, it was established in Section 7 that the slow-moving anthocyanins were relatively stable on extended storage, and certainly for a period of about three weeks, the extracts used in this investigation remained in storage. It was also found, as reported in Section 7 that several anthocyanins of barley were increasingly labile as the acid levels increased as the acidified alcoholic extracts of anthocyanins were concentrated *in vacuo*. It should be noted also that during the three-week storage period, the amber bottles remained in darkness in the refrigerator and that the extracts were chromatographed soon after volume reduction. The anthocyanins therefore remained in an environment of high acidity only for a short time. Inasmuch as the stage III extracts which were concentrated and chromatographed under similar conditions show distinctly the presence of slow-moving anthocyanins, the absence of the latter from stage I extracts does not appear to be a consequence of anthocyanin degradation.

Apart from the evidence presented above, the studies described elsewhere, for instance the absence of the slow-moving anthocyanins at stage I extracts of Gopal (Fig. 5.3), the deacylation of anthocyanins and the presence of several new anthocyanins toward maturity (Sec. 7)

support a dynamic view of anthocyanin development in barley tissues. The conclusion receives support from work with grape tissues and cultured petals of Impatiens balsamina. Bockian et al. (1958) found that the order of pigment development in maturing grapes was: malvidin diglucoside, malvidin monoglucoside, delphinidin glucoside, petunidin glucoside, and free malvidin. They suggested that the occurrence of free malvidin towards maturity was contrary to the hypothesis that in the developing tissues, anthocyanins are formed at the expense of anthocyanidins. The Russian workers Nutsubidze and Gulbani (1959) found that free anthocyanidins are formed during the period of rapid growth of the vegetative tissues of grape vines. The anthocyanins in the leaves are formed towards the end of growth period. The malvidin and peonidin glucosides appear first in greatest concentration and are followed by those of delphinidin and petunidin. Klein and Hagan (1961) reported the monoglycoside appeared before the diglycosides in Impatiens balsamina.

No free anthocyanidins were detected in this investigation (see Sec. 10.3, band I, and Sec. 23). It may be added that the results of anthocyanin hydrolysis also support the step-wise elaboration of anthocyanins.

The anthocyanin changes occurring in the separate tissues of caryopsis past early hard dough stage could not be investigated because peeling becomes difficult. It was, however, noted in Section 4 that anthocyanins in certain tissues, for instance, lemmas and awn tips were observed tofade under field conditions upon maturity. The results with awn tips suggest that even the disappearance of anthocyanins may be step-wise (Fig. 8.2): the anthocyanins which develop late may disappear early. In general, little is known of the catabolic pathways of

anthocyanins. The only work in the area is that of Paech and Eberhardt (1952) who showed that an anthocyanin 'decolorizing' enzyme, which was absent in young leaves of Cydonia maulei was active in older leaves. Biogenetical considerations necessitate that the catabolism of anthocyanins in awn tips must be explored further. Perhaps too much emphasis should not be placed on the catabolic breakdown of anthocyanins reported above because of the in vitro instability of anthocyanins to be dealt with in subsequent sections of the thesis (see Sec. 11, 12, 13, 15, 19, 23 and 24).

Although the anthocyanin patterns in all of the maternal grain tissues of Gopal were basically similar (Sec. 5 and Fig. 5.3), the patterns in the Black Hulless (Fig. 5.1) showed some variation from tissue to tissue especially with respect to the presence of slow-moving anthocyanins. The absence of these in certain tissues is, on the basis of the dynamical view, understandable, because the tissues were not always equivalent on the basis of color development.

It was concluded from earlier studies (Secs. 5 and 7) that the anthocyanin distribution in the several maternal tissues within and between the purple varieties, Black Hulless and Gopal, was broadly similar. However, comparison of Figs. 8.1 and 8.2 shows that the pattern in different tissues of Gopal and Black Hulless changes from stage to stage in time. Also, in the 1958 season Gopal tissues (Fig. 5.3) did not show as many anthocyanins as were found in 1959 (Figs. 8.1 and 8.2). This is expected if the stepwise elaboration of anthocyanins does occur; otherwise the variation is ascribable to the degradation of anthocyanins during handling.

The implications of the stepwise biogenesis of anthocyanins in studies on gene action, and intermediary metabolism are fundamental, and the issue cannot be determined hastily. Although the work on grape pigments

supports the barley findings, the results of the different grape workers are not in accord. For instance there is the unresolved point regarding the order of appearance of anthocyanidins. Again Bockian et al. (loc.cit.) found that malvidin diglucoside appeared before the monoglucoside, yet the current beliefs indicate that sugars are added step by step from mono- to di- to tri- glycoside state (Alston, 1964: p. 197). Durmishidze (1955) found that there was a considerable qualitative and quantitative variation in anthocyanins of several varieties of grape, yet Albach, Kepner and Dinsmoor (1959) analysed the anthocyanins of 125 varieties of grapes and found only quantitative variations. The work with barley to this point begins to point to the fact that current methods of extracting and studying anthocyanins result in considerable degradation. Firm decisions on the stepwise biogenesis would be contingent upon understanding the factors affecting anthocyanin stability. For this reason, this section has been titled 'Preliminary'.

8.3.5. A comparison of the results (Fig. 8.1) obtained when the tissues were extracted with MeOH - conc. HCl (99:1; v/v), and MeOH - conc. HCl (97:3; v/v; Harborne loc. cit.) is informative. Since the tissues used in the extractions did not represent the same physiological age, it can not be stated whether or not the disappearance of certain anthocyanins, when Harborne's solvent was used, is physiologically induced, or solvent induced (due to excess acid). However, the studies described in Section 23 indicate that the difference are likely to be due to the excess concentration of acid in Harborne's solvent.

8.3.6. The fastest moving anthocyanin component (R_f 0.97: see Sec. 5) was not detected in any extract; its absence, as expected, is due to the

extreme lability of the acylated organic residue (Sec. 7.3.7). The second fast-moving component (R_f value range 0.8 to 0.9: Sec. 5) was present only in the spermoderm tissues and gave R_f value of only 0.75 (Fig. 8.1). The high R_f value range found previously (Sec. 5) appears to be due to temperature variations. At higher temperature (30°C) used earlier, the sealing petrolatum melts and some solvent vapours escape thereby affecting the vapour equilibrium within the chromatography jar, and in turn the rate of movement of the solvent on the paper. This fast-moving component was absent from other tissues.

The work was repeated in 1960. The collection of tissues took a period of about three months. When the extracts were processed and chromatographed later, the fast-moving anthocyanins did not appear in most cases. Thus, the absence of fast-moving anthocyanins may not be due to physiological age but entirely due to time-induced degradation that ensues in storage. The absence in awn tips, however, could be due to physiological age. These facts were not appreciated clearly until the data included in Sections 5 and 7 were recompiled with reference to the time taken in extraction, processing and storage prior to the chromatography.

8.3.7. The Color Competence of Pericarp

The delineation of color competence of pericarp layer (cf., Sec. 3, 4, 5 and, also Tables 27.I to 27.XX) has been problematical. It was shown previously (Fig. 5.1) that pericarp contained the same number of anthocyanins as did the spermoderm. However in this study only a few anthocyanins were recovered. The reasons for this discrepancy were noted in the peeling process. Since the pericarp tissue begins developing

pigments (if at all) only around the hard dough stage, the stage at which the peeling of pericarp near the apical end becomes difficult, the anthocyanins of pericarp shown elsewhere in Fig. 5.1 are likely derived from the adhering spermoderm. The presence of a few anthocyanins recorded in Fig. 8.2 could have been due either to the transference of color by fingers in the peeling process, or to the minute adhering spermoderm tissues. It is, however, entirely possible that the color may develop inherently in the pericarp tissue around the hard dough stage. It is notable that although the spermoderm of 36-B1 B1-21 does develop anthocyanins at the slit, the pericarp remains absolutely colorless.

The difficulty of peeling pericarp near the apical end suggests that adposition of this tissue against spermoderm, occurring as a consequence of grain maturity, begins from the apical end.

8.3.8. These studies confirm the previous findings (Sec. 5) that delphinidin is absent from the maternal grain tissues. Also, no leucoanthocyanins were detected in the colorless stage of tissues, both when the tissues or their extracts were hydrolyzed directly.

8.3.9. The observations on the probable deacylation of anthocyanins during development, and the elaboration of new anthocyanins toward maturity (Sec. 5 and 7) indicated that anthocyanins are actively involved in intermediary metabolism. The ephemeral presence of anthocyanins in the spermoderm at the the lemma-palea slit (36-B1 B1-21) further supports the involvement of anthocyanins in metabolism. Since the region of the spermoderm covered by the lemma-palea does not develop anthocyanins, it is possible that genetic expressivity of anthocyanins is influenced by the wavelengths of light

filtered by lemma and palea.

8.3.10. It is genetically significant that only two anthocyanins, one a strong and the other very weak, were recovered from the lemma veins of 36-B1 B1-21. Inasmuch as the anthocyanin expression in veins is determined by an independent set of three dominant gene pairs (Buckley, 1930), it appears necessary that separate analysis of both the lemma veins and the interveinal region of lemma should have been done for Gopal and Black Hulless, which develop anthocyanins in both the tissues.

8.3.11. These studies have established that at least two brownish pigments of the slow-moving brown streak, observed originally in the combined tissues of mature caryopsis (Mullick et al., 1958) are derived from the spermoderm layer. The nature of these pigments remains to be determined.

8.3.12. Elution and Purification of Anthocyanins

The results shown in Fig. 8.3 have been included only to show the complexity of barley anthocyanins. It is well established that acylated anthocyanins split only in two components upon rechromatography in butanolic solvent owing to the ease of hydrolysis of their ester linkage (Harborne, 1959a, 1960). One of the split components is still acylated, while the other deacylates, and is, generally, polyglycosylated. The presence of more than two anthocyanin spots obtained from each anthocyanin band is suggestive of the greater complexity of barley anthocyanins. At first it was thought that the extra components were due to admixture from neighbouring anthocyanin bands. However, the work included elsewhere (Sec. 19) has clearly shown that the nature of barley anthocyanins is highly complex. The elution and purification work, indirectly, supports

the complexity of barley anthocyanins observed thus far. It is important to indicate that this type of complexity and anthocyanin 'splitting' was not noted by Metche and Urion (1961) and Urion and Metche (1961) in their studies on the anthocyanin pigments from the variety violet Guimalaye.

8.4. SUMMARY

This preliminary study on the ontogeny of anthocyanins in awns, hulls, and spermoderm of the purple varieties, Black Hulless and Gopal, by the usual techniques, suggests that there is a stepwise biogenesis of anthocyanins. The cyanidin derivatives are formed first, and then the peonidin and/or pelargonidin derivatives. The findings are in accord with the developmental dynamics of anthocyanins in grapes.

Importantly, however, the anthocyanin patterns obtained from this study (1959 season) differ from those of the studies included in Section 5 (1958 season), and from those of the composited pearlings of flinty grain tissues (Mullick et al., 1958). Although the discrepancies are explainable in terms of the developmental dynamics, investigations to this point began indicating the fact that the standard procedures for processing anthocyanins result in considerable anthocyanin breakdown. Inasmuch as the implications of the stepwise biogenesis in studies on gene action and intermediary metabolism are fundamental, firm decisions on stepwise biogenesis would be contingent upon understanding the factors affecting anthocyanin stability. This study, accordingly, is entitled 'preliminary'.

The anthocyanin competence of the pericarp tissue and the necessity for studying separately the anthocyanin patterns from lemma veins and lemma interveins is discussed.

Also reported are the first attempts at elution and purification of anthocyanins from the spermoderm. Each of the eluates from ten BAW anthocyanin bands, gave upon rechromatography in BAW, three to five anthocyanins. Thus the anthocyanins of barley are very complex. The causes of this complexity will be dealt with in Section 19.

ANTHOCYANINS & RELATED COMPOUNDS

9. DEVELOPMENTAL STUDIES ON LEUCOANTHOCYANINS AND RELATED POLYPHENOLS IN THE MATERNAL GRAIN TISSUES

9.1 INTRODUCTION

The chemico-genetical studies of Stephens (1948, 1949) on Asiatic cotton, of Stephens and Blakeslee (1948) on Rudbeckia hirta, of Seyffert (1955) on Impatiens balsamina, and of Coe 1955b) and recently of Reddy (1963, 1964) on the aleurone layer of maize show that genetics of anthocyanins and leucoanthocyanins is intimately related, and in several cases leucoanthocyanins may act as the direct precursors of anthocyanins. Several chemical studies, for instance, those of Simmonds (1954b) on Musa acuminata, of Pigman et al. (1953) on spruce and hemlock, of Paech and Eberhardt (1952) on red cabbage, of Stansbury et al. (1950) on the testa of peanut kernels, and of others have indicated close relationship

between anthocyanins and leucoanthocyanins. Yet there are other biochemical studies, for instance, those of Stephens (1951) on cotton, of Alston and Hagen (1958) on Impatiens balsamina, and of Feenstra (1960) on Phaseolus vulgaris, which show that leucoanthocyanin disappearance and anthocyanin appearance are not related.

Although leucoanthocyanins do occur in malt (Harris, 1956) and barley (Mullick, Faris, Brink and Acheson, 1958), their inheritance in barley has not been investigated. Thus to provide a basis for the leucoanthocyanin-anthocyanin precursor relationship in barley, an 'ontogenetic' study was undertaken to determine if leucoanthocyanins did develop in the maternal tissues of purple grains before the development of anthocyanins, or in the maternal tissues of anthocyanin-free stocks at any stage of development.

It was intended originally to undertake the study of flavonoids related to anthocyanins, and their C_6-C_3 precursors, but the work on the anthocyanin chemistry became so involved and prolonged that no detailed attention could be given these compounds. Nonetheless, in the course of the studies on the ontogeny of leucoanthocyanins, several polyphenolic compounds were detected on chromatographing the acidic hydrolysates of tissue extracts. The chromatographic distribution of these compounds in the separate tissues of the caryopsis at different stages of development of a few barley stocks will be presented in part. Although several workers, namely, Cook and Pollock (1954), Harris (1956), Seikel and Geissman (1957), Harris and Ricketts (1958), Sumere, Hilderson, and Massart (1958), Seikel and Bushnell (1959), Seikel, Bushnell and Birzgalis (1962), Reynolds, Atterton, Kirsop and Pool (1961), and Meredith (1961, 1963) have studied flavonoids and polyphenols of barley by

chromatography, the developmental features and, also the incidence of these compounds in the separate tissues of the caryopsis has not been investigated previously.

9.2 MATERIALS AND METHODS

9.2.1. Extraction

This investigation was carried out in the summer of 1959 using the same materials which were grown for the studies reported in Section 8. The varieties, Black Hulless and Gopal (purples), the iso-lines 36-B1 B1-21 (blue) and 33-b1 b1-13 (white), and Gatami (black), were chosen for this study. The analysis was conducted on awn tips, hulls, pericarp and spermoderm at different stages of development. The tissues of the purple varieties were collected prior to the development of anthocyanins. The tissues of the other three varieties, which, in general do not develop anthocyanins except in awn tips, were collected in reference to the physiological state of kernel maturation, viz.

stage I - soft dough
stage II - mid dough
stage III - hard dough

These stages roughly correspond to the successive stages of anthocyanin development described in Section 8.1.1. The tissues were extracted according to the method described in Section 8.1.2 using 1% conc. HCl in methanol. The extracts were processed prior to chromatography not by the method described in Section 8.1.3, but by the method described in Section 5.1.

9.2.2. Purification

9.2.2. Hydrolysis

The procedure for the hydrolysis of the tissue extracts was the same as that described in Section 5.1, except that the period of hydrolysis was extended to half an hour.

In a large number of cases, the tissues containing no anthocyanins were hydrolysed directly in 3N HCl by the method of Bate-Smith (1954). Further processing was carried out as described in Section 5.1.

9.2.3. Chromatography

The chromatography was carried out in Forestal solvent as per conditions described in Section 8.1.5, except that the extracts were banded on Whatman paper No. 1, with several concentrations. The concentration giving the best resolution and detectability of the spots or bands was used. Spotting did not give clear-cut results. The chromatobands, as usual, were examined in visible and ultraviolet light without treatment, after exposing to ammonia fumes and after treating with 1% aluminum chloride in ethenol (Geissman, 1955).

9.3. RESULTS

The unextracted awn tips, hulls, pericarp, and spermoderm tissues of all varieties and isolines, at different stages of development, and their acidified methanolic extracts were hydrolysed. The hydrolysates of the anthocyanin-free tissues and their extracts always gave reddish brown colors, without any bluish tinge, in the iso-amyl alcohol layer. Although the visual examination of the brownish red color did indicate the absence of leucoanthocyanins, to be sure, the hydrolysates of the acidified

TABLE 9.I.
COLOR REACTIONS AND R_f VALUES OF THE CHROMATOSPOTS FROM THE HYDROLYSATES OF THE AWNS, HULLS, PERICARP AND SPERMODERM EXTRACTS

Spot No.	Avg. R_f value x 100		Colors				
			Untreated		NH_3 Fumes		$AlCl_3$
	Ascent	Descent	V	UV	V	UV	UV
1	100	100	Br	Bl Bk	NC	NC	-
2	98	-	BrG	BrR	NC	NC	-
3	98	-	Y	BrY	NC	NC	-
4	98	-	Y	Y-GY	Y	dbY	GY-Gf
5	98	-	lY	Y	lY	Of	-
6	96	-	lY	lBlf	bY	bGBlf	Blf
7	95	-	Dy	OR	bY	Of	-
8	95	94	C-lY	C	C	GBlf	-
9	95	91	lY	Yf	bY	GYf	GYf
10	93	89	YBr	lBlf*	NC	bBlf	Blf
11	90	86	Y	lBlGf	Y	CrBlf	-
12	84	-	C	bBlf	lY	bGBlf	-
13	80	78	C-lY	CrBlf	NC	bCrBlf	GBlf
14	75	-	C	lBlf**	Y	lGYf	GYf
15	62	-	C	C	C	C	Y
16	59	51	--------Cyanidin----------				
17	37	-	C	Blf	C	Blf	-

* Blue violet absorption under short wave
** Invisible under short wave

alcoholic extracts of blue and white isogenic lines, 36-BlBl-21, and 33-blbl-13, and of black variety, Gatami, were chromatographed. These hydrolysates gave a total of 17 spots; the visible and ultraviolet colors of the untreated, ammonia treated, and aluminum chloride treated spots are shown in Table 9.I. The distribution of these spots in different stocks and tissues at different stages of development is shown in Table 9.II. The results of the purple varieties have been excluded in the interest of brevity. However, all maternal grain tissues, as well as basal leaf sheathsof the 'purples' prior to the appearance of anthocyanins

TABLE 9.II

CHROMATOSPOTS FROM THE HYDROLYSATES OF SEVERAL GRAIN TISSUE EXTRACTS FROM 36-B1B1-21, 33-b1b1-13, AND GATAMI AT THREE STAGES OF DEVELOPMENT

Spot No.	36-B1B1-21											33-b1b1-13						Gatami									
	Awns			Hulls			Pericarp			Spermoderm		Awns		Hulls		Pericarp		Sermod.		Awns		Hulls		Peric.		Spermod.	
	I	II	III	I	II	III	I	II	III	I	II	I	III	I	III	I	III	I	III	I	III	I	III	I	III	I	III
1	+	+	t	+	+	-	⊕	+	+	t	t	+	+	+	+	⊕	+	+	-	+	+	+	+	+	+	t	t
2	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	-	-	±	-
3	-	-	-	-	-	-	+	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	+?	+?	-	+?
4	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	⊕	t	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+
7	-	-	+	-	-	⊕	+?	+?	+?	-	-	-	-	-	-	+?	+?	-	-	-	-	-	-	-	-	-	-
8	-	+	-	-	-	-	-	+	+	-	⊕	+	+	+	+	-	⊕	-	+	+	+	⊕	⊕	-	-	-	-
9	+	+	-	+	-	-	⊕	-	-	+	t	-	-	+	-	-	-	+	t	-	-	+	-	-	+	-	-
10	+	+	⊕	+	+	⊕	-	+	+	+	⊕	+	+	+	+	+	⊕	+	⊕	+	+	+	+	⊕	t	t	t
11	+	⊕	t	⊕	t	t	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
13	-	-	-	-	+	⊕	-	-	-	-	+	-	-	+	+	-	?	-	+?	-	-	-	-	-	-	-	-
14	-	⊕	t	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+?	-
15	-	-	-	-	-		-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
16	+	+	-	-	+	+	-	-	-	-	t	-	-	-	-	-	-	-	-	?	-	-	-	-	-	-	-
17				-	-	-	-	-	-	-	-	⊕	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

⊕ major spot

? doubtful

were hydrolysed directly.

The leucoanthocyanins were not recovered from any maternal tissue of any variety at any stage of development, either by the direct hydrolysis of the tissues by the method of Bate-Smith (loc. cit.), or from the hydrolysates (Table 9.I) of the tissue extracts. Tables 9.I and 9.II, however, do show the occurrence of cyanidin in the blue isoline at stage I and II of awn tips, at stage II and III of hulls, and in traces at stage II of spermoderm (traces, because anthocyanins in the spermoderm develop at the lemma-palea slit around stage II; cf., Secs. 3 and 4). The occurrence of cyanidin in these tissues is due to anthocyanins (Sec. 4) and not leucoanthocyanins.

Although no identification was undertaken, several spots included in Table 9.I give color reactions characteristic of flavones, flavonols and related flavonoids and cinnamic acid analogues. The distribution characteristics of these spots at different stages of development of the same tissue, between the maternal tissues of the same variety, as well as different varieties, as summarised in Table 9.II, will be elaborated briefly.

Spot No. 1 occurs in all cases. It is probable that a part of the brown color may be due to sugars, because sugars upon acidic hydrolysis turn brown and show cream color under ultraviolet light (Bate-Smith, 1954). The brownish spot, however, is blue black under U.V. (Table 9.I). Although the blue black spot was found to be surrounded with the creamish halo, its nature is not understood.

Spot No. 2 occurs predominantly in the early stages of all tissues. It is notably absent in the pericarp tissues of all varieties. The color reactions indicate that it is due to chlorophyll. It will be

noted from R_f values in Table 9.I, that a large number of spots cluster in this region. It is due to the superimposition that the color of this spot appears brownish red under ultraviolet. However, in some tissues particularly in the early stages of spermoderm, this spot is visibly clear green, and under ultraviolet fluorescent pink. The concentration of this spot changes drastically with maturity. The spot is absent from several tissues at stage III of kernel ontogeny.

Spot No. 3 was found only at stage I of pericarp from 36-B1B1-21. A spot at this R_f value was also present in the pericarp and spermoderm of Gatami, however, its color reactions appear to be different from that of the spot No. 3.

Spot No. 4 appears to be a flavonoid and was found exclusively in the pericarp of the white and blue isolines. In the blue isoline, this spot, notably, does not develop at stage I but develops at stage II and III. On the other hand, in the white isoline, this spot is present in large quantity at stage I and disappears during the later stages of development. Although the developmental features of this spot appear to be reversed in the white isoline, no definite conclusion can be drawn because it is difficult to collect the tissues of the different stocks at identical physiological age. Nonetheless, it is clear that there is a definite developmental pattern of this spot within the isolines.

Spot No. 5 was found exclusively in the awn tips of the black variety, Gatami. It was absent at stage I but was present only at stage III. It is notable that melanic pigments develop in awn tips at stage III (cf., Sec. 4; Appendix: Sec. 27).

Spot No. 6 was also found only in the pericarp and spermoderm tissues of the black variety, Gatami. It was notably absent at stage III

of the pericarp.

Spot No. 7 was present only in the blue and white isolines and absent from the black variety. In the blue isoline, it is present only at stage III of the awns, and hulls but absent from the spermoderm. At all developmental stages of the pericarp tissue from the blue and white isolines is present a spot at the R_f value of the spot No. 7. However, the color reaction of this spot does not appear to be similar to that of the spot No. 7.

Spot No. 8 occurs in all the three stocks. In the blue isoline, it does not develop in all the four tissues at stage I and is absent in the hulls at all stages of development. In awn tips, it develops at stage II but disappears at stage III. In pericarp and spermoderm, it is present during later stages of development. Although this spot does not develop in any tissue of the blue isoline at stage I, it does develop in the awn tips and hulls of the white isoline at stage I. The developmental features in the pericarp and spermoderm of both the isolines are, however, similar. The spot is notably absent at stage III of the awn tips and hulls of the blue isoline but it is present at stage III of all the four tissues of the white isoline. In the black variety, the spot is present in relatively large amounts at both stages of awn tips and hulls, but it is absent from pericarp and spermoderm.

Spot No. 9 gives color reactions of a flavone or a flavonol It occurs in several tissues of the three stocks at certain stages of development but is absent both from awn tips and spermoderm of Gatami, and pericarp and awn tips of 33-blbl-13. In the blue isoline, it is present in all the four maternal tissues at stage I but disappears in every case by stage III. The hulls of all the three colorants behave

in the same manner; the spot, in all cases, is present at stage III as a major spot. These observations clearly indicate that in the pericarp this flavonoid, and probably its glycosidic derivatives (because it was obtained by acidic hydrolysis), is metabolised early in development in the blue isoline and accumulates in the black variety at stage III when melanins are being elaborated.

Spot No. 10 appears to be a C_6-C_3 organic acid. It occurs widely in all maternal tissues of the three colorants at almost all stages of development. It increases in quantity with maturity (stage III) in all tissues of the blue and white isolines. However, the developmental pattern is reversed in the black variety. This is particularly clear from the pattern in spermoderm and pericarp of Gatami, which show its maximum incidence at stage I; the spot disappears as melanin forms.

Spot No. 11 occurs in the hulls of the three colorants at all stages of development. In the blue isoline, its incidence is maximum at stage I. The spot also occurs in the awns of the blue isoline.

Spot No. 12 was detected only in the hulls of the black variety.

Spot No. 13 is present in certain tissues of the blue and white isolines but is absent from all tissues of the black variety. In the hulls of blue isoline, this spot does not develop until stage II, but in the white isoline it is present both at stage I and III. The spot, also, develops in the spermoderm of the blue isoline at stage II. However, the correspondence of this spot with that found in the pericarp and spermoderm of the white isoline is doubtful.

Whereas spot No. 11 is localised in the hulls of all the three colorants, the spot No. 14 is present only in the awn tips of the three colorants. In the blue isoline, its presence is dynamic with maximum

quantity at stage II. The correspondence of this spot with that found in the spermoderm of Gatami is doubtful.

Spot No. 15 is a colorless spot and is detectable only after aluminum chloride treatment. Although the spot was only detected in the hulls and awn tips of the white isoline, its absence from other varieties is not conclusive because of its colorless nature.

Spot No. 16 is due to cyanidin. Its identification is based on cochromatography with synthetic cyanidin, and visible and ultraviolet colors. Cyanidin is present in certain tissues of the blue isoline, but only at those stages of development which show the presence of anthocyanins. For instance, on the basis of field observations, it was shown in Section 4 that in the blue isoline (Appendix: Table 27.XIII), the awn tips develop anthocyanins only at early stages of development, and degrade by stage III; the hulls, on the other hand, are anthocyanin free at stage I but do develop anthocyanins only in the veins at stages II and III. These visual observations are fully borne out by chemical analysis as shown in Table 9.II. Further, the pericarp tissue does not develop anthocyanins. The spermoderm, however, does show the presence of cyanidin in traces at stage II, because at this stage anthocyanin develops in traces at the slit bounded by lemma and palea (cf., Sec. 4). Although anthocyanins do develop in the awn tips of Gatami at early stages of development, the presence of cyanidin in this tissue has been marked 'doubtful' in Table 9.II because the reddish color of the extract had suffered degradation during processing.

Spot No. 17 was detected only in the awns of the white isoline.

9.4. DISCUSSION

This inquiry on the development and localisation of leucoanthocyanins and related polyphenols was undertaken for biogenetical reasons. It is obvious from the introductory remarks that the issue of leucoanthocyanin-anthocyanin relationship, despite a large number of studies in several plants, still remains doubtful. Circumstantial evidence from structural viewpoint suggests that leucoanthocyanins do not act as precursors of anthocyanins. For instance, the structures of naturally occurring leucoanthocyanins thus far established happen to be flavan-3, 4-diols. "There is at present no proof, and," presumably from chemical viewpoint, "indeed, little probability," as stated by Clark-Lewis (1962), "that conversion of flavan-3, 4-diols into anthocyanidin is a normal biosynthetic pathway." The natural leucoanthocyanins, structures for which have been unambiguously established, apart from the 3,4-diol substituents, either carry an hydroxyl substituent at position-8 or no substituent at position-5. That no anthocyanidins with such hydroxylation patterns have been found in nature has led to the belief that leucoanthocyanins do not act as the precursors of anthocyanins. This view is also strengthened from other investigations. Stephens (1948, 1949) and Alston and Hagan (1955) first showed that leucoanthocyanins act as precursors of anthocyanins. However, in their later studies (Stephens, 1951; Alston and Hagan, 1958), they failed to find any temporal correlation between the presence and absence of leucoanthocyanins and anthocyanins in the tissues. Feenstra (loc. cit.) also reached the same conclusions.

Evidence that leucoanthocyanins may, very probably, be the

precursors of anthocyanins has been provided recently by the chemico-genetical studies on the aleurone pigments of maize. Of all plants, maize has been studied most intensively in regard to the genetics of anthocyanin production. A large number of single and double mutant stocks have been obtained, some of which in certain combinations accumulate leucoanthocyanins, and in other cases, anthocyanins. While studying these mutant stocks by the inter-tissue complementation technique (Reddy and Coe, 1962), a technique that has been used for the study of gene action sequence in anthocyanin biosynthesis, Reddy (1963, 1964) has shown clearly that "there is a common gene-controlled pathway for the synthesis of leucoanthocyanidin and anthocyanin."

The complete absence of leucoanthocyanins, from the maternal tissues of barley grain, whether they do or do not develop anthocyanins, establishes that leucoanthocyanins are not involved in the biosynthesis of anthocyanins, at least, in the maternal tissues. Whether the leuco-anthocyanins present in the aleurone (Sec. 6) act as the precursors of the aleurone anthocyanins, yet remains to be demonstrated.

Since 1959, owing to their involvement in beer chill hazes, the localisation of leucoanthocyanins, or anthocyanogens, so called because they yield anthocyanidins upon hydrolysis, has been the subject of several studies. A group of workers at the Brewing Industry Research Foundation, England showed in 1959 (Hall, Harris and Ricketts) that anthocyanogens were exclusively localised in husks (hulls), which were obtained by sifting the crushed malt. Pollock, Pool and Reynolds (1960) continued the localisation studies in the same laboratory and found that no anthocyanogens were present in the husks obtained by manual peeling. This was confirmed by Meredith (1961). The discrepancy in the earlier work was due to the

adhering fragments of the aleurone tissue to the husks (Pollock et al., ibid.). These workers did not identify histologically the nature of the adhering fragments; they stated that the fragments were only due to aleurone. Obviously, aleurone was not used in a specific sense because fragments of pericarp, spermoderm and endosperm were included (Sec. 3).

Pollock and Pool (ibid.) also studied the localisation by decorticating the hulls and pericarp in 50% sulfuric acid. They found that the "decorticated grain (endosperm, aleurone, embryo, and testa)" contained 96% of total anthocyanogens present in the whole grain. Since endosperm obtained after pearling the outer coverings contained only 27% of the total anthocyanogens, Pollock et al. (ibid.) concluded that bulk of the anthocyanogens were localised in the aleurone. The localisation was also studied by Meredith (1961) by pearling. The "hulls, flour, and pearled grain were separated and cleaned by sifting." Since the major quantity of anthocyanogens was found to be localised in the flour, Meredith concluded that the "endosperm layers adjacent to the hull are, therefore, the main source of anthocyanogens" and supported the findings of Pollock et al. (ibid.) that anthocyanogens were localised in the aleurone layer.

It is notable that these workers did not determine if the spermoderm (cf. Sec. 3) and also, pericarp were, indeed, free of anthocyanogens. Pollock et al. (ibid.) have also established that a large quantity of anthocyanogens leach from the decorticated barley. Thus, the discrepancy of the 4% anthocyanogens in the decorticated grain suggests that since no anthocyanogens are present in hulls, the 4% discrepancy could have been due to the localisation of anthocyanogens in the pericarp; the anthocyanogens in the pericarp in turn could have diffused during the

sulfuric acid steeping.

It was shown elsewhere (Sec. 3 and 8) that pericarp, contrary to the prevalent belief, contains, at best only very small amounts of anthocyanins and that the bulk of anthocyanins were localised in the spermoderm. Thus, if anthocyanogens and anthocyanins have common biogenetic relationships, the presence of anthocyanins in extremely large quantity in the spermoderm of Black Hulless (Sec. 3) supports the suspicion that leucoanthocyanins might be present in the tissue. A clear-cut demonstration of the absence of leucoanthocyanins from the spermoderm is, therefore, mandatory to the conclusion that leucoanthocyanins are exclusively localised in the aleurone layer. That the spermoderm and other maternal tissues do not contain leucoanthocyanins at any stage in the developing grain has been conclusively established in this study. The conclusion confirming that leucoanthocyanins are localised exclusively in the aleurone and endosperm as concluded by others (Pollock et al., ibid; and Meredith, ibid.)

Although this study was undertaken originally to investigate the incidence of leucoanthocyanins, the ancillary information derived from the same hydrolysates demonstrates the qualitative dynamism of these polyphenolic compounds, at different stages of plant development. Recently El-Basyouni and Towers (1964) have demonstrated quantitative dynamism of phenolic acids of wheat during growth under controlled environments. Although our study is not quantitative, the chromatographic technique permits conclusions on the absence and presence of different compounds shown in Table 9.I. The results of this study indicate that several spots either develop late in development (stage III) viz., spot No. 5 (Gatami), spot No. 7 (36-B1B1-21), or early in development (stage I) viz., spot No.

6 (Gatami-pericarp), spot No. 9 (all tissues of blue and white lines), or only at stage II, viz., spot No. 14 (36-BlBl-21-awn tips). The observations indicate that the chemico-genetical studies on the parents and their hybrids must be carried out at the same physiological age and state of the individuals to draw dependable conclusions on hybrid substances. This view receives support from the studies of Urban (1959), who observed that the biochemical state of flavonoids is greatly influenced by diurnal variations. The variations point out the need for care in time of harvest even to the hour of the day. El-Basyouni and Towers (ibid.) eliminated the diurnal variation by using controlled environments. Thus, further ontogenetic studies of the kind reported in Section 8 should be carried out under rigidly controllable conditions.

The results also indicate that some spots, e.g., 1,2,9,10, etc., occur in all the three, namely white, blue and black stocks. Other spots, e.g., 4,5,11,12,14, are tissue specific, and some, e.g., 5, 12, 15 are stock specific. While still others are found both in the blue and the white, but absent from the black stock. For instance, the flavonoid spot No. 4 develops early in the white, late in the blue and not at all in the melanic variety. Similarly spots No. 7 and 13, which are present in the blue and white lines are absent from the black variety. Although it will have to be ascertained in later studies whether these spots are totally absent (and this appears to be highly unlikely) from the melanic stocks even at very early stages of development, their absence from the melanic and presence in the non-melanic stocks at the corresponding states of development likely suggests that their biogenesis, in the black, may be blocked at phenylalanine or tyrosine stage (Sec. 4: Fig. 4.2) and the amino acids may be channeled towards melanin formation. The accumulation

of the organic acid spot No. 10 in the non-melanic and early disappearance from the melanic stocks is circumstantial evidence for the foregoing contention.

It was previously thought that the chemistry of anthocyanins and melanins may be quite unrelated (Mullick, Faris, Brink and Acheson, 1958). However, on the basis of several field observations (Sec. 4), the observations made on the blackening of anthocyanin isolates (Sec. 8.2.4., and 19.3.3), and also on the basis of recent advances made on the biochemistry of phenolic compounds (Neish, 1960), it was suggested elsewhere (Sec. 4) that the chemistry of anthocyanins and melanins may, in fact, be related in biogenesis. The observations mentioned in the preceding paragraph further support the suggestion of the biogenetic association of the melanic and flavonoid pigment systems in barley.

Further studies on the separate tissues of caryopsis are necessary to understand the physiological role of the outer coverings of caryopsis, and the molecular basis of permeability (Secs. 21 and 22), both so vital from the brewing standpoint.

9.5. SUMMARY

It was shown by Mullick, Faris, Brink and Acheson (1958) that leucoanthocyanins are present in the composited pearlings from the pericarp and aleurone tissues of barley. Hall, Harris and Ricketts (1959), being not aware of the above finding, showed that leucoanthocyanins were present in the husks. Pollock, Pool and Reynolds (1960), and Meredith (1961) established by indirect methods that leucoanthocyanins were present not in the husks but in the aleurone. The latter workers, however, did

not demonstrate that the leucoanthocyanins were absent from the pericarp and spermoderm tissues. In this study, the pericarp and spermoderm tissues of several variants were separated by manual peeling at several stages of kernel maturation. The study establishes that the tissues do not develop leucoanthocyanins, whether they eventually do or do not develop anthocyanins. Hence, the leucoanthocyanins are present only in the aleurone and endosperm tissues and they are not involved in the biogenesis of anthocyanins in the pericarp and spermoderm tissues.

The ancillary information derived from the above study shows that there is a qualitative dynamism of polyphenolic compounds in awns, hulls, pericarp and spermoderm tissues at different stages of kernel development.

DEVELOPMENT OF METHODS FOR CHARACTERIZING OF ANTHOCYANINS

(Sections 10 to 18)

Having examined the qualitative distribution of anthocyanin pigments in several tissues at different stages of development in different barley stocks, the characterization of the anthocyanins was undertaken. Contrary to the initial beliefs, the anthocyanins of barley were found to be highly complex and also labile. Thus, new methods had to be developed to secure their stability and to identify them. This is not to imply an independence of the basic techniques of other workers but, very few of them could be directly and validly applied without modification. An account of the impediments and the methods developed will be presented from Section 10 through Section 17.

In Section 18 are presented the problems associated with the glycosidic analysis of anthocyanins, eluted from chromatography paper, and a method for resolving them.

METHODS FOR CHARACTERIZING ANTHOCYANINS

10. PREPARATIVE CHROMATOGRAPHY OF ANTHOCYANINS

The preparative chromatography of the extracts which were used in the studies described in Section 10 to Section 19 will be presented in this section. In addition to discussing the sources of materials and the preparation of extracts, the basis for choosing a solvent for the preparative chromatography, and the chromatographic stability of the extracts will also be discussed.

10.1. MATERIALS

About 300 plant and grain tissues at different stages of development, obtained from several barley stocks, were collected originally for the characterization of anthocyanins and related flavonoids. The tissue collections came from varieties planted at the University Farm on May 13, 1960 and May 23, 1960. The collection of the 300 tissues was started in

early July, 1960 and completed in September, 1960, A large number of the tissue extracts were lost, to the direct objectives of the studies, in attempts to understand the lability of anthocyanins, the importance and nature of which is illustrated from two anthocyanin collections, namely, the Field Collection No. 130 and 138, obtained from the basal leaf sheaths of the purple variety, Gopal. The leaf sheaths of the Collection No. 130 (planted May 23) were collected on July 11, 1960 and those of the Collection No. 138 (planted May 13) on July 12, 1960. In both cases, the sheaths were collected at the stage of maximum color development.

10.2. METHODS

10.2.1. Extraction and Processing

The tissues, immediately after harvesting, were extracted in 1% conc. HCl in methanol, in amber bottles kept over ice, as described in Section 8.1.2. Because the collection of the large number of tissues (ca. 300) entailed considerable work, the extraction and processing could not be undertaken simultaneously. The amber bottles containing the tissues and the solvent remained in a refrigerator (-15°C) until the collection work was finished in September, 1960.

Following the preliminary low temperature extraction, and filtration through glass wool, the tissues were allowed to dry at room temperature for 24 hours. The dry weight of the tissues was determined. The tissues, if the anthocyanins were not extracted completely, were reextracted in fresh solvent. The extracts were combined and processed further to eliminate the non anthocyanin contaminants as described in Section 8.1.3. The processing of the 300 extracts was undertaken in October, 1960 and com-

pleted in February, 1961.

The extracts of the tissues showing maximum color development, were reduced to a standard volume based on the dry weight of the tissue following the preliminary extraction. Good chromatographic resolution was obtained when the extracts of different tissues at the maximum color stages were roughly standardised as shown in Table 10.1.

Of the several methods explored to determine the dry weight of the tissue, the one mentioned above satisfied the needs of mild extraction pro-

TABLE 10.I

STANDARDIZATION OF EXTRACTS FROM DIFFERENT TISSUES AT THE STAGE OF MAXIMUM ANTHOCYANIN DEVELOPMENT

Tissue	Volume of the extract per gram dry weight of the tissue at room temperature following preliminary extraction
Basal Leaf Sheaths	12.5 ml
Auricles	5.0 ml
Awns	5.0 ml
Lemmas & paleas (Hulls)	2.5 ml
Pericarp	2.5 ml
Spermoderm	6.0 ml
Caryopses (seeds minus hulls)	0.3 ml

cedures used in this study. Since methanol dehydrates the tissues, a good repeatability of dry weight ($\pm$ 2%) at room temperature was observed. The value of the procedure lies in the amenability for reextraction of the tissues, when the extraction is not complete in the first extraction. The procedure is at best semi quantitative for tissues showing a very small quantity of anthocyanins.

10.2.2. Chromatography

The processed extracts remained in the freezer for several months until the chromatography of all extracts was completed. In general, 0.3 to 0.5 ml of the standardized extract of tissues representing the stage of maximum anthocyanin development was sufficient to give good resolution. The extract was banded usually in two equal portions on Whatman paper No. 3 with a special (banding pipette) developed in this laboratory (Sec.10.2.3.). Other chromatographic considerations are mentioned in Section 8.1.3. The principle solvents used for anthocyanin chromatography (Harborne, 1959a; and Hayashi, 1957), both for preparative and characterization work, are listed in Table 10.II.

TABLE 10.II

SOLVENTS USED FOR ANTHOCYANIN CHROMATOGRAPHY

Solvent System	Abbreviation	Ratio v/v	Phase Used	Time*** Required Hours
n-Butanol-Acetic Acid-Water	BAW*	4:1:5	U	17
n-Butanol-2N HCl	Bu-HCl**	1:1	U	
Water-Conc. HCl	Aq-HCl	97:3	S	5-5½
Water-Acetic Acid-Conc HCl	HAc-HCl	82:15:3	S	6½-7
Water-Acetic Acid-Conc.HCl	3:1:8	3:1:8	S	7-7½

U= upper phase S= single phase

* For ascending chromatography on Whatman paper No. 3 for preparative purposes, the solvent was aged, at least for 24 hours. However, for R_f value determinations on Whatman paper No. 1 by descent, for characterization work, the solvent was aged for three days before use.

** After mixing the solvent components, and as soon as the phases had separated, the lower phase of the mixture was collected and immediately used for advance equilibration of papergrams. The upper phase

(solvent proper) was used 24 hours after the initial mixing.

*** The development time of the solvent at 20°C ± 1°C.

The BAW solvent was chosen for the initial separation of anthocyanins in extracts (Sec. 10.3.1). The extracts were banded on Whatman paper No. 3 and run for 3 days by ascent in Kurtz-Miramon chromatography cabinets (12" x 12" x 24") which could accomodate 20 sheets (9" x 22½") at one time. The sheets were secured with 'teflon' clamps.

For the determination of R_f values for characterization, purified anthocyanins were spotted on Whatman paper No. 1, unless otherwise specified, and run at 20°C ± 1°C by descent. The entire chromatographic assembly was kept as horizontal as possible with spirit levels and sufficient weight was placed on the top of the lid to keep the system air-tight. The R_f values were measured from the leading edge of the spot with a 'Chromatogrid' (California Corporation for Biochemical Research).

10.2.3. A Banding Pipette

The banding of extracts on chromatography paper was carried out using a pipette designed by the writer (Fig. 10.1). The pipette is sturdy, simple and cheap to construct and was as satisfactory as the costly, and fragile banding pipettes sold commercially (RSCO). The major advantage of the pipette over other pipettes is apparent from the manner of banding as illustrated. Since the band is applied using the rounded convex undersurface of the capillary, the surface of the paper undergoes minimal modification with no scratching or tearing even with repeated applications. Smoothness of delivery eliminates local overloading, however, to this end it must be ensured that the extract does not dry on the convex under-

surface in between the repeated applications. The width of the band is easily controlled by the speed and angle of application. Delivery can be made quantitative by graduating the pipette. The unit is easily cleaned.

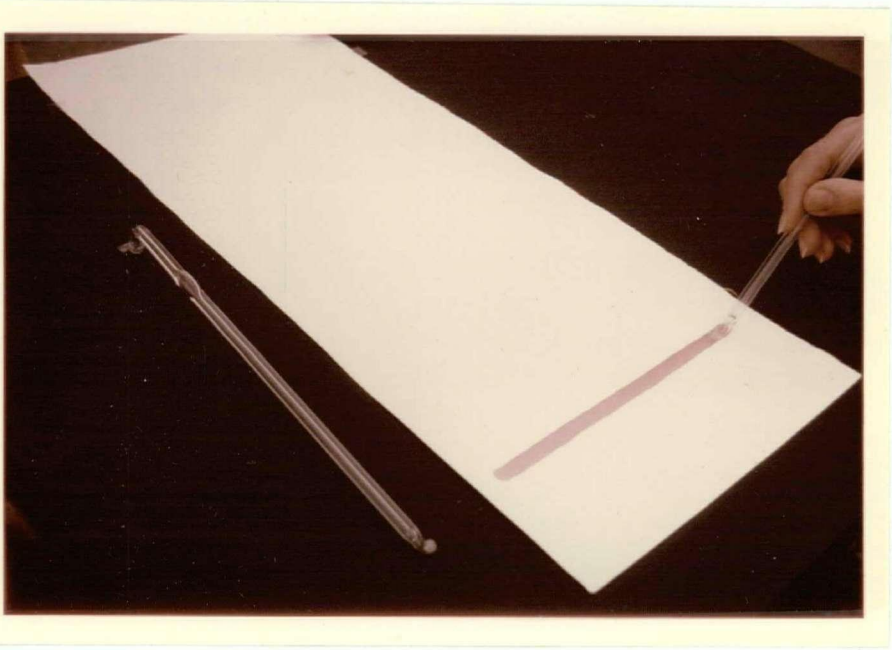


Fig. 10.1.
The banding pipette in use. Note that the convex undersurface of the capitallary is applied to the chromatography paper in banding.

A pipette, 12" in length, drawn from barometric tubing (I.D. 0.065"; O.D. 0.30"), with a bulb blown in the stem about 3½" from the top to act as a reservoir for extract, gives a satisfactory rate of flow for most extracts. The upper end is bent at an angle of 150° to make suction easy. To the lower or applicator end is fused one half inch of thermometric capillary tubing (I.D. 0.013"; O.D. 0.24") bent at an

included angle of 120°. The capillary end is ground at right angles to the capillary tube and a small notch is filed vertically in the ground glass surface.

10.3. RESULTS

10.3.1 Choice of a Solvent for the Preparatory Chromatography

The solvent best suited for preparative chromatography was first to be determined. Harborne (1958, 1959a, 1960) used the Bu-HCl solvent for the preparative runs (i.e., initial separations of anthocyanins from extracts), and purified the eluted anthocyanins in one or more of the first four solvents listed in Table 10.II, viz., BAW, Bu-HCl, HAc-HCl and Aq-HCl. In order to determine the best solvent for the first chromatography, the anthocyanin extracts of the Collections No. 138 and 130 were chromatographed on Whatman paper No. 3 in the 6 solvents listed in Table 10.II, and also in 5% acetic acid (5% -- HAc), in 1% methanolic HCl (MeOH - HCl), and the orthophosphoric acid solvent of Lesins and Lesins (1958). The chromatographic results in a few of the solvents, for Collection No. 138, are shown in Fig. 10.2. The results shown are only those obtained by descending chromatography.

The Bu-HCl solvent shows only two major bands (R_f 0.08, 0.35), a minor band (R_f 0.43), and a weak band (R_f 0.12). The upper half of the band at R_f 0.08 turns blue upon storage and, therefore, this band could be a mixture. The other major band being very diffused may also be a mixture of anthocyanins. The HAc-HCl solvent gives two major bands (R_f 0.32, 0.58), two minor bands (R_f 0.08, 0.37) and a weak anthocyanin

Fig. 10.2
Chromatographic resolution of the extracts from basal leaf sheaths of Gopal (Field Collection No. 138) in the Bu-HCl, HAc-HCl, Aq-HCl, MeOH-HCl and 5% HAc solvents by descent.

trail extending over a large area (R_f 0.58 to 0.90). The resolution of the components in the 3:1:8 solvent was similar to that of the HAc-HCl solvent. The Aq-HCl gives two major but highly diffused bands (R_f 0.30, 0.43), and also a diffused area extending from (R_f 0.43 to 0.65). The resolution in the MeOH-HCl solvent is very poor. The 5% HAc solvent also gives highly diffused bands (the solvent front of the 5%-HAc chromatogram is not shown).

The chromatography of the extracts of Collection No. 138 and, also, 130 in the BAW solvent, by ascent, is shown in Fig. 10.3. The chromatogram No. 138A was developed by descent for two days; the other three chromatograms in Fig. 10.3, namely, 138B, 130A, 130B were developed by ascent for three days. The extracts of the Collection No. 138 gives maximum number of discrete anthocyanin bands in the BAW solvent. The BAW solvent was accepted as superior to all other solvents for the initial

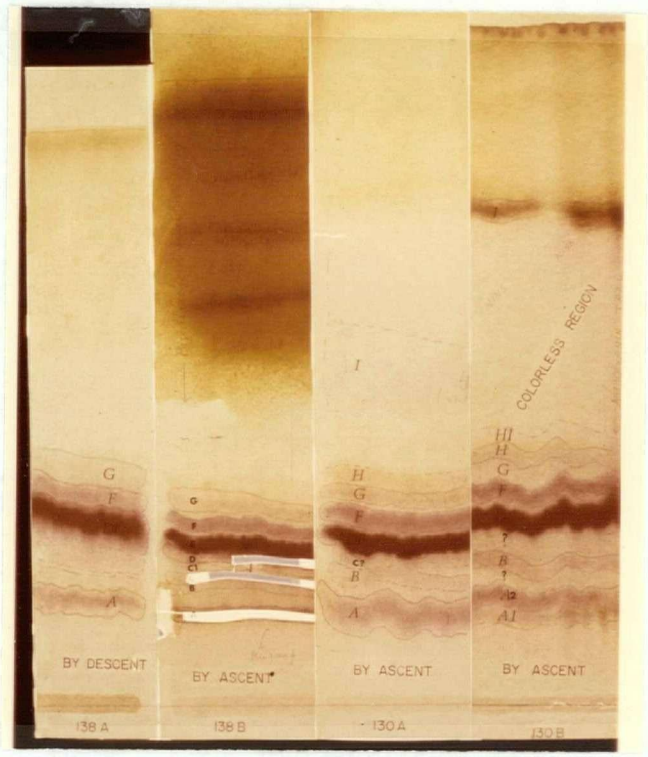


Fig. 10.3.
Chromatography of the basal leaf sheaths of Gopal (Field Collections No. 130 and 138) in the BAW solvent. Chromatograms 138B, 130A, 130B, developed for 3 days by ascent, give better resolution than chromatogram 138A developed for 2 days by descent.

separation of barley anthocyanins. Further, the comparison of chromatograms 138A with 138B, 130A, and 130B shows clearly that the sharpness of resolution was greatly increased when the time of development was extended to three days from two days. Accordingly, the preparative chromatography of the anthocyanin extracts, in all further investigations was undertaken in the organic phase of the BAW solvent for 3 days by ascent.

10.3.2. Chromatographic Stability of Anthocyanin Extracts Upon Storage

It was shown in Section 7 that anthocyanins in the processed ex-

tracts of basal leaf sheaths underwent degradative modifications. Inasmuch as the extracts of the Field Collections No.130 and 138 remained in storage for a long time in the course of extraction and processing (Sec.10.2.1), it appeared desirable to examine the stability of these extracts. This will be illustrated from the chromatograms shown in Fig. 10.3.

TABLE 10.III

CHROMATOGRAPHIC CHARACTERISTICS OF THE ANTHOCYANIN BANDS OBTAINED FROM BASAL LEAF SHEATHS OF THE PURPLE VARIETY, GOPAL, IN THE BAW SOLVENT (FIELD COLLECTIONS 130 AND 138)

Band No.	Average R_fx100	Colors		Derivative of**
		Visible	Ultraviolet	
A	16	Reddish purple	Violet purple	Cyanidin
B	20	Reddish pink	Mauve	Cyanidin & Peonidin
C	23	Reddish orange	Weak?	Pelargonidin?
D	27	Bluish magenta	Purple to magenta	Cyanidin
E	29	Deep magenta	deep magenta	Cyanidin
F	32	Pink	Bright pink	Peonidin and Cyanidin
G	35	Orange red	Mauve?	Pelargonidin
H	37	Bluish red	?	Cyanidin
I	67	Pink	Pink fluorescent	Cyanidin
J	75	Reddish brown	Deep dark ??	

* cf. Table 10.IV.

** See Section 13.2.2 for details of identification.

The chromatograms shown in Fig. 10.3, namely, 138A, 138B, 130A and 130B were developed on October 13, 1961, September 19, 1961, August 27, 1961, and November 7, 1961, respectively. Thus, commencing from the time of original extraction, i.e., July, 1960 (Sec. 10.2.1), the extracts re-

mained in storage for over a year. A total of 10 anthocyanin bands (A-J) were detected on chromatograms; their chromatographic characteristics are summarized in Table 10.III. Although partial identification data of each anthocyanin band will be presented later (Sec. 13), the aglycones of each band have been included in the table for ease of reference. The R_f value of each anthocyanin in Fig. 10.3 is given in Table 10.IV. The average R_f values included in Table 10.III represent the average of only four chromatograms (Table 10.IV: Column 7). In Column 8 (Table 10.IV) are included

TABLE 10.IV

R_f VARIATIONS OF ANTHOCYANINS FROM CHROMATOGRAMS NO.* 138A, 138B, 130A, AND 130B IN THE BAW SOLVENTS

	R_f x 100 (Whatman Paper No. 3: Ascending)						
	Chromatogram Number*						
Band No.	138A	138B	130A	130B	Range of Variation	Average	Overall Average**
A	20	16	12	18	12-20	16	11
B	23	19	15	22	15-23	20	19
C	27	21	20?	?	20-27	23	
D	31?	24	?	?		27	22
E	35	26	24	31	24-35	29	25
F	39	29	27	34	27-39	32	28
G	42	32	29	36	29-42	35	32
H	43?	?	31	38	31-43	37	35
I	?	?	50-66	50-68	----variable----------		
J	-	-	-	75	-	-	66

* Refer Fig. 10.3.
** Determined from 60 chromatograms.

the overall average R_f values of these bands which were derived from 60 chromatograms. A perusal of Table 10.IV and Fig. 10.3 at once brings out

the wide variation in R_f values that occurs during the preparative chromatography. The R_f values, for instance, band A, were observed as low 0.08. Nonetheless, the resolution of all bands was relative and no difficulty was encountered in identifying the major bands. It appears that, the longer the time of development, the lower were the R_f values by ascent. The time of development was not strictly adhered to in the preparative chromatography; the period of 3-day development could have varied by as much as ± 6 hours. However, on the basis of this experience, the periods of development for different solvents, as specified in Table 10.II, in characterization work described elsewhere (Section 19) were strictly adhered to.

On the basis of total experience, it may be stated that the major bands A, E (cyanidin derivatives), F (peonidin derivative), and G (pelargonidin derivative) resolve sharply with the three-day development in the BAW solvent. Difficulties with the clearcut detection of other anthocyanin bands were encountered usually. In general, only one band is readily detectable between the bands A and E. This is particularly the case when the extracts are chromatogrammed by spotting rather than banding (See Figs. 7.1 and 7.2: Gopal, and Black Hulles basal leaf sheaths). Banding improves, if not resolution, at least, detectability (see also Sec. 13.2.4.). The anthocyanin bands that appear in this region are generally very weak, and their resolution is not uniform (Fig. 10.3). For instance, in the chromatogram 138A, there is an anthocyanin region between the bands A and E, which is weak and diffused, but which, on careful examination, can be resolved into 2 to 3 bands. However, because of the shorter period of development, the resolution in this chromatogram is poor. Whereas the band E resolves as a single band in chromatogram 138A, it resolves distinctly into two bands (D and E; both cyanidin derivatives) in chromatogram 138B. The

splitting occured in a number of chromatograms. Although the band D, as compared to the band E, was bluish to begin with, it turned to the magenta color of the band E within a few days on chromatograms. The reasons for the irregular presence of the band D are not understood. Frequently, the band D did not separate from the band E. Thus, the band D and E were generally eluted together as a mixture (see also Sec. 19).

Immediately below the spot D (Chromatogram 138B), is a weak anthocyanin region, sometimes denoted as band C_1, which could be occasionally distinguished (see Section 13). Below this region is the nearly orange-red band C (cut out); the hydrolysis gave cyanidin and weak pelargonidin spots. The band C was as weak as the band B (chromatogram 138B). The band B gave peonidin and cyanidin. The presence of the mixture of different anthocyanins in one band is indicative of the poor resolution in the region between the bands A and E. The difficulties of resolution of the bands from the caryopsis tissues in this region were far more pronounced (Fig.19.3). Thus, the problem of collection of equivalent bands from different chromatograms are self-apparent.

As stated earlier, the basal leaf sheaths of the Field Collections 130, and 138 were obtained at the stage of maximum color development, except, that the materials were collected from the plantings of different dates. The chromatograms 130A and 130B show the same four major bands, A,E,F, and G, present in chromatograms 138A and 138B. The band DE in general, was eluted as a mixture. The resolution of anthocyanins at the region between the bands A and E is again very poor. Although the chromatogram 130A shows bands B and C (abnormal resolution), the chromatogram 130B shows only one band, B, which relatively is quite pronounced. However, the regions between bands A and B and between B and E do contain diffused

anthocyanins. Also, the splitting of band A (chromatogram 130B) into two bands is notable. These features of resolution make difficult the clear cut delineation of the bands from different chromatograms for elution and identification.

The resolution of the anthocyanin bands from the region G and above is not clear cut. The bluish red band H (cyanidin derivative) is present in the 130 series. Frequently, the band is partly superimposed on the orange band G. In some chromatograms, its intensity was, at least, as strong as that of the band G, while in others it was barely detectable. The spot, in general, was absent from the Collection No. 138, but in chromatogram 138A, it can be detected, partly superimposed, on the band G. Thus, the differential presence of the band H in the extracts of 130 and 138 collections may not be due to physiological consequences but, may possibly be due to degradation occurring in storage or chromatography.

Occasionally, an additional band, denoted as H1,was also observed in the chromatograms of the extracts of Collection No. 130.

The band I was obtained largely from the extracts of the Collection No. 130: in Collection No. 138, it was barely detectable only in some chromatograms, in others it was altogether absent. Even in the Collection No. 130, its resolution and detectability was very haphazard. The diffused area is largely due to cyanidin, which appears to have originated as an artefact of extraction and processing (see Section 23).

The band J is highly labile and disappears during chromatography in most chromatograms. It is to be noted that even in the chromatograms shown in Fig. 10.3, it is present only in chromatogram 130B. Its presence in the 130B may not be due to the time induced degradations during storage because it was absent from several chromatograms developed in the same run.

10.4. DISCUSSION

10.4.1. Choice of a Solvent for the Preparatory Chromatography

Although the Bu-HCl solvent is widely used for the preparatory chromatography, with barley anthocyanins it gave only 4 anthocyanin bands, as did the several other solvents. The BAW solvent, which gave 7-10 discrete anthocyanin bands was accepted as superior. The differential resolution of anthocyanins in different solvents was, indeed, puzzling and disconcerting when it was first encountered. It is, however, clear that the number of anthocyanin bands obtained on a chromatogram gives no indication that the same number of anthocyanins exist *in vivo*. The discrepancies in resolution appear to be due to the resolution of two or more anthocyanins at the same chromatographic locus, rather than to anthocyanin breakdown suffered in the course of chromatography in the BAW solvent. In any event, the results clearly indicate the importance of a careful choice of a solvent for the preparatory chromatography; it is probable that no single solvent may give good resolution for all anthocyanins obtained from different plant materials.

10.4.2. Stability upon Storage

The chromatography of the stored extracts, undertaken on different dates demonstrates that the major anthocyanin bands do not undergo any significant change upon storage. Some minor variations in resolution, detectability and, most probably, due to degradative modifications do occur upon storage of the processed extracts. The chromatogram 130B, which was developed 73 days later from chromatogram 130A, still shows almost all the bands present in the chromatogram 130A. the anthocyanins from caryopsis

tissues (Fig. 19.3), and, from several other tissues showed a similar range of stability upon storage. Of course, upon prolonged storage some degradation must ensue (See Sec. 19). These observations on stability of anthocyanins in extracts 130 and 138 substantiate the essential stability of the slow-moving anthocyanins, obtained upon the degradation of the fast-moving anthocyanins (Sec. 7).

It was shown in Section 7 that basal leaf sheath extracts of Black Hulless and Gopal obtained from the November, 1958 collection (Fig.7. 1) contained several fast-moving acylated anthocyanins and that the acylated anthocyanins were highly unstable upon storage of the extracts. Consistent with these expectations, the fast-moving anthocyanins were not recovered from the extracts of the Field Collections No. 130 and 138 (Fig. 10.3). Since chromatography could not be undertaken immediately after extraction, it cannot be definitely stated whether these extracts did contain the fast-moving anthocyanins initially. It is, however, noteworthy that the pattern of anthocyanin distribution obtained from the basal leaf sheath extracts of Black Hulless, and Gopal when the fast-moving anthocyanins had degraded upon storage for 15 days, and 3 days respectively (Fig. 7.2 and Table 7.1) parallels that of the anthocyanins shown in Chromatogram 138B (Fig. 10.3.). These observations, therefore, imply that the fast-moving anthocyanins could have been present initially in the extracts. Since the fast-moving anthocyanins are labile and the slow-moving anthocyanins relatively stable, it was decided to direct prime attention to the nature of the slow-moving anthocyanins. Several difficulties were encountered upon elution and flash evaporation and these will be described in the next section.

10.5 SUMMARY

In this section the details of extraction, processing, stability in storage and preparatory chromatography of anthocyanins used in Sections 10 to 16 and 19 are described. The extracts were banded with a specially designed pipette (Fig. 10.1). Of the nine solvents tried for the preparative chromatography of Gopal basal leaf sheath anthocyanin extracts (Field Collections 130 and 138, the BAW solvent gave the best resolution (7-9 bands). The other solvents gave less than four bands. Thus, the number of bands obtained on chromatograms is no sure indication that the same number of anthocyanins exist in vivo, a point that merits careful attention in biochemico-genetical investigations.

Consistency in resolution of each of the nine BAW anthocyanin bands (designated A to I) is scrutinized. Difficulties of collecting equivalents of the bands, particularly B and C, are indicated. The range of R_f value variations in the preparatory chromatography is presented.

METHODS FOR CHARACTERIZING ANTHOCYANINS

11. INSTABILITY OF BARLEY ANTHOCYANINS DURING ELUTION AND FLASH EVAPORATION

The instability of chromatographically isolated anthocyanins and anthocyanidins during elution and flash evaporation certainly constituted a major difficulty in the advancement of this study. A brief account of the impedimenta will be presented in this section.

11.1. MATERIALS

The BAW anthocyanin bands of the Field Collections No. 130 and 138 (Fig. 10.3), obtained from a large number of chromatograms as described in Section 10, were used in this investigation.

11.2. METHODS

11.2.1. Elution and Purification

Equivalent BAW anthocyanin bands, collected from several chromat-

ograms, were chopped with scissors, packed tightly into glass columns and eluted in a virtually dark room with an eluent such as methanol containing 1% to 2% HCl (Asen. Siegelman and Stuart, 1957), or water-methanol-acetic acid (25:70:5, v/v) (Harborne, 1958). The anthocyanins from the chopped pieces did not elute completely, particularly, from the margins. The pieces were, therefore, ground in a Wiley mill and again packed into columns, and reeluted as described above. The eluates were combined, concentrated *in vacuo*, at 50°C, centrifuged, and finally flash evaported to dryness at 50°C.

The eluents mentioned above were latterly abandoned in favor of methanol containing 1% conc. HCl, and the elution was carried out directly from the paper bands. Moreover, the eluates were concentrated *in vacuo* at the lower temperature of 30°C, centrifuged if necessary, and chromatographed. The eluates after concentration were chromatographed in the HAc-HCl solvent (Table 10.II). The anthocyanins were again eluted, concentrated and rechromatographed, whenever possible, in some of the solvents listed elsewhere (Table 10.II).

11.2.2. Reexamination of Chromatograms

The chromatograms were exposed to acid vapours in a moist environment in chromatography cabinets prior to reexamination under visible and ultraviolet lights.

11.2.3. Notations for Designating Anthocyanins

The following system of notations for designating anthocyanins is used throughout the thesis, except in Section 13. The anthocyanins of the preparatory run when obtained from plant tissues, such as, basal leaf

sheaths, will henceforth, be denoted by the consecutive letters of the English alphabet, the letter A beginning from the anthocyanin band nearest to the starting line (see the notation of the bands in Fig. 10.3). If these bands split into different components, upon rechromatography of their eluates, in the HAc-HCl solvent, the component bands will be denoted by the addition of Arabic numerals to the BAW band notations. For instance, in Table 11.I, the BAW band A 'split' into four components in the HAc-HCl solvents. The four components, beginning from the starting line, are denoted as A1, A2, A3, A4. Similarly, the BAW band E, upon splitting into 5 components in the HAc-HCl, has been denoted as E1, E2, E3, E4, and E5.

In contrast to the notations used for basal leaf sheath anthocyanins, the spike and grain tissue anthocyanins of the preparatory run in BAW, are denoted by the Arabic numerals, beginning from the anthocyanin nearest the starting line. The split bands in the HAc-HCl run will be denoted by the addition of consecutive letters of the English alphabet. Thus, the BAW band No. 5 (Sec. 19) split into over 12 components in the HAc-HCl run (Fig. 19.5). These are denoted as 5A, 5B, through 5L, and so on.

In order to establish the origin of each anthocyanin, the Collection number of the extract is added to the anthocyanin notation after a hyphen. For example, the notation E-138 means that the anthocyanin E is obtained from the preparatory run (BAW) of the extracts of Collection No. 138. The notation E3-138, indicates that this anthocyanin was obtained from the extracts of Collection No. 138 after chromatography in the two solvents, which, always, will be BAW and HAc-HCl, unless otherwise specified (See Sec. 13). Further, the anthocyanin was obtained as the component No. 3 (beginning from the starting line), when the eluates of the BAW band E were rechromatographed in the HAc-HCl solvent.

11.3. RESULTS

When the anthocyanin eluates obtained by the procedures described in Section 11.2.1 were flash evaporated to dryness at 50°C, degradation of anthocyanins occurred in several eluates. Although, the eluates of the BAW bands D, E, and in some cases A (Fig. 10.3) remained visually stable upon drying, the other eluates; notably, from the BAW bands B, C, H, and G, yielded yellow degradation products. The degradation to yellow materials, as estimated visually, varied generally from 20% to 50%, and occasionally up to 100%. The maximum degradation occurred in eluates obtained from weak anthocyanin bands such as B, C, H, I, J, etc. Although, some anthocyanins did crystallize to brownish colors upon flash evaporation of the eluates obtained from strong bands such as A, F, G, etc., the flash evaporated materials also contained the yellow degradation products surrounding the crystalline anthocyanins. Rechromatography had to be undertaken to separate the anthocyanins. Degradation occurred again whenever eluates were evaporated to dryness in vacuo. A large number of anthocyanin extracts obtained from different tissues and stages of development of different genetic stocks (Sec. 10) were thus lost, and progress greatly impeded.

It was observed from earlier studies (Sec. 8), that degradation was not as pronounced if the eluates were only concentrated but not flash evaporated to dryness. Thus, in the later work, presented below, the anthocyanin eluates were only concentrated and not dried. The elution was carried out with methanol containing 1% conc. HCl directly from the paper strips; the other eluents, particularly those containing aqueous fractions, were not used because they took a longer time for flash evaporation. In the likelihood that the 50°C temperature for flash evaporation was high, the

TABLE 11.I

DATA FROM PREPARATIVE CHROMATOGRAPHY AND HAc-HCl PURIFICATION OF THE ANTHOCYANINS FROM GOPAL BASAL LEAF SHEATHS (FIELD COLLECTION 138) BY ELUTION

Preparative Chromatography: BAW					Purification: HAc-HCl			
Band No.	Asc. R_f^P	Desc. R_f^P	Colors		Component No.	Desc. R_f^P	Colors	
			V	UV			V	UV
A^r	10	7	Purple	Deep Violet purple	A1	10	Magenta	Near-red fluorescence
					A2	32	Magenta	Mauve to magenta
					A3	59	Rose red	Purplish
					A4	68	Rose purple	Near mauve (weak)
C^s	15	8	Red to orange red	Red to mauve	C1	9	Pinkish	Reddith fluorescence
					C2	29	Magenta	Magenta
					C3	56	Magenta to purple	Near mauve
					C4	70	Orange red	Near mauve
D^t	19	13	Mauve to purple	Violet mauve	D1	8	Pink	Near-red fluorescence
					D2	27	Magenta to purple	Deep dull purple
					D3	?	a long weak trail	

TABLE 11.I--(continued)

Preparative Chromatography: BAW					Purification: HAc-HCl			
Band No.	Asc. R_f[p]	Desc. R_f[q]	Colors		Component No.	Desc. R_f[p]	Colors	
			V	UV			V	UV
E[u]	23	13	Rose red	Deep purple	E1[v]	12	Pink	Near-red fluorescence
					E2	27	Mauve	Purple to magenta
					E3[w]	35	Rose red	Dull magenta
					E4	38	Pink	Bright pink
					E5	51	Mauve	Mauve ?
F	25	17	Pink	Bright pink	F1	11	Light pink	Pink fluorescence
					F2	28	Mauve	Mauve
					F3	36	Pink	Pink
G	28	20	Orange red	Dull red	Degraded during elution			

p R_f values x 100, determined on Whatman paper No. 3 by ascent.

q R_f values x 100, determined on Whatman paper No. 3 by descent.

r The BAW band A remains purple-violet during and for a few hours following development; later it becomes bluish red.

s The resolution of the BAW band C, in general, was inconsistent. Occasionally it resolved into two weak bands. It is probable that one of the bands is equivalent to the BAW band B-130 (Table 13 III) and B-400s (Table 19.I), The orange red shade may be due to superimposed copigment.

TABLE 11.I--(continued)

t	The BAW band D generally disappears during development; separates occasionally from E when development period is extended.
u	Very intense major band.
v	Crystallized in a refrigerator. Crystals insoluble in 1% aqueous HCl at room temperature.
w	Crystallized in a refrigerator. Crystals soluble in 1% aqueous HCl at room temperature.

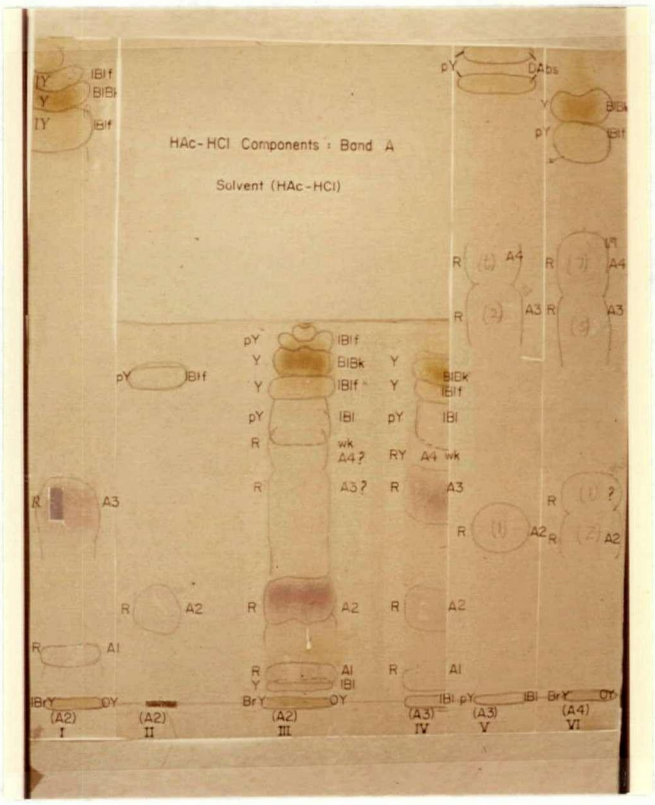
lower temperature of 30°C was used.

The eluates obtained from the BAW bands (Fig. 10.3) of the Field Collection No. 138, obtained under the above conditions, were chromatographed by banding for purification in the HAc-HCl solvent. The chromatographic data is summarised in Table 11.I. It will be noted that except the eluate from band D-138, all other eluates 'split' into three to five anthocyanin components. All anthocyanin eluates showed varying degrees of breakdown products, such as, yellow bands and other substances detectable under ultraviolet light on the chromatograms. Although, the anthocyanin G-138 degraded completely in the course of elution, in another attempt (Sec. 12), it was recovered in a partially degraded state. All chromatograms developed in the HAc-HCl solvent showed large quantities of dull brown yellow materials,which remained at the starting line, and also showed yellow band(s) near the solvent front (see later).

The anthocyanin components of each band obtained in the HAc-HCl run (Table 11.I) were eluted again with methanol containing 1% conc. HCl. The eluates after concentration were left in a freezer (-15°C) and aliquots re-chromatographed in several solvents to determine if the splitting of anthocyanins would occur still, and also to determine the extent of degradation. The results of the HAc-HCl bands A2, A3, and A4 upon rechromatography in HAc-HCl are shown in Fig. 11.1 (chromatograms I to VI). The eluates of A1 degraded in the course of elution and flash evaporation, and are, therefore, not shown in Fig. 11.1. The chromatograms I, V and VI were developed immediately after processing the eluates of A2, A3, and A4; the solvent fronts of these chromatograms had just run off. The chromatograms II, III, and IV were developed about two days later; the chromatogram III and IV were run using the same eluates as were used for the chromatogram I and V respectively, and the chromatogram II was developed by stitching (see Sec. 13) the segment excised from the anthocyanin spot A2 (see window in chromatogram I), in order to avoid elution. The anthocyanin spots in Fig. 11.1 are denoted by the letter R (red); the visible colors of the other spots are on the left and the ultraviolet on the right of each spot.

It is clear from the results that all anthocyanins again split upon rechromatography and that the eluates also contain several degradation products. For example, the A2-138 breaks down into two distinct anthocyanin components A1 and A2 (shown in Fig. 11.1, by error, as A3); and at least 2 to 3 yellowish breakdown products (Chromatogram I). However, when the same eluate A2-138 was chromatographed, after two days, using about twice the concentration applied in chromatogram No. I, (Chromatogram III), it showed, in addition to A1 and A2, also the presence of a distinct, though weak, anthocyanin trail leading to the R_f values of the anthocyanin components A3

Fig. 11.1.
Chromatograms I to VI show the lability, associated with the usual elution and flash evaporation procedures, of the HAc-HCl components from the BAW band A. The HAc-HCl components used are (A_2), A_3), and (A_4). The equivalent components recovered on HAc-HCl rechromatography are shown without brackets, viz., A, A_2, A_3, etc. Breakdown products from highly purified anthocyanins are prominent. (Erratum: Chromatogram I, A_3 should read A_2).



and A4 (Table 11.I). In addition to common yellow breakdown products, which are present in chromatograms I, and III, the chromatogram III also shows the presence of a pale yellow (light blue under UV) component, R_f 0.80, superimposed partially on the spot A4, and a yellow (light blue under UV) component R_f 0.06, superimposed partially on the spot A1. These components are not seen on chromatogram I. Both the chromatograms, i.e., I and III, also show the presence of a large quantity of brownish yellow substance (s) which did not move from the starting line. It will be noted that the excised spot A2-138 upon rechromatography, however, does not show any detectable split of the anthocyanin, and also shows only one light blue fluorescent component

(R_f 0.88). The resolution of anthocyanins by stitching, and hence by avoiding elution, will be described in detail in Section 13.

The chromatogram V shows that the anthocyanin A3-138 splits at least into two distinct anthocyanins, A2 and A3, and also into a barely detectable trail of the anthocyanin A4. The figures in the centre of the spots indicate an estimate of the relative concentrations of the anthocyanins. It is clear that the anthocyanin A3 does not show the anthocyanin component A1 even in trace amounts. However, when the eluate was rechromatographed after two days, using large amounts, traces of the component A1 also appear (chromatogram IV), and also the component A4 becomes distinctly visible. The degradation products, which were not clearly detectable in the chromatogram V are seen in abundance in chromatogram IV.

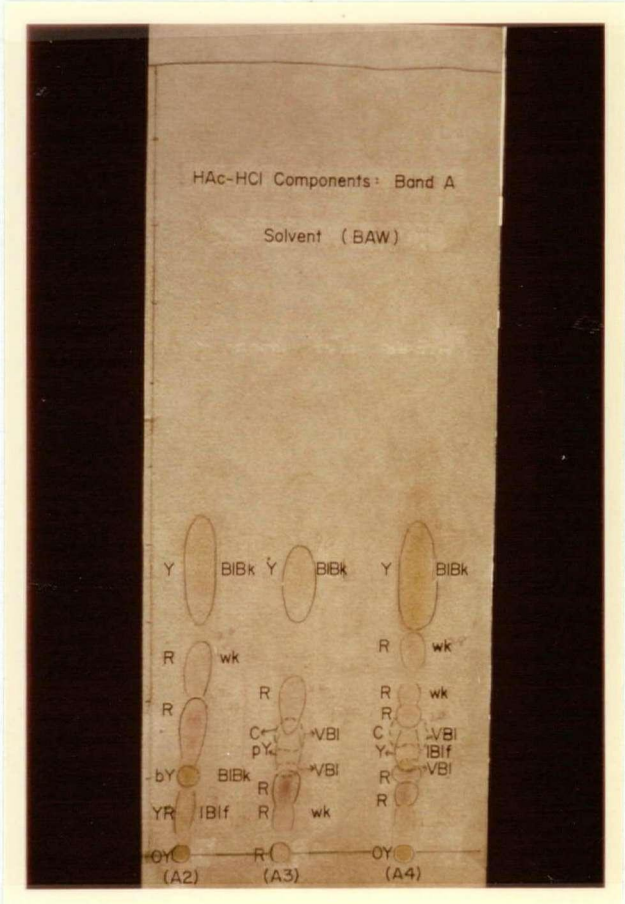
The chromatogram VI shows that the anthocyanin A4-138, upon rechromatography in the HAc-HCl solvent, splits into 4 anthocyanin components, probably, equivalent to the anthocyanin A2, A3, A4. The equivalent of the question - marked anthocyanin component, immediately above the anthocyanin A2, was not detected in other eluates. The numbers in the centre of the spots are estimates of the relative concentration ratio of the different anthocyanins. It is clear that the chromatogram VI does not show the presence of the anthocyanin A1. It will be noted that the chromatograms IV, V, and VI also show the presence of the yellowish components that are stationary at the starting line.

The eluates from the HAc-HCl bands A2, A3, and A4 (Table 11.I), which were rechromatographed in the HAc-HCl solvent (Fig. 11.1) were also rechromatographed in the BAW solvent by spotting. The results are shown in Fig. 11.2, and are broadly similar to those obtained from rechromatography in the HAc-HCl solvent (Fig. 11.1). The eluate of the anthocyanin A2-138

shows one major anthocyanin (R_f 0.20) and two very weak anthocyanin spots (R_f 0.08, and 0.28), none of which resembles the spot A1 (Fig. 11.1). The spot at R_f 0.08 is confounded with a spot of light blue fluorescent substance. Two distinct, bright yellow and yellow, degradation products (R_f 0.11 and 0.43, respectively) are also present. The eluate of the anthocyanin A3-138 also splits into two anthocyanin spots (R_f 0.11 and 0.23), the yellow spot (R_f 0.40) and other substances detectable under ultraviolet light. The eluate of the anthocyanin A4-138 shows three major anthocyanin spots (R_f 0.10, 0.12, and 0.20), two minor anthocyanin spots (R_f 0.23, 0.28) and a weak reddish

Fig. 11.2.

Shows the lability, associated with the usual elution and flash evaporation procedures, of the HAc-HCl components from the BAW band A. The HAc-HCl components used are (A_2), (A_3) and (A_4). The equivalent components recovered on rechromatography, on this occasion using the BAW solvent, are shown without brackets. Breakdown products from highly purified anthocyanins are prominent.



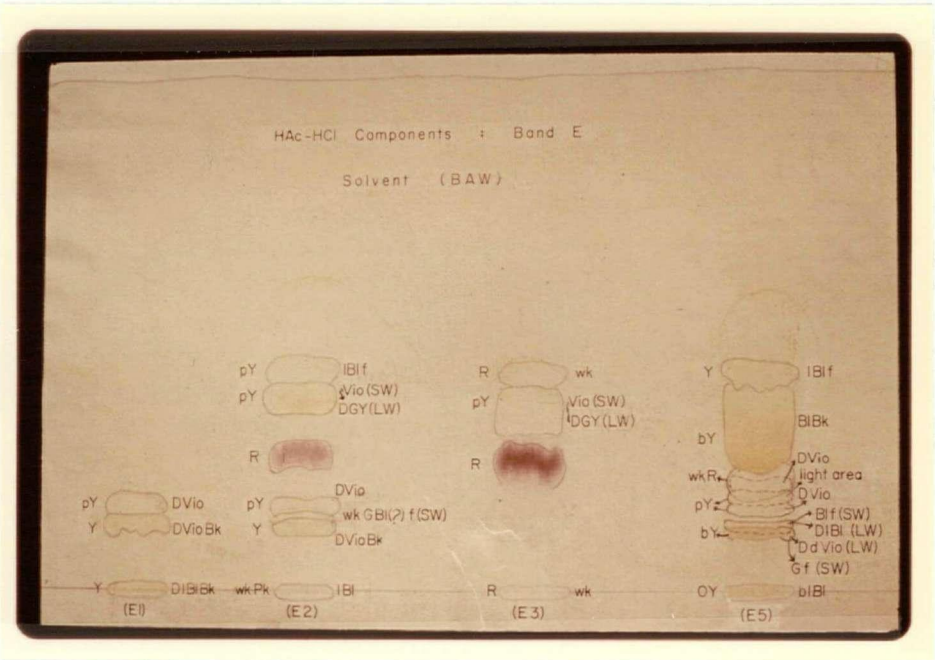


Fig. 11.3.

Rechromatography in BAW of the components (E_1) to (E_5) (obtained from the HAc-HCl chromatography of the BAW band E) showing the comparative stability of E_1 , E_2 and E_3 and the marked instability of E_5 .

yellow trail (R_f 0.07) which, probably, is an anthocyanin. Additionally, it shows two yellow spots (R_f 0.43, and 0.17) and two spots showing violet blue absorption (R_f 0.18 and 0.13: shown by dotted lines), which are also present in the eluate of the anthocyanin A3-138. In addition, all the eluates show distinctly the stationary components at the starting line; they are orange yellow in the case of A2-138, and A4-138 and weak red in the case of A3-138.

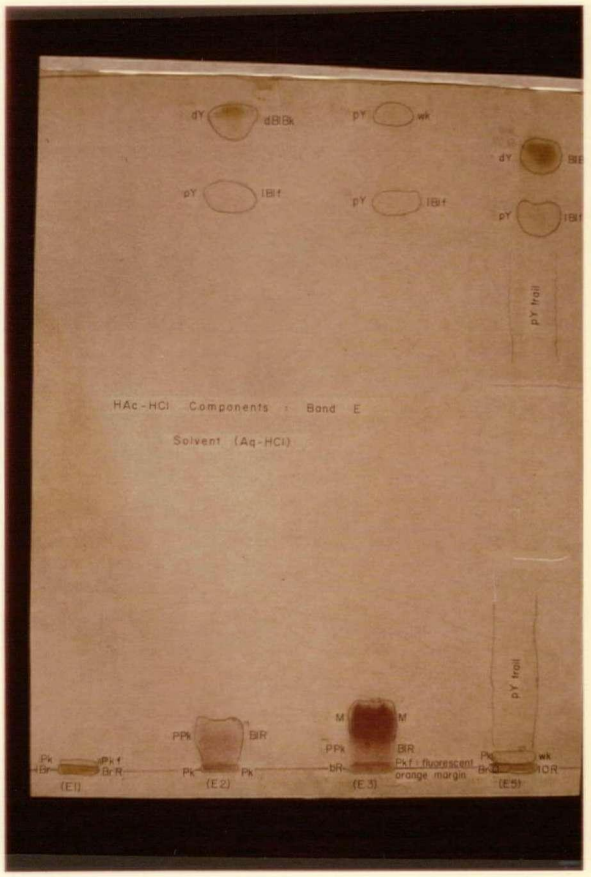
The HAc-HCl anthocyanin components of the BAW band C (Table 11.I) were available only in small amounts. The component C1-138 and C4-138 suffered complete degradation upon elution and flash evaporation. Similarly,

the components D1-138 and F1-138 (Table 11.I) also suffered degradation during processing.

It was observed that the degree of degradation was not the same for all anthocyanin eluates following flash evaporation. This is illustrated with the rechromatography of the HAc-HCl components of the BAW band E (Table 11.I), in BAW (Fig. 11.3) and Aq-HCl (Fig. 11.4). The results of the HAc-HCl components, E1-138, E2-138, E3-138 are shown in Fig. 11.3. Although the eluate of the component E1-138 was visually red, it split into two components, a red and a yellow, at the time the band was applied; the red component remained in the centre and the yellowish component diffused around the red. However, within a few hours of development in the BAW, the red color disappeared. The chromatogram only shows two yellowish spots, one at R_f 0.15 (dull violet black absorption) and the other at R_f 0.19 (dull violet absorption). Additionally, it will be noted that a yellow component remained at the starting line. The eluate from E2-138 (Fig. 11.3) also shows the presence of the same two yellowish components, which were present in the eluate from E1-138. However, in between the yellowish components is also present, in E2-138, a weak greenish blue fluorescent (R_f 0.15) spot seen only under short-wave ultraviolet radiation. The eluate also shows one distinct anthocyanin spot (R_f 0.29) and two pale yellow spots(R_f 0.40 and 0.46). It was noted in Table 11.I that the spot E2-138 was, in fact, the lower part of the major spot E3-138. It was designated as a separate spot because it showed a slightly more bluish tinge than the spot E3-138. The chromatogram E3-138 (Fig. 11.3) also shows one anthocyanin spot at R_f 0.29). The anthocyanin E3-138 does not show much splitting but for a very weak spot at R_f 0.44. Whereas the stationary component of the eluate E2-138 is pink, it is barely visible, though reddish, in the eluate of E3-138. The eluate from

Fig. 11.4.

Rechromatography in Aq-HCl of the components (E_1) to (E_5) (obtained from the HAc-HCl chromatography of the BAW band E) showing the comparative stability of E_1 , E_2 , and E_3 , and the marked instability of E_5 . Additionally the failure of certain colored components to move from the starting line in this solvent is to be noted. The degradation of E_1 later shown to be an aglycone, is shown in this chromatogram and in chromatogram 11.3.



the anthocyanin E5-138 carries large quantities of yellow degradation products. Only one anthocyanin spot (R_f 0.23) is barely visible; it is confounded with two ultraviolet absorbing substances (Fig. 11.3). The major degradation product is the bright yellow spot (R_f 0.40).

The chromatography of the HAc-HCl components of the BAW band E-138 in Aq-HCl (Fig. 11.4) supports the results shown in Fig. 11.3. Although the anthocyanin E1-138 (pink fluorescence) degraded completely in BAW, it remained somewhat stable in Aq-HCl (R_f 0.017). The anthocyanin E2-138 in in Aq-HCl gives a lower R_f value (0.07) than the component E3-138 (R_f 0.10).

As already indicated, E2-138 is slightly bluer than E3-138. However, the color of the trailing portion of E3-138 matches that of E2-138, both in visible and ultraviolet light. Although E2-138 and E3-138 give identical R_f values in BAW (Fig. 11.4) and HAc-HCl (Table 11.I), they maintain the R_f value difference in Aq-HCl, when the development of the chromatogram was continued for over a day letting the solvent front drip off. The eluate from the anthocyanin E5-138 parallels the degradation shown in BAW (Fig. 11.3). It shows a weak red spot (R_f 0.02), a long weak yellow trail (shown by the pencilled lines) and some yellow spots near the solvent front. Notably, the colors of the stationary substance at the starting line show striking difference in the BAW and the Aq-HCl solvents.

11.4. DISCUSSION

The chromatographic results demonstrate the type of instability encountered in barley anthocyanin complexes following elution and flash evaporation. Even when anthocyanins were processed simultaneously under equivalent conditions, some showed greater instability than the others. This is clearly illustrated with the results of the HAc-HCl eluates of the anthocyanin E5-138 in relation to the eluates of other anthocyanin components of the BAW band E-138 (Figs. 11.3 and 11.4). The instability of the HAc-HCl components A1-138, E1-138 and F1-138 either in the course of elution or in chromatographic development, particularly in the BAW solvent, is noteworthy. Whereas the anthocyanin G-138 degraded completely in this study, it was recovered in a partially degraded state as described elsewhere (Sec. 12). The instability is, therefore, unpredictable.

The observation that the anthocyanin A2-138, when applied in small quantity showed only two anthocyanins and when applied in larger quantity

showed at least four anthocyanins (Fig. 11.1) indicates the importance of quantity of eluates applied and the chromatographic detectability. That the anthocyanin A2-138, did not split by stitching led to the belief that the splitting of anthocyanins and also degradation to yellow products was entirely due to the techniques used in elution and flash evaporation. It was, however, found later (Sec. 13), when large scale stitching chromatography was undertaken, that discrepancies in the detectability were, also in part, due to the differences in quantity of the anthocyanin present in the band segment stitched. The chromatogram No. I (Fig. 11.1), from which the excised segment for the chromatogram No. II (Fig. 11.1) was originally obtained, shows that the spot A2-138 was reasonably strong, yet it was not strong enough to show presence of other components in detectable amounts on the chromatogram because, the anthocyanin splitting on the paper as compared to the solution is proportionately, minimal (Secs. 13 and 15). These observations show pitfalls in visual detection in chromatography.

The eluates showing pronounced degradation, invariably, showed the presence of yellowish brown stationary materials at the starting line. If the degradation was minimal, as was the case with anthocyanin eluates E2-138, and E3-138, the color of the stationary material was red. The reddish non-moving materials were also observed commonly upon chromatography of the partially hydrolysed anthocyanins in later studies. The nature of the red, reddish brown and brownish yellow stationary materials does not appear to have been investigated. Nonetheless, the non-moving materials certainly constitute a sizeable fraction of the anthocyanin breakdown products. The magnitude of the stationary materials is such that a study of them prior to quantitative studies of anthocyanins is imperative. A few further comments on the stationary materials will be made in Section 15 and

19.3.3. Spectral characteristics of degradative materials other than the stationary materials will be elaborated in Section 12.

It was shown in Section 8.2.4. that the anthocyanins of the spermoderm extracts also split into several components upon elution and rechromatography (Fig. 8.3.). Those eluates also showed varying degrees of yellow breakdown products and the stationary materials. Thus, the breakdown as well as the splitting of anthocyanins was broadly similar to the one presented in this section. The preponderance of anthocyanin breakdown encountered in the course of early work, as described in this section, appears primarily to be due to total evaporation in vacuo. The question as to whether or not the degradation is due to flash evaporation or to the solvents used or both may find answer in subsequent sections (particularly section 15, and 23).

In spite of the degradation, the R_f values of different anthocyanins could still be determined in most cases and the eluates could still be hydrolysed to determine the parent anthocyanidins. However, owing to the splitting of anthocyanins, sufficient quantities of isolates for the glycosidic and spectral analysis could not be obtained. Because the anthocyanins, immediately after elution, appeared to be quite stable, and because the anthocyanins could not be dried (because of degradation), it was decided to explore the possibility of studying their spectra after direct elution with methanol containing 0.01% conc. HCl, the solvent used by Harborne (1958) for spectral identification of anthocyanins. The results of this study are described in the next section.

11.5. SUMMARY

It was demonstrated in Section 8, that the spermoderm anthocyanins are complex and split into several components upon rechromatography. The basal leaf sheath anthocyanins (Field Collection 130, and 138) also split, likewise, into several components following elution and flash evaporation. In addition, the anthocyanins of barley are highly labile because the usual techniques of handling give rise to varying degrees of breakdown. The breakdown to yellow products, in general, occurred only in the final stages of flash evaporation. The eluates obtained from weak anthocyanin bands suffered greater breakdown than those from the strong bands. The differences in stability may be largely due to the differences in quantity than the differences in structure of anthocyanins because the instability was genally unpredictable.

A part of the breakdown product, upon chromatography, remains stationary at the starting line. It will be demonstrated in Section 19.3.3 that the breakdown to the stationary materials is due to the polymerization of anthocyanins.

METHODS FOR CHARACTERIZING ANTHOCYANINS

12. SPECTRAL EVIDENCE FOR THE INSTABILITY OF ANTHOCYANINS

In the preceding section it is shown that anthocyanin drying could not be undertaken owing to breakdown that occurred with flash evaporation. However, the pigments must be available in a pure, dry state so that the spectra can be studied in the solvent, namely, methanol containing 0.01% conc. HCl (Harborne,1958). Since the eluates appeared to be stable prior to flash evaporation, the feasibility of eliminating drying (by flash evaporation) and eluting anthocyanins directly with Harborne's spectral solvent, was examined. The anthocyanins again degraded upon elution and processing. In this section, the spectral characteristics of certain degradation product or products of anthocyanins and anthocyanidins in the wavelength range 330-600 mu will be presented.

12.1. MATERIALS AND METHODS

The BAW bands* of the Field Collections No. 130 and 138 (Fig. 10.3) were eluted with 0.01% conc. HCl in methanol directly from the paper strips. The spectra of the eluates was drawn in the wavelength range 330 -600 mu against a solution obtained from blank areas of the chromatograms. The elution and spectral analysis were completed on the same day in about 16 hours. The elution of all anthocyanin bands took 8-10 hours depending upon the rate of elution of each anthocyanin. The spectral analysis, commenced immediately after elution, took about 6 hours.

The anthocyanidin, E3-138, was obtained by the hydrolysis of the crystalline anthocyanin E3-138 (Table 11.I), and the anthocyanidins G-138* , J-130 and F-130 by the hydrolysis of the corresponding anthocyanin eluates. The hydrolysates were purified by chromatography in the Forestal solvent by the usual procedures (Harborne, 1958) except that elution was carried out with methanol containing 1% conc. HCl. The eluates were dried *in vacuo*. The other anthocyanidins, namely DE3(Sec. 19.3.7.2), 7H and 5L (19.3.7.3), were prepared from the corresponding anthocyanins by the method described in Section 19.2.3.

All spectral measurements were made with Beckman DK2 spectrophotometer in 1-ml silica cuvettes using 0.01% methanolic HCl.

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*The orange colored BAW band G-138 (Table 11.I) resolves as a mixtures of anthocyanins. The band was first eluted with methanol containing 1% conc. HCl, concentrated *in vacuo*, and chromatographed in the HAc-HCl solvent. The orange component separated on the chromatogram was designated as G4x-138. The component is designated in this section as G-138 for expediency.

12.2. RESULTS

Eight anthocyanins, A-138, B-130, C-138, D-138, E-130, F-130, G-138 and H-130, were eluted simultaneously under similar conditions. At the time, the spectra were drawn, the colors of the eluates of the bands A-138 and F-130 changed to reddish yellow and that of the band G-138 to orange yellow; the color of eluates of the bands B-130, C-138 and H-130 changed almost completely to yellow. The spectra of the last eluates were, therefore not drawn; that of the other eluates are shown in Fig. 12.1.

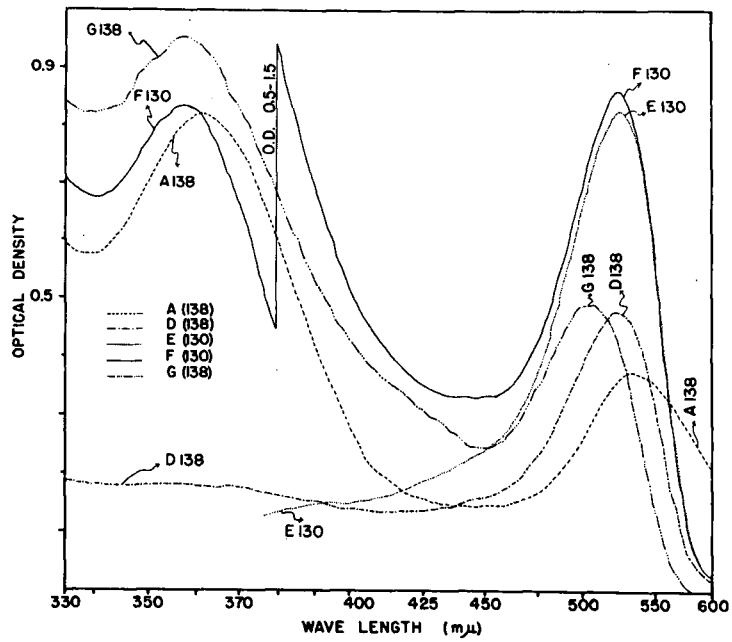

Fig. 12.1
Absorption spectra in 0.01% methanolic HCl of the anthocyanin bands A, D, E, F, and G from leaf sheaths of Gopal. A, F, and G are partially degraded but show the characteristic flavylium peaks. D and E are stable and do not show the degradation peak, ca. 365 mu.

The partially degraded anthocyanin eluates A, F, and G (Fig. 12.1), apart from their characteristic flavylium salt peaks (528, 519 and 504 mu respectively), show λ_{max} ca. 362, 356 and 358 mu, and λ_{min} ca. 336, 340 and 340* mu, respectively. The anthocyanins D and E, however, show only the usual spectra obtained from stable anthocyanin solutions.

It was observed that the 'yellowness' of the anthocyanin eluates increased upon standing. The conversion to yellow materials occurred even at night while the eluates stood in incandescent light in the course of spectral measurements. By morning, the eluates had become pale yellow. It was observed later, in other cases, that the pale yellow solutions became almost colorless upon further standing. It is shown elsewhere that color change is a guide to a series of degradative spectral modifications, which occur when eluates stand under room conditions for any length of time (Mullick and Brink, in press).

That the spectral peaks around 360 mu do not characterize the anthocyanin molecule but are characteristic of degradative modifications was appreciated in the course of spectral studies on anthocyanidins. The spectra of only a few anthocyanidins will be presented to illustrate the point.

Fig. 12.2 shows the spectra of cyanidins obtained from hydrolysis of the anthocyanins E3-138 (Table 11.I), and DE3 (Table 19.VIII.DE). Both of these anthocyanins are chromatographically identical and are

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*Although the λ_{min} value ofthe anthocyanin G-138 is not distinct, the value 340 mu was obtained from an identical anthocyanin eluate G-130. However, the anthocyanin G-130 showed far greater degradation: the flavylium peak was almost swamped by the degradation peak.

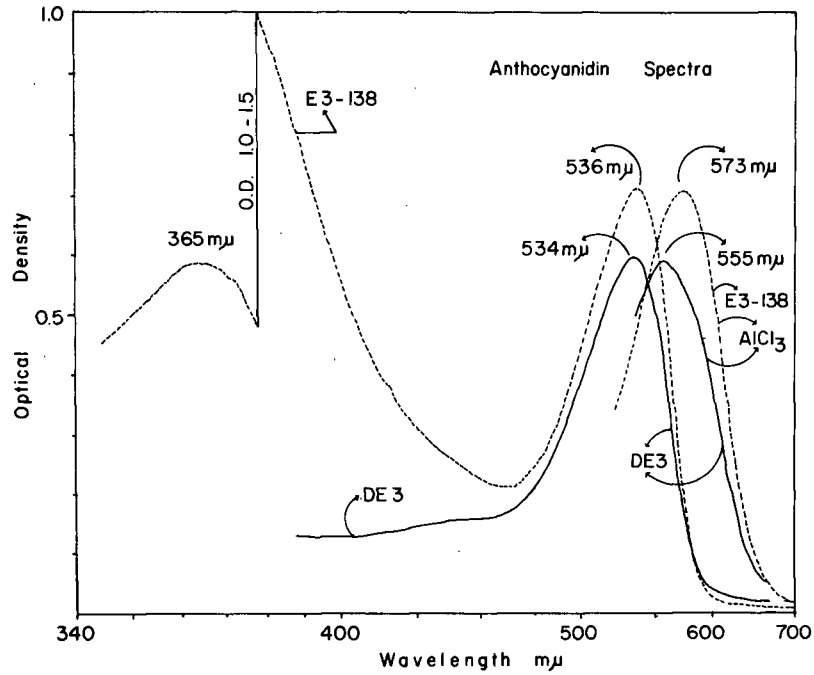

Fig. 12.2
Spectra in 0.01% methanolic HCl of cyanidins obtained from the hydrolysis of identical anthocyanins from bands E3-138 and DE3 from basal leaf sheaths of Gopal. Note the peak at 365 mu resulting from degradation.

obtained from basal leaf sheaths of the purple variety, Gopal. The cyanidin, E3-138, was obtained in earlier work by chromatographic purification (Harborne, ibid.) and the other cyanidin, DE3, by the method which was finally used throughout this study (Sec. 19.2.3). The cyanidin, E-138, became reddish yellow in solution, and shows two peaks at 536 mu and ca. 365 mu. The cyanidin, DE3, remained clear pink in solution and shows only one peak at 534 mu. The aluminum chloride shifts (Fig. 12.2), which confirm the identification, are notably variable (cf., Harborne, ibid.).

A similar situation is shown in Fig. 12.3 with respect to pelargonidins obtained from the hydrolysis of the anthocyanins 7H, and G-138. Although the anthocyanin 7H was obtained from grain tissues

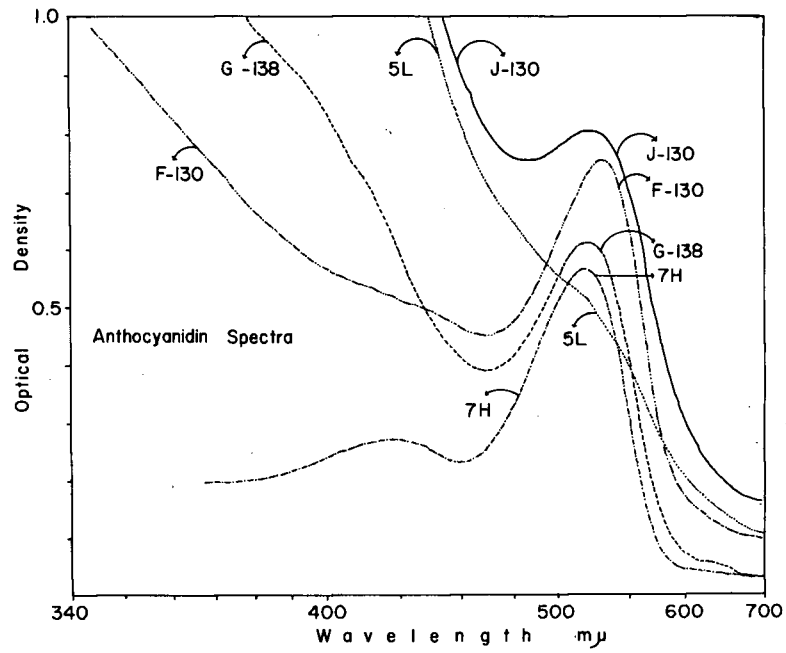

Fig. 12.3
Spectra in 0.01% methanolic HCl of pelargonidins derived from the hydrolysis of bands 7H and G-138, of peonidin derived from band F-130, of cyanidin derived from band 5L and of an anthocyanidin derived from an unknown anthocyanin band J-130. The degradation peaks ca. 365 mu, are masked but are probably present (see Fig. 8.2).

(Sec. 19.3.7.3), and the anthocyanin G-138 from the leaf tissue, both are chromatographically identical. It will be noted that whereas the pelargonidin, 7H, which remained orange red in solution, shows the usual spectral features obtained from stable solutions of pelargonidin, the pelargonidin, G-138, which became orange yellow, shows the increasing absorption below the wavelengths around 440 mu. No aluminium chloride shift was obtained with the two pelargonidins. The anthocyanidin, F-130, a peonidin, also, did not show the aluminum chloride shift. However, it did show increasing absorption below the wavelengths around 440 mu. In several cases, it was noted that as the anthocyanidin solutions became

yellowish, the absorption due to degradative transformations became so pronounced that the flavylium peaks were confounded as is shown for anthocyanidins J-130 and 5L.

12.3. DISCUSSION

The results show that although anthocyanins were stable immediately upon elution, with time, degradation occurred in several cases. Direct elution with the spectral solvent* proved to be unsatisfactory. Although the eluates were protected against light during handling, remaining in darkness during elution, exposed only to incandescent light at night during spectral analysis, and only exposed at most for a few hours in half light as they were handled in the laboratory, degradation occurred. Harborne (1958), using the same solvent did not encounter much degradation in the course of spectral analysis, presumably over a short period of time. Since the eluates in our case were also stable immediately upon elution, the degradation must have occurred upon standing over a period of time. Consistent with the belief that high acid concentration, such as 1% HCl, extracts and preserves anthocyanins and anthocyanidins (Robinson and Robinson, 1933), the breakdown in 0.01% conc. HCl was assumed, at the time, to be due to the low quantity of the acid. It was

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*Anthocyanin instability, at the time this study was carried out had arrested further progress on anthocyanin identification. This investigation was undertaken, as a last resort at that time, to determine if some parameters for identification of anthocyanins could be established by spectral means. Obviously, the acidity of the spectral solvent would have increased during elution due to evaporation of methanol, making the spectral comparisons not strictly comparable to those obtained by Harborne (ibid.).

believed that Harborne (ibid.) chose low concentration of acid* because of the requirements of the aluminum chloride test (Geissman and Jurd, 1955).

That the spectral peaks around 360 mu represent the degradative transformations of anthocyanins and anthocyanidins, occurring on standing in solution, is shown by the spectra of cyanidins (Fig. 12.2) and pelargonidins (Fig. 12.3). It is clear from the spectra presented elsewhere (Figs. 18.2. and 18.3) that the degradative peaks do not arise from chromatography paper during elution. The anthocyanidins purified by chromatography show the degradative peaks, presumably because of the extra time taken for elution and drying. Although anthocyanidins prepared without elution (Sec. 19.2.3) remained generally stable, some degradation nonetheless occurred when either the quantities of anthocyanidins obtained in hydrolysates were small, or when the original anthocyanin isolates could not be isolated in stable state. The anthocyanin 5L, for example, suffered partial degradation during isolation: the spectra of the hydrolysed isolates (Fig. 12.3), therefore, show marked absorption in the degradative spectral range.

The conclusion that the peaks around 360 mu are due to degradation is reinforced from the results presented in Section 14: the results show that spectra of anthocyanins, drawn directly from paper (without elution), do not show the degradative peaks nor any increasing absorption below the wavelengths 440 mu. The degradative peaks were absent because anthocyanins remain relatively stable on the paper. Again, the spectra

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*It is not clear whether Harborne (1958) used methanol containing 0.01% conc. HCl or 0.01% HCl as the spectral solvent: both ranges of concentration are mentioned in the publication. (See also Jurd, 1962; p. 133)

from crystalline anthocyanins do not show the degradative peaks. Thus, although the spectra of anthocyanins shown in Fig. 12.1 would not be given immediately after elution, it can be safely assumed that the degradative peaks must have appeared as a result of standing.

It was observed that several partially degraded anthocyanins and anthocyanidins did not show the degradative peaks around 360 mu, but they did show increasing absorption in the degradative region of the spectrum (see Fig. 12.3). It appears that such increasing absorption represents a further stage in degradation. Although it is well known that anthocyanins are unstable, the mechanisms and causes of time-induced degradations are not clearly understood.

The results also indicate that the degradative peaks did not appear in all anthocyanins. Did the anthocyanin D and E remain stable because they were present in larger quantity as compared to other anthocyanins (see Fig. 10.3)? If the differential stability is due to quantitative differences, then the spectrum of the anthocyanin E-130 should have shown some absorption around 360 mu: it was shown in Section 11 that this anthocyanin does show some breakdown products. The differential stability of eluates does not appear to be due to 'standing' alone because all anthocyanins were eluted and processed simultaneously under similar conditions. In any event, the reasons for differential breakdown are not known. Literature on the causes of breakdown of anthocyanins is scanty: only the breakdown of the strawberry anthocyanin, pelargonidin-3-glucoside has been investigated in any detail (Lukton, Chichester and Mackinney, 1956; Tinsley and Bockian, 1960). But the spectral analysis of the breakdown of this anthocyanin has been done at long time intervals and conditions of experimentation are far more removed than those

obtained during chromatographic elution and processing, and hence no leads can be drawn from their work.

Anthocyanin instability is of more concern than has been generally appreciated and may adversely influence interpretations in several ways. It is clear that owing to the presence of degradative peaks, the ratio of O.D. 440 mu/O.D. λ_{max} as a means of distinguishing 3-monoglycosides from 3,5-diglycosides (Harborne, loc. cit.) is practicable only in the case of crystalline anthocyanins or those chromatographically purified anthocyanins which show no degradation, or those which have been purified through nylon or polyclar-AT columns (Sec. 18.1.3) by milder methods of processing (Sec. 16). The polyamide purification is suggested to eliminate both the paper-derived 'yellow materials' (Figs. 18.2, 18.3, 18.4, and 18.5) and the 'degradation products' of anthocyanins, both of which influence the absorption below the wavelengths around 440 mu.

That the peak around 360 mu is due to degradation products of anthocyanins does not appear to have been recognised in the literature. Thus none of the quantitative studies on anthocyanins, has taken into consideration the amount of anthocyanin degradation represented by the peak around 360 mu. The spectra of anthocyanins (wavelength range 400-600 mu) reported by several workers such as Thimann and Edmondson (1949), Jorgensen and Geissman (1955), and others, who carried out quantitative studies on anthocyanins show the presence of increasing absorption below the wavelength range 440 mu. The spectra reported recently by Knoll (1963) do show the presence of the peak around 360 mu. Thimann et al. (ibid.) believed that "the high absorptions in the near ultraviolet (around 400 mu) are probably due to accompanying flavone pigments." Jorgensen et al. (ibid.) ascribed the presence of the high absorptions (their spectra look

very similar to the anthocyanidin J-130: Fig. 12.3) as due to aurone and flavone glycosides. Since the instability of anthocyanins has been acknowledged in the above two publications, it is likely that a portion of the high absorptions could also have been due to the degradation of anthocyanins. Although the above workers did not use chromatography for elution and purification, under which conditions the degradation is generally more pronounced, Knoll (ibid.) did purify the anthocyanins by chromatography and ascribed the increasing absorptions around 360 mu to the presence of interfering flavonoid pigments and not to the degradation of anthocyanins. The above observations clearly indicate that spectral studies on the time-induced degradative transformations of anthocyanins must be continued further to establish a sound basis for calculating pigment concentrations until dependable methods for ensuring anthocyanin stability are found. The peaks around 360 mu may not be sufficient for establishing the extent of degradation because several other unreported spectral observations on the disappearance of the peaks around 360 mu indicate that upon standing, degradation into other products also occurs.

New light on the probable nature of the peaks around 360 mu has been recently shed by the work of Jurd (1963). He shows that synthetic flavylium salts lacking a 3-substituent, in the pH range 1-4, undergo reversible transformations to the corresponding 2-hydroxychalcones. The peaks around 360 mu shown in Fig. 12.1 appear to be very similar to those of Jurd (ibid.). At the time this work was carried out, the reversibility of the reaction over a 2-hour period (cf., Jurd, ibid.) was not studied. In this connection, it is noteworthy that although the synthetic flavylium salts lacking a 3-substituent do chalconize, Jurd (1964) found that natural anthocyanins, in the pH range 1-4, however, do not chalconize but

give rise to pseudo base modifications. Thus, the situation with anthocyanins appears to be quite involved and calls for further investigations.

Until further understanding of the nature of degradation products is obtained, the losses of anthocyanins occurring due to peaks around 360 mu and also, more importantly, the amounts of the stationary materials that remain at the starting line upon chromatography of anthocyanin eluates (Sec. 11) must be provided for in any quantitative work.

The studies in Sections 11 and 12 establish that anthocyanins of barley are highly unstable. Why did most anthocyanins degrade partially or completely, while others could be recovered in a stable state? Are the degradation and also the splitting of anthocyanins into several components due to the inherent complexity of barley anthocyanins, or are the degradation and the splitting merely hydrolytic or degradative artefacts arising out of the shortcomings of the techniques of our handling? These two points could be adequately established if anthocyanins could be chromatographed without the intervention of elution and flash evaporation. This was achieved by sewing the anthocyanin band segments on new sheets of chromatography paper. The results will be presented in the next section.

12.4. SUMMARY

The anthocyanins could not be obtained in a pure dry state for spectral studies because of their breakdown following elution and flash evaporation (see Sec. 11). Notwithstanding the breakdown, the R_f values of anthocyanins could still be determined. In order to reinforce the chromatographic characterization, the anthocyanins of the Field Collections

130, and 138 were eluted for spectral characterization directly with the spectral solvent (methanol containing 0.01% conc. HCl). The elution and spectral analyses (wavelength range 330-600 mu) were completed in a continuous operation lasting about 18 hours. The spectra from several eluates gave, in addition to the corresponding flavylium peaks, well-defined peaks around 360 mu. Additional evidence from the spectra of anthocyanidins is presented to establish that the peak around 360 mu, probably due to a chalcone, represents a degradative modification of anthocyanins, a fact which does not seem to have been recognized in the literature.

METHODS FOR CHARACTERIZING ANTHOCYANINS

13. THE SEWING TECHNIQUE AS AN AID IN CHARACTERIZING BARLEY ANTHOCYANINS

The instability of barley anthocyanins hindered progress in characterizing anthocyanins as shown in the two preceding sections. It is well established that although anthocyanins are unstable in solution they are quite stable on paper. Sewing* excised anthocyanin bands after preparative chromatography directly on fresh chromatopaper appeared to hold excellent possibilities for the elimination of elution (i.e., the solution) and flash evaporation, and hence for the elimination or reduction of the degradation. In this section data are presented to show

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*The sewing technique has also been used by Yang et al. (1960), in the paper chromatographic determination of rutin in tobacco. Also see review by Lederer and Lederer (1957, p. 129).

whether the instability and splitting of anthocyanins (Secs. 11 and 8) are due to the shortcomings of the published or standard techniques for anthocyanin study, or due to special properties of barley anthocyanins. A clear-cut answer was essential to extend the basic objectives of the research program.

In this section, partial characterization data for the basal leaf sheath anthocyanins, obtained from the field grown materials, by R_f values determined exclusively by sewing technique, will be presented. Value of the data is seen in qualitative comparison of anthocyanins obtained from the field, and greenhouse (See sec. 19) grown materials).

In keeping with the overall objectives, it also appeared desirable, not only as a means of eliminating elution, but also as a means of saving labor, to determine if anthocyanins could be characterized partially, at least, for comparative work such as is called for in hybrid analysis, without elution from paper. In comparative work the characterization potential of R_f values obtained by sewing could be reinforced not only by the spectral studies on paper (Sec. 14), but also by the identification of aglycones by the method of anthocyanin hydrolysis on paper developed in this laboratory (Sec. 20).

13.1. MATERIALS AND METHODS

The anthocyanin bands of the basal leaf sheaths of the purple variety, Gopal (Field Collection No. 130 and 138; ex. Sec. 10) obtained from the BAW run (Fig. 10.3) were used in this investigation.

13.1.1. Sewing Technique

Small anthocyanin bands ca. 1" long were excised and sewn by hand, and long bands, ca. 8" long, by machine to fresh sheets of chromato-paper. Nylon thread was used for sewing because cotton thread yielded blue fluorescing contaminants. Good resolution is obtained when the entire surface of the band segment, particularly the edges, are assured of intimate contact with the new chromato-paper. If the anthocyanins on the bands were weak, as many as 2 to 6 band segments, half on the upper and half on the under surface of the new paper were stitched. The excised bands must be sewn on paper of the same thickness and texture to obtain good resolution.

13.1.2. Chromatography of Anthocyanins

Chromatography was carried out on Whatman paper No. 3 in the solvents described elsewhere (Sec. 10.2.2; Table 10.II) by descent. In general, the chromatograms were developed in formica-lined two-way "RSCO" chromato-cabinets.

13.1.3. Chromatography of Anthocyanidins

The anthocyanin eluates were hydrolysed as described elsewhere (Sec. 18.1.4) and identified by chromatography in the solvents included in Table 17.I.

13.1.4. Notations Used for Designating Anthocyanins

Although the system of notations described elsewhere (Sec. 11.2.3) is used throughout, it had to be modified slightly for designating

the anthocyanins shown in Table 13.III. The modification was necessitated because the BAW bands of the Collection No. 130 (Fig. 10.3), were re-chromatographed in the BAW solvent, instead of the usual HAc-HCl solvent. The purified BAW bands were rechromatographed further in the Aq-HCl solvent. Thus, the anthocyanin band obtained finally following the purification in Aq-HCl, for example, the anthocyanin Ala (Table 13.III) will be designated Ala-130 (BAW(2)-Aq-HCl). The additional notation is self-explanatory: it denotes that the anthocyanin Ala-130 was obtained following two purifications in the BAW and final purification in the Aq-HCl solvents. The system of notation, otherwise, is basically the same as that used in Section 11.2.3.

13.2. RESULTS

13.2.1. Anthocyanins of the Field Collection No. 138

The chromatographic results of the BAW anthocyanin bands (see Fig. 10.3 and Table 11.I) of the basal leaf sheaths of Gopal (Field Collection No. 138) by sewing technique in four solvents, namely BAW, Bu-HCl, Aq-HCl, and HAc-HCl are shown in Figs. 13.1, 13.2, 13.3 and 13.4 respectively; the R_f values, colors, and relative concentrations of the anthocyanins, as estimated visually, in the four solvents are summarised in Table 13.I. Different numbers of excised band segments, depending upon the intensity of the anthocyanin, were stitched; the number of band segments stitched is indicated in parentheses in the figures.

It will be noted that whereas the BAW band A-138 shows only one distinct anthocyanin upon rechromatography in the BAW solvent (Fig. 13.1), it shows at least two very distinct spots in the HAc-HCl (Fig. 13.4), three

TABLE 13.I

R_f VALUES RELATIVE CONCENTRATION[1] AND COLORS OF THE BAW ANTHOCYANIN BANDS FROM GOPAL LEAF SHEATHS (FIELD COLLECTION 138) BY THE BAND STITCHING TECHNIQUE

BAW	BAW Re-run[3]				Bu-HCl[3]				Aq-HCl[3]				HAc-HCl[3]			
Band	R_f x 100	Rel. Conc.	Colors V	Colors UV	R_f x 100	Rel. Conc.	Colors V	Colors UV	R_f x 100	Rel. Conc.	Colors V	Colors UV	R_f x 100	Rel. Conc.	Colors V	Colors UV
A	12		PVio	VioR	7	(20)	P	VioMv	6	(2)	RP	Wk	27	(1)	Wk	
					11	(1)	WkR	WkVio	30	(20)	1P	Vio	58	(20)	PR	VioMv
					21	(1)	WkR	dMv	51		wk		80	?	?	?
C	15	(10)	Mv	wk	7	(3)	PR	dPk	7	4	PR		27	(1)	PR	wk
	20	(1)	wk		22	(2)	Mv	Mv	31	4	Mv	wk	66	(2)	Mv	?
									44		weak, not definite					
D	24		M	dMP	28		M	dP	10		M	dP	34		M	dP
E	24		DM	DM	30		DM	DM	10		DM	DM	36		DM	DM
F	35		Pk	bPk	34		Pk	bPk	12		Pk	bPk	40-45		Pk	bPk
G	23	(1)	PkMv	DMv	27	(1)	PkMv	DMv	7	(1)	PkMv	DMv	32	(1)	PkMv	DMv
	37	(3)	OR	DMv	41	(3)	OR	DMv	16	(2)	OR	DMv	44	(2)	OR	DMv

[1]Estimated values

[2]See Table 4.II for abbreviations

[3]All R_f values were determined on Whatman paper No. 3.

Fig. 13.1.
Features shown by rechromatography of the BAW bands of Gopal leaf sheaths (Collection 138) in the BAW solvent. Number of pieces stitched at each starting point is given.

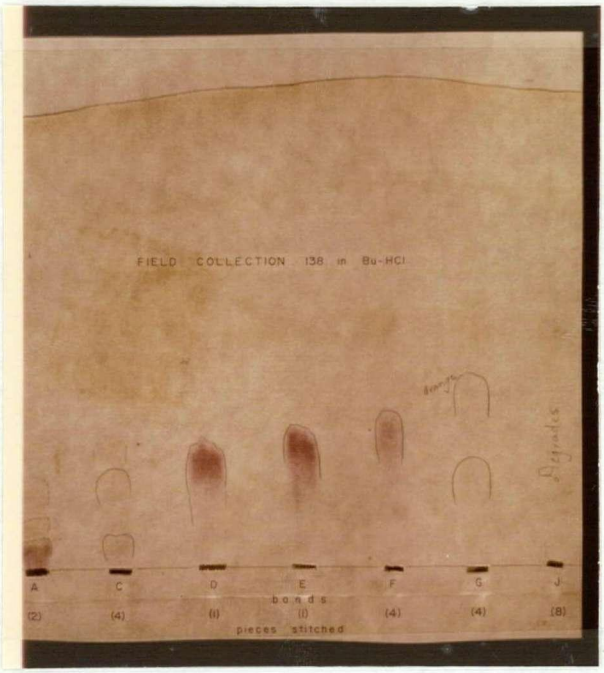

Fig. 13.2.
Features shown by rechromatography of the BAW bands of Gōpal leaf sheaths (Collection 138) in the Bu-HCl solvent. Number of pieces stitched at each starting point is given.

spots in the Bu-HCl (Fig. 13.2), and also three spots in the Aq-HCl (Fig. 13.3). However, when this band was chromatographed following elution, it gave rise to four spots in the HAc-HCl solvent (Table 11.I). The band B, being too weak, was not chromatographed, though it could have been chromatographed by the concentration technique (see Sec. 16) developed later on. The characteristics of the band C, are similar to those of band A; two spots were given in BAW, 2 to 3 spots in Bu-HCl, 3 to 4 spots in

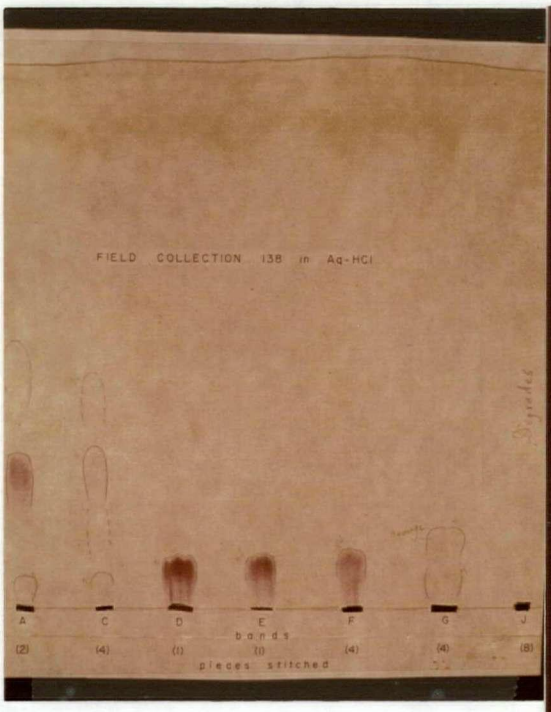

Fig. 13.3.
Features shown by rechromatography of the BAW bands of Gopal leaf sheaths (Collection 138) in the Aq-HCl solvent. Number of pieces stitched at each starting point is given.

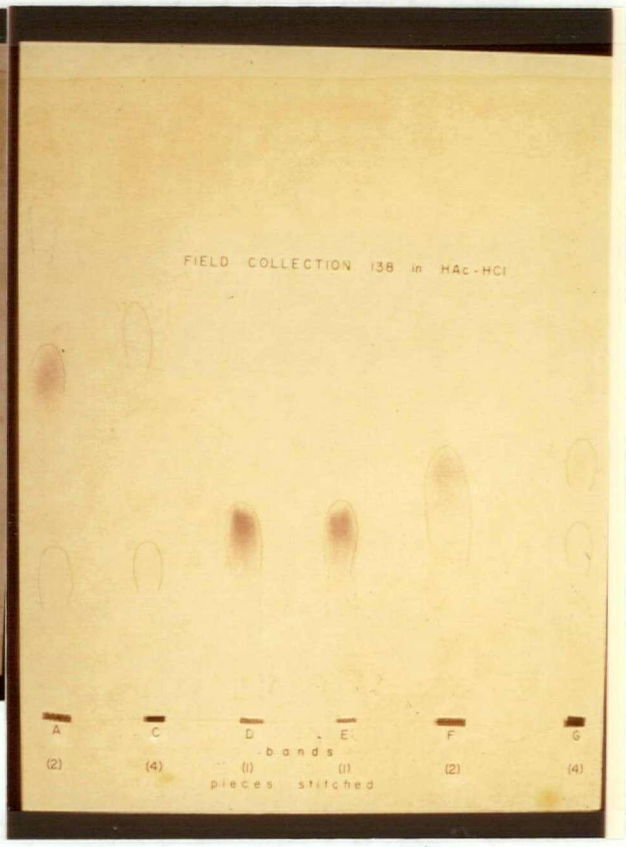

Fig. 13.4.
Features shown by rechromatography of the BAW bands of Gopal leaf sheaths (Collection 138) in the HAc-HCl solvent. Number of pieces stitched at each starting point is given.

Aq-HCl, and only two spots in the HAc-HCl by the stitching technique (see Figs. 13.1 to 13.4). However, 4 spots were given in HAc-HCl by the elution technique (Table 11.I). Whereas the bands D, E, and F gave only one spot in all the four solvents by stitching, they gave 2, 5, and 3 spots respectively in the HAc-HCl solvent by elution. Although the spot G degraded completely in early attempts by the elution technique, it gave 2 spots, one an orange red, and the other a bluish red, in each of the four solvents by stitching.

These patterns initially were puzzling. It was, however, observed that many of the differences were partly due to the difficulties in the visual detection of the spots. It was observed that when 2 segments of the band F were stitched, it gave only one trailing spot. However, when 4 segments were stitched, two additional spots were given in addition to the major trailing spot (see Fig. 13.8 as well). The band F by elution gave only two components (cf., Table 11.I). Shown in Fig. 13.8 are the results, when one and six segments of the band F-130 (cf., Fig. 13.7) were stitched in the HAc-HCl solvent: up to six spots were obtained when six segments were stitched. Thus, differences in spot numbers may be often due to variations in the quantity of anthocyanins 'per unit area'* of the segment. The results of the Field Collection No. 130, presented in Section 13.2.3. support the view.

13.2.2. Anthocyanidins of the field Collection No. 130

The anthocyanidins obtained from the BAW bands A, B, D, E, and H (Fig. 10.3), and the HAc-HCl components F1, F2, G1, and G2 (Table 11.I) of the Field Collection 130 are shown in Table 13.II. The anthocyanidins were characterized by chromatography in several solvents. It will be noted that the anthocyanin bands B, F, G, and H resolve as a mixture of anthocyanins, because each of the bands on hydrolysis yields two anthocyanidins. It is clear from Table 13.II that cyanidin, peonidin, and pelargonidin are the aglycones of basal leaf sheath anthocyanins obtained from the field

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*The expression is used here rather loosely because the detectability is improved when a longer band is stitched compared to a small band: evidently the quantity per unit area is the same.

TABLE 13.II.
ANTHOCYANIDINS FROM THE HYDROLYSATES OF THE BAW ANTHOCYANIN BANDS FROM GOPAL BASAL LEAF SHEATHS (FIELD COLLECTION 130).

BAW Band	R_f x 100								Identification
	Forestal	FA-Harborne	FA-4NHCl	Propionic	Iso-PrOH	5:1:5	HAc-HCl	BAW	
A	52	26	38	30	48	38	11	60	Cyanidin
B	50		37					59	Cyanidin
	66		50					66	Peonidin?
D	50	24	36	29	39	36	11	65	Cyanidin
E	50	22	36	39	48	38	11	59	Cyanidin
F1		22	37					55	Cyanidin
F2		28	50	41	49	49		67	Peonidin
G1	48	22	36						Cyanidin
G2	69	32	52	46	60			82	Pelargonidin
H			37		39				Cyanidin?
			52		56				Pelargonidin?
I	48	22	36	30	50	38	10		Cyanidin

grown materials. It is noteworthy that the orange component of the band G-130 is a pelargonidin derivative. The identifications are also applicable to the anthocyanins of the Collection 138. The identification of the band B is slightly doubtful because of the scarcity of the isolate.

13.2.3. Anthocyanins of the Field Collection No. 130

The extracts of the Field Collection No. 130 which, as indicated elsewhere (Sec. 10), were also obtained from the basal leaf sheaths of

Gopal. The sheaths of both the Collections represented about the same stage of pigment development except that the stocks of the Collection No. 138 were planted 10 days ahead of the stocks of Collection No. 130. The chromatographic results of the BAW bands of the Collection No. 130 (Fig. 10.3) in three solvents, namely, BAW, Bu-HCl and HAc-HCl by the sewing technique are shown in Figs. 13.5, 13.6 and 13.7 respectively; the relevant chromatographic data in the 4 solvents is summarized in Table 13. III.

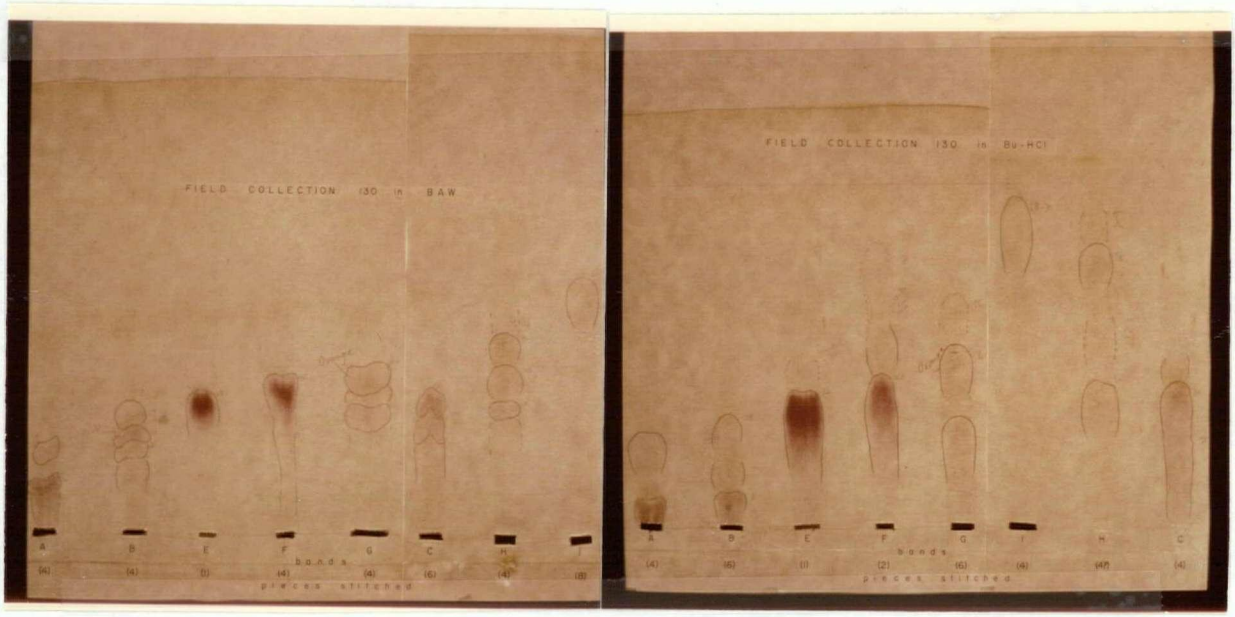

Fig. 13.5.
Features shown by rechromatography of the BAW bands of Gopal leaf sheaths (Collection 130) in the BAW solvent. Number of pieces stitched at each starting point is given.

Fig. 13.6.
Features shown by rechromatography of the BAW bands of Gopal leaf sheaths (Collection 130) in the Bu-HCl solvent. Number of pieces stitched at each starting point is given.

The R_f values in the Aq-HCl (Table 13.III) are only approximations because the solvent fronts ran off the sheets. Most of the stitched bands, upon chromatography, yielded trails; in such cases the spot circles are left

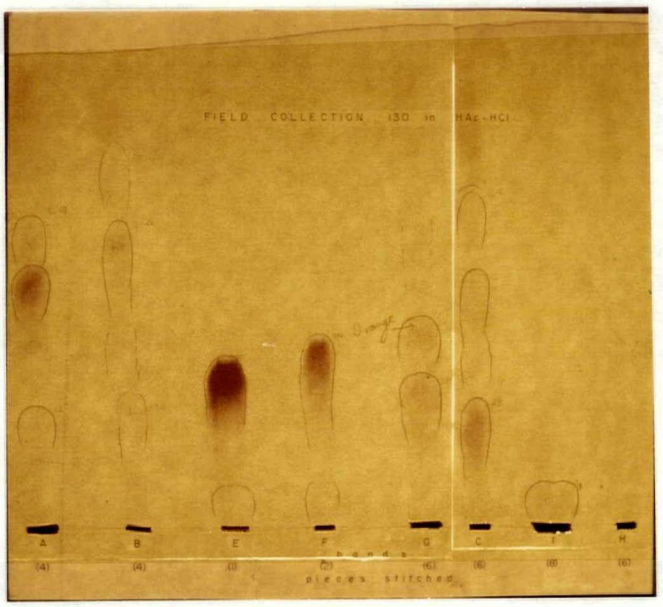

Fig. 13.7.
Features shown by rechromatography of the BAW bands of Gopal leaf sheaths (Collection 130) in the HAc-HCl solvent. Number of pieces stitched at each starting point is given.

open toward the trailing end because the trails are too weak to determine their limits. The trails usually extend over a longer distance than shown in the figures.

The results of the Collection No. 130 may now be compared with those of the Collection No. 138. The figures included in parenthesis, in the text, indicate the number of spots obtained from the Collection No. 138 in the corresponding solvent by stitching. It will be noted that in the case of the BAW band A-130, four band segments were stitched as against two stitched for the band A-138 in all the solvents. Upon rechromatography in the BAW solvent (Fig. 13.5), the band A-130 gives 2 spots (instead of one); the trailing of the major spot, in fact, suggests the probable presence of a third spot. In the Bu-HCl solvent (Fig. 13.6) it gives only two spots (instead of 3). In the Aq-HCl solvent (Table 13.III), it gives 3 spots (also, 3). But in the HAc-HCl solvent (Fig. 13.7), it gives 4 spots (instead of 2), which in R_f values match the R_f values of the spots obtained by the elution technique (Table 11.I).

TABLE 13.III

R_f VALUES, RELATIVE CONCENTRATION,[1] AND COLORS[2] OF THE BAW ANTHOCYANIN BANDS OF GOPAL LEAF SHEATHS (FIELD COLLECTION 130) BY THE BAND STITCHING TECHNIQUE

	BAW Re-run[4]				Bu-HCl[4]				Aq-HCl[3,4]				HAc-HCl[4]			
			Colors				Colors				Colors				Colors	
BAW Band	R_f x 100	Rel. Conc.	V	UV	R_f x 100	Rel. Conc.	V	UV	R_f x 100	Rel. Conc.	V	UV	R_f x 100	Rel. Conc.	V	UV
A	12	8	PVio	P	7	20	PVio	P	SF ran off.				10	wk	wk	wk
	20	1	1P	P?	24	1	wk	wk	3 spots @				26	1	R	R
									ca. R_f 7,				56	20	PVio	P
									32 & 51				65	4	B1P	wk
B	20	2	B1R	B1R	8	10	PkMv	?	SF ran off.				10	t		
	24	5	Pk?	Mv?	15	1	R?	?	3 spots				26	1	M	
	27	1	R?	wk	28	1	M?	?					66	10	PkMv	
	30	wk	R?	wk									74	wk	wk	wk
C	24	1	B1R		A long trail to				SF ran off.				27	10		
	30	3	R?		R_f 30				3 spots				52	3		
													70	1		
E	31		M	dM	32	100	M	dM	12		M	dM	9	1	R?	Rf?
					40	1	R?	?					35	100	M	dM
					70	wk	R?	?								
F	36		Pk	dPk	37		Pk	dPk	14		Pk	bPk	39		Pk	bPk
G	29	1	Mv	DMv	27	3	Mv	DMv	9	1	Mv	DMv	30	1	Mv	DMv
	39	2	OR	DOR	44	5	OR	DOR	17	1	O	DOR	42	1	OR	DOR
H	28	2	Mv		30	5	Mv		5	2	Mv		33	3	Mv	
	35	1	?		58	3	Mv		20	1			42	1	Mv	
	42	2	?										64	1	Mv	
I	54		R	wk	68		Pk	Rf	1.5		Pk	Pkf	10		R-Pk	Rf

[1] Estimated values

[2] See Table 4.II for abbreviations

[3] Solvent front (SF) ran off: only approximate R_f values are given.

[4] R_f values determined on Whatman paper No. 3.

The BAW band B is weak and quite complex. The band is a mixture of two anthocyanins, derived from cyanidin, and probably, peonidin (see Table 13.II). Upon rechromatography, it gives 3-4 spots in BAW (Fig. 13.5), probably, 3 spots (note the trail) in Bu-HCl (Fig. 13.6), 3 spots in Aq.HCl (Table 13.III), and 4 spots in the HAc-HCl solvent (Fig. 13.7).

In the case of the BAW band C-130, 6 segments were stitched in each of the BAW and Bu-HCl solvents and 4 segments in the HAc-HCl solvent as against the 4 band segments stitched in each of the four solvents in the case of the BAW band C-138. Upon rechromatography, the band C-130 gave two spots (note the trailing spot) in the BAW (Fig. 13.5) solvent. Although the band C-138, also gave two anthocyanins, it appears from the R_f values (cf., Fig. 11.3 and Fig. 13.5), that there may be basic differences: the major spot of C-138 is at R_f 0.15, whereas that of C-130, at R_f 0.30. It is likely that the two spots of C-138 are equivalent of the lower trail (R_f 0.24) of the band C-130. Thus, the spot at R_f 0.30 (C-130) may be present as a contaminant in the band C owing to the poor resolution of anthocyanins in this region (Fig. 10.3). The contamination due to poor resolution is further supported by the chromatographic comparisons of the BAW bands, B-130, C-130 (Fig. 13.6) and C-138 (Fig. 13.2) in the Bu-HCl solvent. The major component (R_f 0.07) present in C-138 is absent from C-130 but it is conspicuously present in B-130. Thus it is clear that the band C-138 carries in it a contaminant from the band B-138 owing, probably to poor resolution in the preparative BAW chromatography. It will also be noted that in Bu-HCl, the band C-130 gives a major spot at R_f 0.30 which is absent from the band C-138. This spot may be a contaminant from band D (see later). Whereas in the HAc-HCl solvent, the band C-130 gave 4 spots, the band C-138 gave only 2 spots. The component at R_f 0.52 (band C-130:

Fig. 11.7) is probably a contaminant from band D-130 (see Fig. 13.7). It is, however, interesting that the R_f values of the band C-130 and C-138 in HAc-HCl obtained by stitching (Fig. 13.7) and by elution (Table 11.I) match closely.

It was pointed out in the preceding section that the band D did not split from the band E-130 (Fig. 10.3) in the preparative chromatography. However, in one case, the lower-most region of the band E, which showed a bluish tinge like that of the band D, was rechromatographed in the HAc-HCl solvent. It gave 2 spots (Fig. 13.7) at R_f 0.29 and 0.52, which again, match the R_f values of the spots E2-138 and E5-138 obtained by the elution technique (Table 11.I). It will also be noted that the component, R_f 0.29 is similar in R_f value, to the spots D2 and E2 (Table 11.I), and the component, R_f 0.52, matches the R_f values of the spot C3-130 (Fig. 13.7). Although the situation is confounded by several factors, it appears that the lowermost region of the band E-130 may be part of band D. Further work is essential to establish this point; meanwhile, the observations on the bands B, C, and D clearly indicate that the anthocyanins between the bands A and E in the BAW preparatory chromatography (Fig. 10.3) do not resolve properly. The observations indicate importantly that stitching as a tool in comparative chromatography, is very valuable because it eliminates elution as a step in processing.

In the case of the BAW bands E-130 and also E-138, only one band segment, because of the presence of large quantity of anthocyanins, was stitched in each of the four solvents. In all cases, only one major spot was recovered. However, the band E-130 shows conspicuous trailing both below and above the major spot. The bands E-130 and E-138 both show an additional presence of a weak spot at R_f 0.70 in Bu-HCl, and R_f 0.10 in

HAc-HCl (band E-130 only). In later studies, band E-130 following elution, drying over phosphorus pentoxide, and rechromatography in the HAc-HCl solvent, however, showed as many components (over 12) as those given by the band DE-400 elsewhere (see Sec. 19, Fig. 19.2).

The chromatographic features of the peonidin band F, pelargonidin band G and cyanidin band H are broadly similar to those of the other bands and will be dealt with in greater detail when the work on characterization of anthocyanins will be described (Sec. 19). However, it may be noted that band I gives only a weak spot in BAW, a relatively stronger spot in Bu-HCl and the strongest spot in HAc-HCl when the same number of band segments were stitched. This spot gives pink fluorescence and has been identified as cyanidin (Table 13.II).

The anthocyanin extracts from the basal leaf sheaths of the Field Collections 130, and 138, both of which were obtained from the same variety, Gopal, gave identical patterns (Fig. 10.3), except for some minor differences. Notwithstanding their chromatographic identity, the corresponding bands of the two collections show discrepancies in the number of spots obtained following chromatography in the four solvents, both by sewing and by elution techniques. The discrepancies were, indeed, puzzling and disconcerting. Although the prime cause of the discrepancies will be dealt with in Section 15, it was indicated briefly early in this section that the discrepancies in the number of detectable spots obtained by sewing are partly due to both the quantity of pigment in the segment, and the number of segments stitched at a time. This is clearly illustrated by the results from bands E, F, G and H (see Fig. 13.8). The number of band segments sewn is shown in parentheses. The results are self-explanatory. As the number of band segments stitched increases from

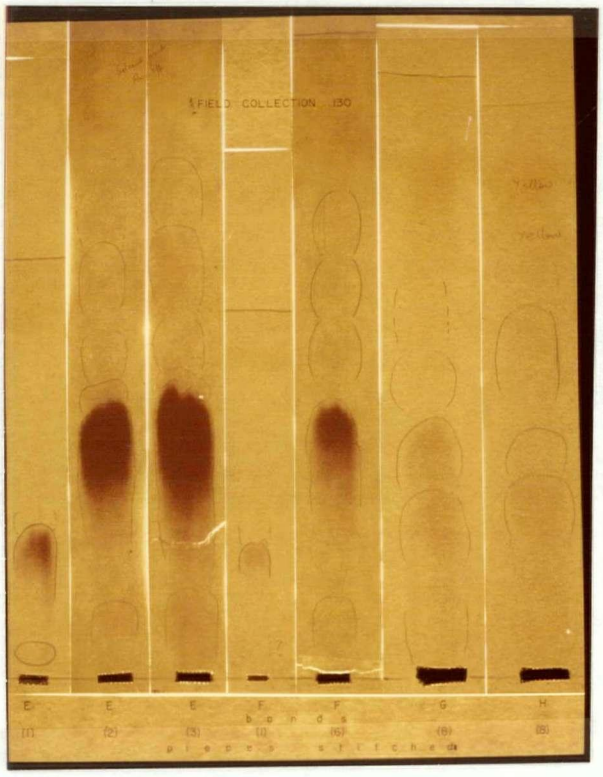

Fig. 13.8.
Influence of number and size of band segments containing anthocyanin complexes E, F, G, and H, sewn at the starting line, on the visibility and number of chromatospots. Gopal leaf sheaths, Field Collection No. 130.

one to three as in E-130 (the first three chromatograms from left in Fig. 13.8), the visual detectability of weak components is greatly increased. The next two chromatograms (Fig. 13.8) show that whereas the band F-130 shows no detectable splitting when only one small segment was sewn, it shows up to six components when six segments were stitched (also see F-130 in Fig. 13.7). On comparison of the results for bands G, and H in Figs. 13.7, and 13.8, it becomes further clear that number and size of the segments greatly influence the detectability of the number of chromatospots. The advantage of working with colored compounds is evident: such minute differences might have missed detection if the compounds were colorless.

It was observed during preparative chromatography by sewing (Sec. 13.2.4) that the detectability of weak components is markedly

improved when the length of the segment sewn is about 10 cm. The improvement is comparable to that of banding over spotting: whereas the leading edges of a band are straight and generally parallel to the starting line; those of a spot are crescent shaped. It is largely because of this difference that banding is superior to spotting (see Sec. 8). The above observations are included as safeguards against the pitfalls of chromatographic comparisons by the sewing technique.

It is noteworthy that chromatograms in Fig. 13.8 show a component around R_f 0.12. The component shows pinkish fluorescence. It was the clear-cut identification of this component that eventually led to an understanding of the causes of anthocyanin splitting on the paper (see Sec. 15).

13.2.4. Preparative and Purification Chromatography of the Field Collection No. 130

The BAW anthocyanin bands, about 20 cm long, of the Collection No. 130 (Fig. 10.3), were excised, and depending upon the intensity of the anthocyanin(s) present, several bands of each anthocyanin were sewn by machine and rechromatographed in the BAW solvent. The results of this preparatory purification and relevant chromatographic characteristics are summarised Table 13.IV. As expected, the bands split into several anthocyanin components, some of which were strong, some medium and others weak. The bands were concentrated in 1% aqueous HCl, and in some cases, in a mixture of 1% aqueous HCl-1% methanolic HCl (1:1, by vol.) in a 'Concentration Chamber' described elsewhere (Sec. 16). The concentration was undertaken to reduce the size, particularly the length of the anthocyanin bands for convenient stitching. The small concentrated bands

TABLE 13.IV.
DATA FOR CHROMATOBANDS FROM FIELD COLLECTION 130: SECTION A. BANDS OBTAINED FOLLOWING STITCHING AND RECHROMATOGRAPHY IN BAW OF THE LONG BANDS OBTAINED INITIALLY FROM PREPARATORY CHROMATOGRAPHY IN BAW; SECTION B. BANDS OBTAINED BY RECHROMATOGRAPHY IN Aq-HCl OF SEGMENTS OF THE BANDS CHARACTERIZED IN SECTION A.

	Section A				Section B			
BAW Band	Comp. No.	R_f x 100[2]	Visible Color[1]	Remarks	Sub-Comp. No.	R_f x 100[2]	Visible Color[1]	Remarks
A	A1	12	R-Vio	major	A1a	29	B1R	major
					A1b	48	RP	major
	A2	20	B1R	medium	A2a	30	B1R	major
					A2b	47	wk	minor
	A3	30	B1R	weak	A3	7	B1R	minor
B	B1	20	B1R	trail	B1	28	wk	long trail; weak spot at R_f ca.43
	B2	25	B1R	major	B2	28	B1R	long trail. Probably @ R_f 7,16 &?
	B3	28	wk	weak	R_f values not available			
	B4	30	wk	weak				
C	C1	24	B1R	trail	C1a	8	B1R	major
					C1b	25	B1R	medium
					C1c	44-50	wk	minor
	C2	31	B1R	major	C2	8	B1R	major
E	E1	25	M	Lower part of E2				
	E2	31	M	major	see text			
	E3	35	Pk	minor				
	E4	750		minor				
F	F1	28	Mv	major	F1	8	B1R	major
	F2	36	Pk	major	F2	11	Pk	major(bright pink in UV)
G	G1	30	B1R	medium	G1a	7	B1R	
					G1b	11	weak	
	G2	40	OR	major	G2	13	OR	
H	H1	29	Mv		H1	6	Mv	
	H2	40	Mv		H2a	6	Mv	
					H2b	17	Mv	
I	I	55	Pk	Pk_f(UV)	I	1.5	Pk	Pk_f(UV)

1. See Table 4.II for abbreviations
2. R_f values were determined on Whatman paper No. 3.

were restitched and chromatographed in the Aq-HCl solvent to determine if the band splitting would occur still. The Aq-HCl solvent for rechromatography was chosen because of its mildness: the solvent is also used for the extraction of anthocyanins. The results of this rechromatography are summarized in Table 13.IV. It may be added that although the anthocyanins showed considerable trailing when the BAW bands, without prior purification were directly stitched in different solvents (Table 13.I and 13.III), the BAW bands, which had been purified at least once upon rechromatography in Aq-HCl (Table 13.IV) or in other solvents gave, in general, excellent resolution.

The complexity of barley anthocyanins will be indicated briefly at this point, by considering the nature of anthocyanin A. It will be noted from Table 13.IV, that the BAW band A splits into 3 components, A1, A2, and A3, upon rechromatography in BAW. Upon further chromatography in Aq-HCl by stitching, the component A1 splits into two major spots, A1a and A1b; the component A2 gives only one major spot (R_f values identical to the spot A1a); the component A3, however, does not split. In addition, it was observed that if the BAW band A was not purified in the BAW solvent, but chromatographed directly, it gave three bands at R_f 0.07, 0.30 and 0.48. As already indicated, the BAW band A, upon hydrolysis, gives cyanidin exclusively. The component A3 is identified as cyanidin-3-glucoside (Sec. 19.2.7.2: note the component is equivalent to the HAc-HCl component A2).

The splitting of the anthocyanin band A into three components, under the mildest conditions of handling known to date, is rather surprising. Normally, the acylated anthocyanins, which are generally polyglycosylated, upon chromatography breakdown, because of partial

deacylation, into two components, the corresponding polyglycosylated form and the original acylated form. It is known that polyglycosylated anthocyanins give low R_f values in BAW and high R_f values in Aq-HCl. Acylated anthocyanins, which are polyglycosylated give slightly higher R_f in BAW than their polyglycosylated form because of the greater solubility of the acyl function (Sec. 5.3). On this basis, the component A1 may be polyhydroxylated as indicated by its low R_f values in BAW. The component A2, which is the same as A2a, may be either less glycosylated or else acylated. It may, at first, appear to be acylated because it gives rise, under extremely mild conditions, to a minor component A2b, which may be polyglycosylated because of its high R_f values (0.48) in Aq-HCl. The above proposition is self-defeating because the component A1, in addition to the polyglycosylated component A1b, also gives a component, A1a, with R_f values equivalent to the assumed acylated component A2a. The other possibility, that the component A1 may be acylated and the component A2 polyglycosylated is ruled out on the basis of the R_f values in the BAW solvent. Thus, the complexity of anthocyanin A is self-evident. This anthocyanin as shown elsewhere (Sec. 19.3.7.2) is a first record.

Table 11.I shows that several other anthocyanins, namely, B, C and E also split into more than two components. Thus, the nature of these anthocyanins, as indicated in the case of the anthocyanin A, may also be quite complex, and hitherto unknown. The band F is probably a mixture of different anthocyanins: the component F2 was identified as Peonidin-3-glucoside on the basis of hydrolysis and R_f values by stitching. The band G is also a mixture of pelargonidin and cyanidin derivatives, the anthocyanins G1a, and G2 were identified as cyanidin-3-glucoside and pelargonidin-3-glucoside, respectively. The band I was identified as

cyanidin. Inasmuch as the results of hydrolysis and R_f values (by stitching) were similar to those reported elsewhere (Sec. 19), the presentation of the characterization data in this section will be omitted. Several other interesting observations were made on the band E. These will be presented in Section 15.

13.3 DISCUSSION

That the anthocyanin complexes of Collection No. 138, upon chromatography, initially showed little splitting into different components by the sewing technique (Fig. 13.1 to 13.4), and sizeable splitting and degradation by the elution technique (see Table 11.I) led to the belief that the anthocyanins obtained by elution were largely the artefacts produced by the techniques of processing (elution and flash evaporation). However, when the results of the anthocyanins of Collection No. 130 obtained by the sewing technique (Figs. 13.5 to 13.7) were compared with those obtained by the elution technique (Table 11.I), virtually no basic differences were noted in the splitting of anthocyanins by either technique. That the pattern differences from Collections No. 130 and 138 obtained by stitching were, primarily, due to the technique was not appreciated until the influence of (a) band stitching (preparative purification, Sec. 13.2.3, Table 13.IV), and (b) size and number of band segments stitched (Fig. 13.8) on resolution and visual detectability of anthocyanins, had been appreciated. Because the splitting of barley anthocyanins occurs under the mildest conditions of handling, and because the patterns of splitting obtainable by stitching correspond to those obtainable by elution, the conclusion appeared warranted that the

of anthocyanins is not as much due to the shortcomings of the elution technique, as due to the inherent nature of the anthocyanins of barley. Although this type of anthocyanin splitting has not been reported for other plant materials, it is likely that the anthocyanins of barley are inherently labile.

The partitioning is so characteristic of the anthocyanins of barley that even when the anthocyanin bands obtained from the BAW solvent are rechromatographed in the BAW, they further split into several components. These observations suggest that a great deal of partitioning must occur in primary extraction. Thus, what is observed in vitro may be far removed from the actual state of anthocyanins in vivo. Although the extracts of the Field Collection No. 130 show several more anthocyanins than those of the Collection No. 138, the conclusion that the differences in anthocyanins may be due to developmental or physiological state of the plant must also be reserved at this stage.

That the splitting of anthocyanins could have occurred originally in the extract may account for the constant variability in resolution of anthocyanins B, C, and D, and occasionally other anthocyanins.

The greater splitting of anthocyanins by elution than by sewing and the greater incidence of the breakdown products, such as fluorescent components, the immobile orange fluorescent band at the starting line, and the appearance of the HAc-HCl component around R_f 0.10 (later on identified as cyanidin, See Sec. 15) also by elution than by stitching clearly indicate that stitching may yet be the mildest technique available for processing and characterization. Indeed, the characterization of the known anthocyanins, such as the monoglucosides of cyanidin, peonidin and pelargonidin, were made by this technique on the basis of R_f values.

The data has been omitted because it was similar to that presented elsewhere (Sec. 19). The characterization potential of the R_f values of other complex anthocyanins obtained by stitching (Fig. 13.1 to 13.7) will be referred to later on when the comparison of anthocyanins grown under greenhouse conditions and field conditions will be presented in Section 19. Although the stitching technique has a definite merit in characterization, the technique can only be used effectively with known compounds and may, therefore, be of value mostly in the comparative work. Because of its comparative amenability, which doubtlessly will be of value in hybrid analysis (because of the scarcity of the materials), in the next section will be presented an improvement in the method for studying anthocyanin spectra on paper, as a means of reinforcing the characterization potential of R_f values determined by stitching.

Since the R_f values of barley anthocyanins, as summarised in Table 13.I, 13.III, and 13.IV, in most instances do not match with the R_f values of anthocyanins thus far isolated from natural sources, it soon became apparent that elution of anthocyanins of barley will have to be undertaken for characterization. Although it was in testimony to the instability of barley anthocyanins (upon elution and volume reduction) that the stitching and spectral analysis on paper were originally undertaken, several observations, as described below, were made in the course of studies reported in this section that furnished several clues for insuring anthocyanin stability.

For instance, although the R_f values by elution and by stitching do match, the intensities of the split anthocyanin spots, obtained by elution and by stitching do not match. Further, that the intensities of the breakdown products are minimal be sewing indicates that the lability

occurs far faster in solution than on the paper; it will be recalled that the anthocyanins adsorbed on the paper remained in contact with the solvent in the Concentration Chamber and later during chromatography to a total of several days. It was the realization of the relative stability of anthocyanins on the paper during constant irrigation with solvents that was one of the major factors in the development of the concentration and elution technique of processing anthocyanins described in Section 16. However, for the successful development of this technique another key question, the appearance of the extra spot (R_f *ca.* 0.10) in the HAc-HCl solvent (Figs. 11.7 and 11.8) which was later identified as cyanidin, as being due to hydrolysis under the recommended conditions of handling, had to be resolved. This key question of hydrolysis will be taken up in Section 15. An appreciation of the influence of the number of band segments stitched on the visual detectability of anthocyanins has increased the dependability of the technique. Variations in results may arise if the band segments are not assured of intimate contact against the paper. Uniform adposition was later obtained by the clamping technique developed in the course of hybrid studies (Sec. 20.2).

13.4 SUMMARY

In order to eliminate elution and flash evaporation, the steps which appeared to be responsible for the breakdown of anthocyanins (Sec. 11 and 12), rechromatography was carried out by the sewing technique. The degradation to yellow products did not occur by the sewing technique. Notwithstanding the mildest conditions, as are obtained in chromatography by sewing, the splitting of anthocyanins, although mild, still occurred.

Thus, there is every reason to believe that the splitting must also occur during preparation of the original extracts and, therefore, what is observed chromatographically *in vitro* may be far removed from the state of anthocyanins *in vivo*.

The chromatographic data of the Gopal basal leaf sheath anthocyanins (Field Collections 130 and 138) in four solvent system by sewing are given. The anthocyanidin(s) from each BAW anthocyanin band, however, was identified by the usual methods. Although the data presented is brief, the value lies in comparison of the anthocyanins obtained from the same sheaths grown under greenhouse conditions (see Sec. 19). The complexity of barley anthocyanins is indicated briefly by considering the nature of the anthocyanin A. It is shown that R_f values of several anthocyanins of barley do not correspond with the known anthocyanins. Thus, it soon became apparent that elution and drying of anthocyanins was a necessary step for further characterization.

Inasmuch as the anthocyanins are known to be 'unstable' in solution and 'stable' on paper, the sewing technique is an excellent addition to the chromatography of anthocyanins. The R_f values determined by sewing match those determined by spotting solutions. At least three simple anthocyanins, such as the 3-monoglucosides of cyanidin, peonidin and pelargonidin are identified on the basis of R_f values determined by sewing.

The technique in combination with an improved technique of studying anthocyanin spectra directly on the paper (Sec. 14), and with a method of hydrolysing anthocyanins on the paper, will find application largely in comparative chromatography such as that of hybrids and their parents.

METHODS FOR CHARACTERIZING ANTHOCYANINS

14. AN IMPROVEMENT IN THE METHOD FOR STUDYING ANTHOCYANIN SPECTRA ON PAPER

Because of the instability of barley anthocyanins following elution and flash evaporation, chromatography by stitching (Sec. 13) and later by clamping (Sec. 20) were developed for the determination of R_f values. The techniques are valuable as a micromethod in comparative (Sec. 13), hybrid (Sec. 20), and other biochemical studies where the supply of materials is limited.

The studies of anthocyanin spectra on paper, by the method of Bradfield and Flood (1952), were undertaken to determine if the characterization of anthocyanins by R_f values, obtained by the stitching technique, could be reinforced by spectral means. The spectral method has been used by others (Bate-Smith, 1954, 1956; Roux, 1957a, 1957b; Roberts et al., 1958) for the study of anthocyanidin spectra.

In the course of comparative work, a large number of weak

anthocyanin spots were obtained, owing to the problem of anthocyanin splitting. The spectra from weak spots cannot be determined by the known methods. Thus, the method for determining spectra from chromatospots could be extended as a tool in comparative characterization, provided the spectra from weak spots could also be determined. A method for determining spectra from weak spots will be presented in this section.

14.1 MATERIALS AND METHODS

14.1.1. Anthocyanins

The basal leaf sheath anthocyanins of the purple variety, Gopal (Field Collection No. 130), were used in this study. The details of planting and extraction are described in Section 10. The anthocyanins were purified in several solvents entirely by machine and manual stitching without the intervention of elution and flash evaporation. The anthocyanins used in this study were those which were purified finally in 1% aqueous HCl after two preliminary purifications in the BAW solvent (Table 13.IV). The system for designating anthocyanins is described elsewhere (Sec. 13.1.4).

Preliminary studies were conducted with the anthocyanin E3-138 which was obtained by the procedures described in Section 11 (Table 11.I).

14.1.2. Spectrophotometry

An area, the size of the common rectangular 1-cm cuvette, was cut out from the anthocyanin band using a metal template so that it snuggly fitted against the slit aperture of the cuvette holder. The rectangular paper strip was made transluscent by a few drops of the

mineral oil 'Nujol' and wrapped in a foil for storage and convenient handling. Two paper blanks were prepared from the corresponding areas of the chromatogram at the same time; all were processed in an identical manner. The spectra was recorded on a Beckman DK_2 Non-linear Ratio Recording Spectrophotometer. The instrument gave best results at the following settings with Whatman paper No. 3, which was used throughout this study, unless otherwise specified:

Sensitivity	between 2 to 3
Time Constant	2
Photo multiplier	20X
Scanning time	5 minutes

The spectra, in a few cases, were also measured in the presence of $AlCl_3$ for the detection of two or more vicinal hydroxyl groups on the same rectangular paper strip which was used for determination of the anthocyanin spectrum. This paper strip was freed of Nujol by liberal washings with ether, and reacted with a few drops of ethanol containing anhydrous $AlCl_3$ (5% w/v) until the color of the anthocyanin turned bluish. After drying, the strip was again treated with Nujol and stored in a plastic wrap (away from light) for determination of the spectrum when convenient. The advantage was believed to be that anthocyanins do not degrade on paper as they do in a solution upon addition of $AlCl_3$. The original blank paper strip was also washed with ether in the same manner except that it was not treated with $AlCl_3$.

14.2. RESULTS

14.2.1. Spectra from Strong Spots (O.D. above 0.5)

Visible and ultraviolet absorption spectra of the anthocyanin

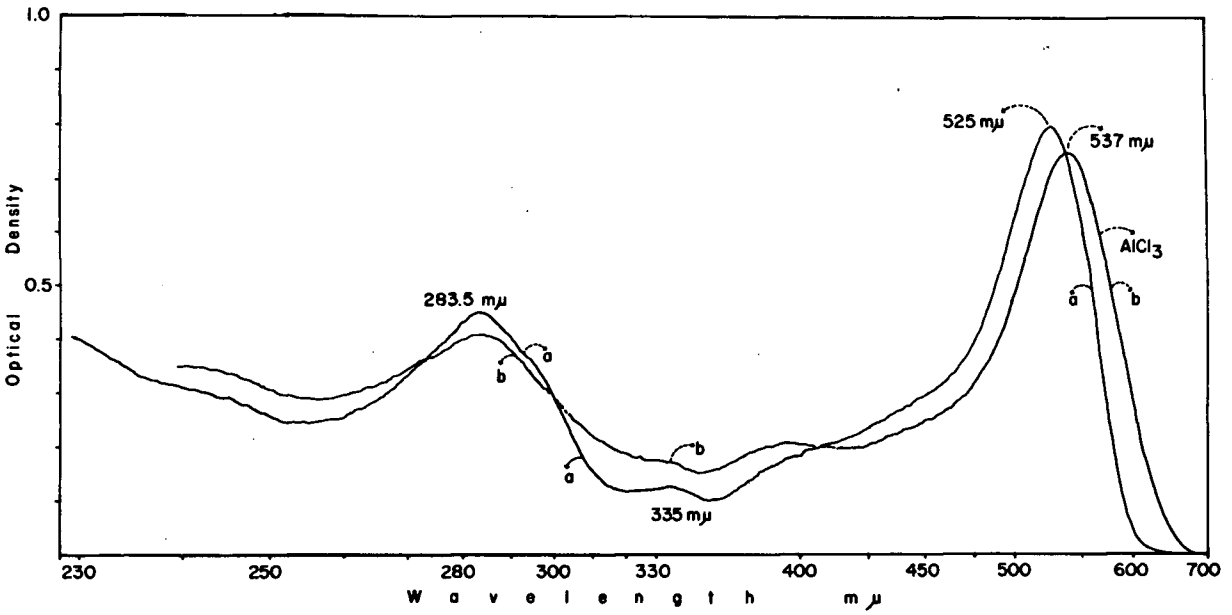

Fig. 14.1
Absorption spectra of anthocyanin E-3-138 from basal leaf sheaths of Gopal drawn directly from paper, (a), and showing the bathochromic shift obtained with $AlCl_3$, (b).

E3-138, both with and without the aluminum chloride treatment are shown in Fig. 14.1. Note the spectra are as 'smooth' as those obtained from solutions. All figures reproduced here are direct tracings of the original spectral records.

14.2.2. Spectra from Medium Spots (O.D. between 0.2 to 0.5)

Initially, the spectra were drawn from strong anthocyanin spots and they gave reproducible results to within $\pm$ 1 mu. However, difficulties were encountered when examining spectra for anthocyanin spots which were visibly a good red and which possessed O.D. values below 0.5. The region of maximum absorption became so 'flat' that the exact value of the $_{max}$ could not be delimited. It was observed that a sharp

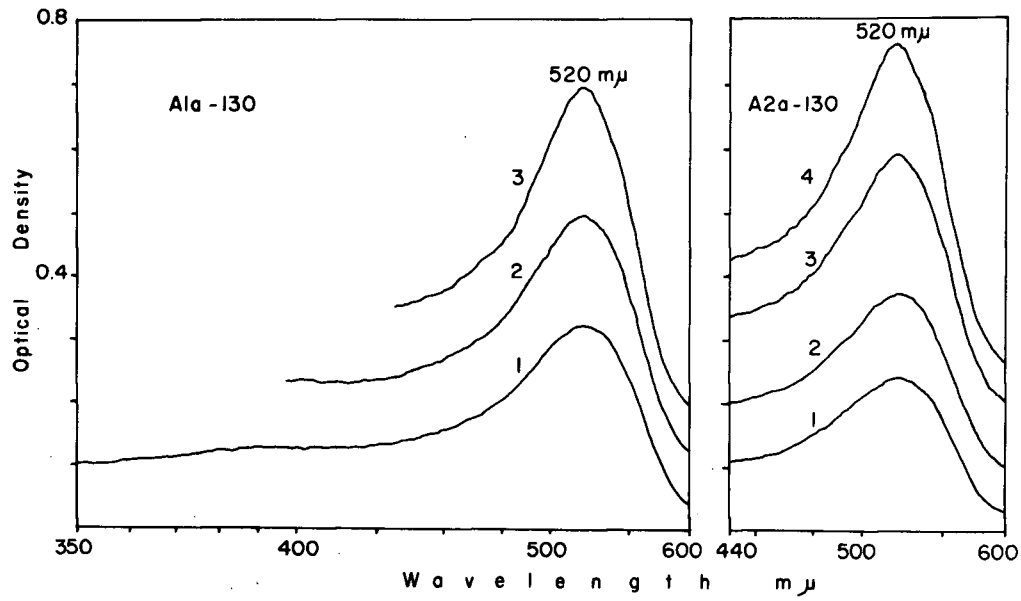

Fig. 14.2
Absorption spectra from the weak anthocyanin spot Ala-130 drawn in series from the original chromatostrip and A2a-130 drawn in series from a comparable chromatostrip. The two series were drawn by suitably setting the zero adjustment control on the Beckman DK2 spectrophotometer.

maximum could be obtained by giving a suitable 'increment to the Zero Adjustment Control Knob. This is shown for the anthocyanin Ala-130 (BAW(2) - Aq-HCl) (Table 13.IV) in Fig. 14.2, which shows three spectra of the anthocyanin obtained from the same paper strip. The Zero Adjustment Control on the DK_2 is used for setting the base line at zero per cent transmittance. After drawing the base line with the paper blanks, the spectrum shown in curve 1 was drawn in the usual manner. The O.D. being 0.32, the λ_{max} could not be differentiated. The curve 2 was drawn by giving a slight increment to the 'Zero' control knob so that the base line at 700 mu was raised to an O.D. ca. 0.1. When this small zero increment was given the base line, the O.D. of the λ_{max} jumped from 0.32 to 0.5, thus giving a sharper peak (Fig. 14.2., curve 2). When the increment to the zero control was further increased, a sharp λ_{max} 520 mu (Fig. 14.2.,

curve 3) was obtained.

This method of obtaining a sharper peak from the flat peak by suitable Zero increment worked very well. Indeed, the λ_{max} of another anthocyanin A2a-130 (BAW(2) - Aq-HCl) (Table 13.IV), which was chromatographically identical to the anthocyanin A1a-130, and which also showed a flat peak at O.D. 0.24, was delimited by the above method, at 520 mu as shown in Fig. 14.2.

14.2.3. Spectra from Weak Spots (O.D. below 0.2)

The accurate delimitation of λ_{max} with the anthocyanin spots below the optical density 0.2 was rather difficult as shown by the spectra of anthocyanins C1b-130 (BAW(2)-Aq-HCl), C1c-130 (BAW(2)-Aq-HCl), G1a-130 (BAW(2)-Aq-HCl) and G2-130 (BAW(2)-AqHCl) in Fig. 14.3.A to 14.3.D. All spectra shown in Fig. 14.3 were drawn at 10-minute scan, except the spectra of anthocyanin C1b-130, which was drawn at a 5-minute scan. Perhaps, sharper λ_{max} could have been obtained with a 5-minute scan since the spectra in Fig. 14.3.A are somewhat sharper than others. The R_f values and colors of the anthocyanins are listed in Table 13.IV.

It is apparent from the spectra, that the maxima, even with Zero increments are not sharp, but can be delimited with an accuracy of $\pm$ 3 mu. Nonetheless, the spectra, doubtlessly, have characterization potential. For instance, it is clear from the results presented in Sections 11 and 13, that the band C-130 and B-130 could not be characterized dependably because the bands were weak and because they showed poor resolution. The band B-130 is a mixture of cyanidin, and probably peonidin (cf., Table 13.II). The nature of the band C-130 is not clearly known. The spectral characteristics of the anthocyanin com-

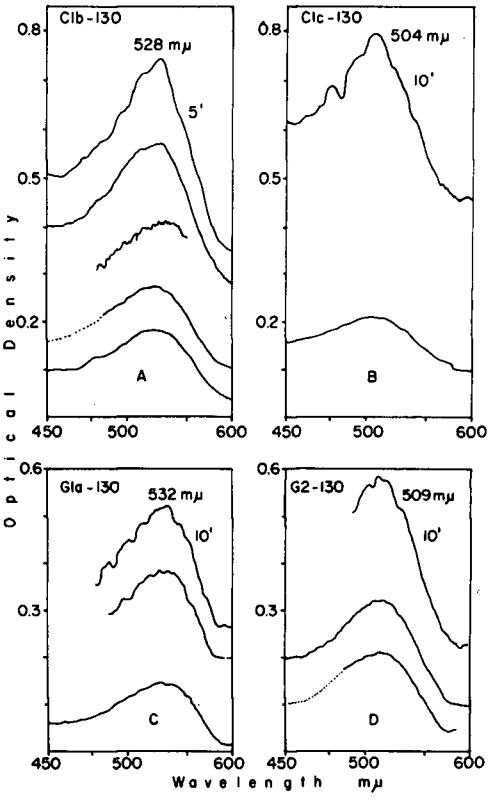

Fig. 14.3
Absorption spectra from some very weak anthocyanin spots obtained from Gopal leaf sheaths. Optical densities were below 0.2 and the series were obtained by adjusting the zero control on the Beckman DK2 spectrophotometer.

ponents of the band C, included in Fig. 14.3 indicate that the anthocyanin Clb-130 (λ_{max} 528 $\pm$ 3 mu) is likely derived from cyanidin, and the anthocyanin Clc-130 (λ_{max} 504 $\pm$ 3 mu) is derived from pelargonidin (cf.,Harborne, 1958). Similarly, the band G-130 was also a mixture of pelargonidin and cyanidin derivatives (Table 13.II); the spectral measurements clearly establish that the anthocyanin G2-130 is a pelargonidin derivative and the anthocyanin Gla-130, a cyanidin derivative. The point of sugar attachment cannot be stated because spectra of known anthocyanins on paper have not been reported before.

It is, however, to be noted that the characterization could not have been possible without the Zero increments; the spectra obtained without the Zero increments are too flat and, therefore, valueless.

In order to obtain concentrated anthocyanin spots, several weak and medium BAW(2)-Aq-HCl anthocyanon spots (Table 13.IV) were concentrated in 1% aqueous HCl in the 'Concentration Chamber' (Sec. 16.2). Efforts were not very successful: during the concentration by ascent, several anthocyanins split and were not considered suitable for further spectral analysis.

14.2.4. Effect of Scanning Time on Spectral Maximum

It was observed that λ_{max} values varied with different periods of scan. Thus, in order to determine the optimum period of scan for the paper strips, the spectra of a few anthocyanin spots were drawn at varying periods of scan, namely 5, 10, 20 and 50 minutes. The results from strong anthocyanin spots E2a-130 (BAW(2)-Aq-HCl), and F2-130 (BAW(2)-Aq-HCl) are shown in Figs. 14.4.A and 14.4.B. The λ_{max} values at different scanning times are noted in the figures; the chromatographic characteristics are summarised in Table 13.IV. It may be added that the anthocyanin E2a-130 (BAW(2)-Aq-HCl) is an equivalent of the anthocyanin E3-138 (Fig. 14.1); the anthocyanin F2-130 is a peonidin derivative (Table 13.II).

The anthocyanin E2a-130 (Fig. 14.4.A)at scanning periods of 5, 10, 20, and 50 minutes gave λ_{max} values at 525, 528, 530 and 532 mu. It is notable that with the increased period of scan, it is not only the λ_{max} which shows a consistent bathochromic shift, but also the optical density of λ_{max} shows consistently a slight increase. The

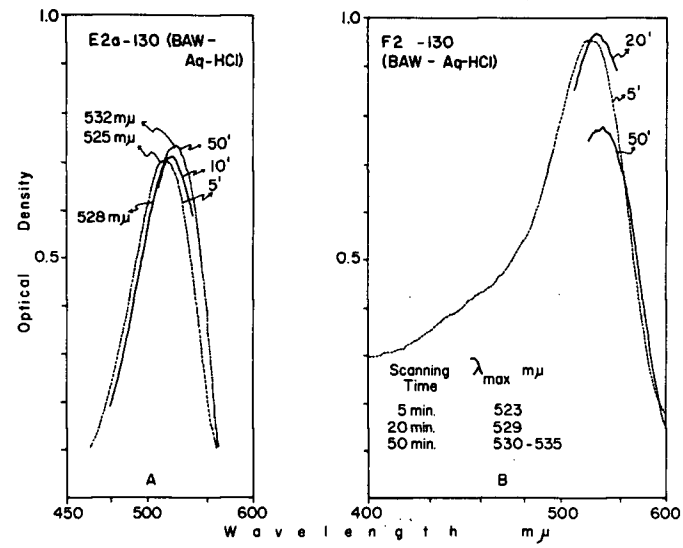

Fig. 14.4
Absorption spectra for the anthocyanin E-2-a-130 and for the anthocyanin F-2-130 drawn to show the effect of different scanning times on λ_{max}.

reproducibility of the λ_{max} obtained at different periods of scan was in general checked by suitable Zero increments or decrements. It was found that whatever the chosen length of a scanning period, the λ_{max} values were reproducible at that scanning period with the usual accuracy. Around the wavelengths of maximum absorption - the region of maximum photometric sensitivity for anthocyanins - the increased period of scan, for example 50 minutes, invariably gives rise to absorption lines, rather than a smooth curve. It was for obtaining smooth spectra that the period of scan was kept at 5 minutes (Sec. 14.1.2).

The spectral features shown in Fig. 14.5.B for the anthocyanin F2-130, confirm the above results.

14.3 DISCUSSION

The results show that spectra from strong anthocyanin spots on paper may be drawn as easily, smoothly and dependably as those drawn from anthocyanin solutions. The difficulties of studying spectra from medium and weak anthocyanin spots were overcome because of the non-liner 'jump' of the O.D. that occurred around the wavelengths of maximum absorption when only a slight Zero increment was given. Theoretically, the non-linear increase of optical density around the λ_{max} by slight Zero increment is expected. In spectral measurements, as the concentration of a substance in solution is increased, increased light absorption occurs at all wavelengths. The increase in absorption, however, is greater (or non-linear) at some wavelengths (which, for the visible spectrum of anthocyanins, are the wavelengths of maximum absorption) than at others. It would seem, on this basis, that the effect of giving increment to the base line by Zero adjustment is obviously the same as increasing the concentration. Thus, the use of slight Zero adjustment, both increment and decrement, appears to be valid in qualitative work.

Although, Bradfield and Flood (loc. cit.), using a manually operated S.P. 500 Spectrophotometer, found that the precision in measurement of light absorption on cellulose (Whatman paper No. 1) was less than $\pm$ 3 mu, the results of this study, using an automatic Beckman DK2 spectrophotometer, indicate that the measurements on cellulose (Whatman paper No. 3) are reproducible with a precision of $\pm$ 1 mu when strong anthocyanin spots are used. Because the time taken for scanning the spectrum on a manually operated spectrophotometer is variable, and because the duration of scan influences the spectral maximum (Sec.14.2.4),

the lack of precision, as reported by Bradfield and Flood (ibid.) could be accounted for on this basis. The studies in Sec. 14.2.4 indicate the desirability of spectral measurements at a fixed scanning period so that the results of other workers may be compared.

Apart from economy in labor and several other advantages pointed out by Bradfield and Flood (ibid.), potential advantage of the technique, in the case of anthocyanins which are labile in solution, lies in the elimination of the elution and flash evaporation. Several spectra of anthocyanin solutions have been reported (see Knoll, 1963; see also Sec.12) which show the presence of degradation products. Thus, when an anthocyanin, isolated on chromatograms, is available only in small quantity, its spectra, to be sure, may first be scanned on paper, the mineral oil may then be washed off with ether and elution undertaken for other spectral and characterization work.

The qualitative value of the technique in characterization of anthocyanins into aglycone types is apparent from the results presented. Differentiation of cyanidin types and pelargonidin types could be made easily on comparing the spectral data obtained from solutions of anthocyanins (Harborne, 1958). Because samples of known anthocyanins were not available, and because λ_{max} values obtained on paper differ from those obtained in solution (Jurd, 1962), it cannot be stated whether the differentiation of anthocyanins as to their glycosidic attachments can be made on the basis of spectral studies on paper, as was done by Harborne (ibid.) in solutions. Nonetheless, the precision in reproducibility of spectra on paper indicates the amenability of the method in hybrid and other comparative studies.

Chronologically, around this time, clues for controlling the

instability of anthocyanins, which provided impetus for this investigation, had been obtained, and these will be described in the following section.

14.4 SUMMARY

Spectral analysis of anthocyanins directly on paper was undertaken originally to extend the characterization potential of the chromatographic data of anthocyanins obtained by the sewing (Sec. 13) or clamping techniques (Sec. 20), and that of anthocyanidins obtained by a technique of hydrolyzing anthocyanins directly on paper (Sec. 20). The spectra of strong spots (O.D. above 0.5) can be easily determined directly on the paper. It was, however, observed that owing to the problem of anthocyanin splitting, a large number of medium (O.D. between 0.5 and 0.2) and weak (O.D. below 0.2) spots were obtained, the spectra from which could not be determined directly on the paper. It is demonstrated in this section that spectra from the medium and weak spots can be determined by suitable adjustment of the Zero control on the Beckman DK_2 ratio recording spectrophotometer. In addition, the influence of scanning period on shifts in λ_{max} values is also presented.

METHODS FOR CHARACTERIZING ANTHOCYANINS

15. CAUSES OF ANTHOCYANIN SPLITTING AND DEGRADATION ON CHROMATOPAPER

It was shown in Sections 11 and 13 that anthocyanins of barley split into several components both by elution and by sewing techniques. It was also shown that marked instability of anthocyanins occurs following flash evaporation (Sec. 11). It is well known that anthocyanins are unstable in the presence of light, oxygen, sugars, amino acids, and near neutral to high pH solutions. In the latter case they undergo transformations to unstable pseudo bases and anhydro bases. In this section, it will be demonstrated that the splitting or instability of anthocyanins occurs additionally due to the hydrolytic breakdown that ensues gradually on chromatography paper.

It was also shown in Section 13 that at least some of the anthocyanins of barley are complex. Thus elution of anthocyanins is necessary for investigating their nature. In this section will also be

presented some observations that eventually aided development of a satisfactory method for elution and purification of anthocyanins.

15.1 MATERIALS AND METHODS

Owing to the instability of anthocyanins during elution and flash evaporation (Sec. 11), the elution of the remaining HAc-HCl bands (Table 11.I) and the BAW bands (Fig. 10.3) was withheld pending development of suitable procedures for insuring anthocyanin stability. Several observations were made on the anthocyanin bands during elution and flash evaporation (Sec. 11), by the concentration technique (Sec. 13.2.4), and by chromatography in several solvents (Tables 10.II and 17.I), by sewing, by clamping and by elution as described under results. Several basic observations were also made in the course of the concentration process (Sec. 13.1.4) on the component bands of the anthocyanin E-130 (Table 13.IV), which were obtained entirely by the sewing technique. Chromatographic elution and purification of the anthocyanin E3-138 was carried out by the procedure described elsewhere (Sec. 11).

The anthocyanins E3-138, and E1-138, used extensively in this study, were obtained in a crystalline state when the concentrated eluates of the HAc-HCl bands (see Table 11.I) were left in a refrigerator (-15°C) for a few days. The crystalline pigment E3 was isolated in a pure state but the crystalline pigment E1 could not be freed completely from yellowish contaminants. The crystalline anthocyanins were placed in vials, dried in a vacuum desiccator over phosphorus pentoxide (Harborne, 1958) at room temperature in darkness for a few days*, and then stored at room tempera-

*Although no storage times were recorded, the vials, plugged with cotton wool, remained in the desiccator for less than a week (cf., Secs. 19.2.2 and 19.3.1).

ture. The crystalline anthocyanins were chromatographed in several solvents after dissolving in 0.01% methanolic HCl. The crystalline anthocyanins E3-138, and also 5D (Table 19.VIII.5), isolated originally in early 1962, were kept tightly corked in darkness at room temperature until July 1965, when rechromatography in the HAc-HCl solvent was again undertaken.

The anthocyanins were hydrolysed by the usual techniques (Sec. 8.1.4). The spectra were determined on Beckman DK_2 ratio recording spectrophotometer in 1-ml silica cuvettes using 0.01% methanolic HCl (Harborne, loc. cit.).

15.2 RESULTS

15.2.1. A Key Observation during Flash Evaporation

In the course of flash evaporation some anthocyanin eluates, obtained with 1% conc. HCl (as described in Sec. 11), showed visible degradation, when the eluate was about to dry; the contents of the flash evaporator flask gave a strong odour of hydrochloric acid. If to this flask, methanol was added quickly, the red color of the eluate was usually reconstituted.* However, if methanol were not added, a great deal of irreversible degradation to yellow products occurred in many cases. The observation indicated that it was, probably, the hydrochloric acid concentrating in the flask as volume reduction occurred, which was responsible for the breakdown of anthocyanins during flash evaporation. After all, when the eluates are evaporated to near dryness, that which

*Color, in part, was due to crude crystallization of anthocyanins. However, this occurred probably in some cases only.

remains in the flask is anthocyanins and constant boiling hydrochloric acid. This was established latterly by titration.

15.2.2 Observations on the Causes of Anthocyanin Splitting - 1962

It was shown that anthocyanins 'split' upon chromatography even when no elution was undertaken (Sec. 13). At the time, when suitable solvent systems for the 'Concentration' technique (Sec. 16) were being explored, it was observed that the splitting of anthocyanins occurred when an anthocyanin concentrant, such as, 1% aqueous HCl (Sec. 13) was used in the Concentration Chamber (Sec. 16). For example, when anthocyanins of the BAW band A-138 or A-130 (Fig. 10.3) were concentrated, a part of the pigment moved readily to the tip of the paper band, a part moved slowly, and a part remained immobile on the paper band. It was, for example, shown in Section 13.2.4 that when the BAW band A-130 was chromatographed by stitching, it gave rise to three anthocyanin components with widely different R_f values or mobilities in the Aq-HCl solvent. Thus, the differential mobility of anthocyanins in 1% aqueous HCl during a concentration process (Sec. 16.2) is obviously due to the differential mobility of each of the split anthocyanins. This observation would have doubtlessly escaped attention, had the anthocyanins been eluted, in the usual manner, without the intervention of the concentration process. It is, therefore, clear that often what is eluted is a mixture of anthocyanins and not pure anthocyanin. This situation was later found to be of general occurrence with several other anthocyanins of barley.

It was mentioned that in the concentration process, a small part of the pigment remained immobile on the paper band, the immobile component showed little movement even after 48 hours in the aqueous

concentrant. It was also noted that the BAW band I-130 (Fig. 10.3 and Table 13.II) also showed little mobility in the aqueous concentrant. Subsequently, the immobility or the "irreversible adsorption" of small quantities of the pigment on paper was found consistently with several other anthocyanin bands. That the anthocyanins cannot be eluted completely from the paper due to some irreversible adsorption, particularly, when the anthocyanins have been left on the paper for a few weeks, has been noted in a recent review by Harborne (1959a).

The pigment component that moved tardily was restudied carefully by the concentration technique (Sec. 16.2) using the most abundant anthocyanin E3-138 (Table 11.I). It was noted that the immobile component from this anthocyanin, after the readily mobile anthocyanin, concentrated at the paper tip, had been snipped off, could be concentrated in a mixture of equal parts of water and methanol containing 1% HCl. Subsequently, the immobile component was found to move faster in 1% methanolic HCl. When the immobile component was examined under ultraviolet light, it showed red fluorescence similar to the component E1-138 (Fig. 15.1). The fast moving component, which was snipped off, of course, gave dull bluish purple absorption in ultraviolet light as component E3-138 (Fig. 15.1). When the concentrated immobile component was chromatographed by the sewing technique (Sec. 13), it gave R_f values identical to those for the component E1-138 (Fig. 15.1).

Following the chromatographic identity of the anthocyanin E1-138 with the immobile component of the anthocyanin E3-138, the splitting of the anthocyanin E3-138 was pursued further. The eluates from the anthocyanin E3-138, even after three purifications in the HAc-HCl solvent, continued to show splitting into the components E1, E3 and a

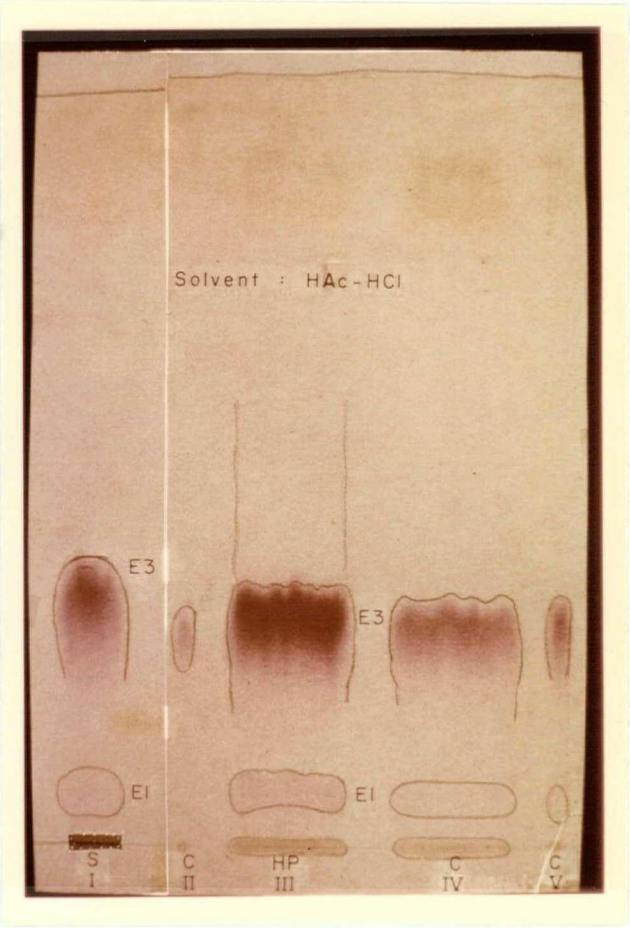

Fig. 15.1
The splitting of anthocyanin E3-138 into at least two components E1, and E3 occurred when the highly purified (HP) eluate without drying (III), or when the band segment by sewing (S), and hence avoiding elution (I), or when the crystalline (C) sample dissolved in 0.01% methanolic HCl (IV) were chromatographed. The effect of anthocyanin concentration, spotted at the starting line, on the visibility of chromatospots is also shown (II, and V).

distinct trail above the component E3. The components from the eluate (without drying), after a third purification are shown in Chromatogram No. III (Fig. 15.1). The spot E3 upon rechromatography by stitching (without elution), also, split into a weak spot E1 as shown in Chromatogram No. I (Fig. 15.1). The splitting into the component E1 and several other components also occurred when the band E-130 was chromatographed by stitching (Fig. 13.8).

In the meantime, the anthocyanin components E3 and E1 were obtained in crystalline state. The component E1 was slightly contaminated with yellowish materials. Whereas the crystalline component E3

was readily soluble in cold 1% aqueous HCl, the crystalline component E1 was insoluble, but soluble in warm aqueous HCl. The crystalline anthocyanin E3 was chromatographed to determine if the crystalline sample would also show splitting (Fig. 15.1, Chromatogram No. IV). The results are self-explanatory: the spot E1, although weak, was distinctly present. Although, the trail above the spot E3 is invisible in Chromatogram No. IV, it is detectable in Chromatogram No. III: the differences in the detectability may be due to quantitative differences in the amount of the pigment applied. If the crystalline anthocyanin is spotted instead of banding, the detectability of the spot E1 depends upon the quantity of pigment spotted. The quantity of the crystalline pigment applied in Chromatogram No. V (Fig. 15.1) was about three times that applied in Chromatogram No. II (Fig. 15.1). Whereas the Chromatogram V shows both the spots, the Chromatogram No. II shows only the component E3, which is reasonably strong but not the component E1. The component E1, obtained by spotting (Chromatogram No. V), appears weaker to the eye as compared to its counterpart obtained by banding (Chromatogram No. IV). It is noteworthy, that the component E3, obtained by spotting (Chromatogram No.V) is certainly stronger than that obtained by banding (Chromatogram No. IV). The observation again emphasizes the influence of quantity on the detectability of minor components and the superiority of banding over spotting. The crystalline component E1-138 upon chromatography behaved exactly in the manner reported previously in Section 11 (Fig. 11.3). The component degraded completely in the BAW solvent and gave R_f value 0.02 in the Aq-HCl and 0.11 in the HAc-HCl solvent. This component was, therefore, likely pure because it did not split.

The spectra of the crystalline pigments is shown in Fig. 15.2.

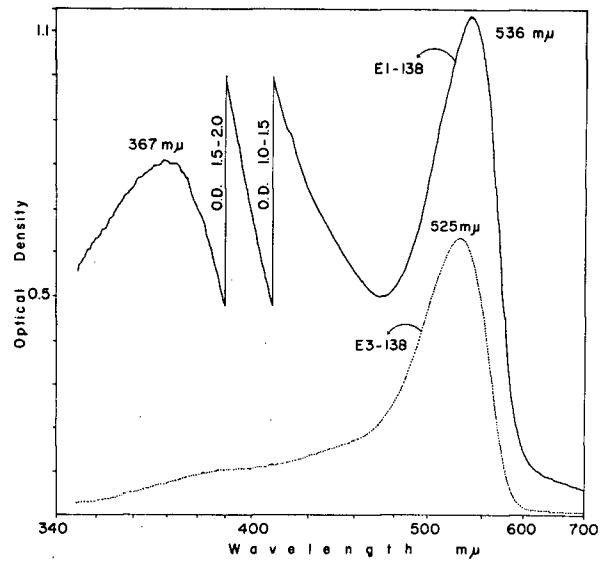

Fig. 15.2
Spectrophotometric differentiation of the crystalline pigments E1 and E3. E1 shows the cyanidin peak at 536 mu and the degradation peak at 367 mu. E3 shows a characteristic λ_{max} at 525 mu of cyanidin glycosides. Spectra were drawn from 0.1% methanolic HCl solutions

The pigment E3-138 gives a distinct λ_{max} at 525 mu. The pigment E1-138 shows two λ_{max} 536 mu and ca. 366 mu. The latter λ_{max} is due to degradation of the pigment (cf., Sec. 12). The λ_{max} 536 mu is, in fact, the λ_{max} of cyanidin as obtained under our conditions. The anthocyanin E3 upon hydrolysis also gave a λ_{max} at 536 mu (see Fig. 12.2). The pigment E1 upon hydrolysis disappeared almost completely. It was, however, chromatographed, without hydrolysis, along with the hydrolysates of the anthocyanin E3 in several anthocyanidin solvents listed in Table 17.I. In every case, the R_f values of the component E1 and the hydrolysates of the component E3 were identical and equivalent to cyanidin.

The results clearly indicate that hydrolysis occurs both on paper and in solution. It was, however, observed that whenever the eluates from the anthocyanin E3-138 were chromatographed along with a spot or a band of synthetic cyanidin, on the same sheet, the cyanidin

spot E1, invariably gave slightly lower R_f values than synthetic cyanidin. However, if the band segments of synthetic cyanidin, and the cyanidin spot E1 were developed in the HAc-HCl and the Forestal solvents by stitching chromatography, the R_f values were found to be identical.

15.2.3. Observations on Anthocyanin Splitting - 1965

In July, 1965 crystalline anthocyanin E3-138, originally isolated early in 1962, was dissolved in 0.01% and 1% methanolic HCl and chromatographed in the HAc-HCl solvent. The results are shown in Fig. 15.3, Chromatograms No. I and III. A comparison of the 1965 and 1962 results (Fig. 15.1, Chromatogram No. IV) shows that whereas the spot E1 is barely visible in the 1962 chromatogram, it is prominent in the 1965 chromatograms. Thus, it is clear, that hydrolysis must have occurred during storage. Both the chromatograms in Fig. 15.3 show reddish 'stationary' materials at the starting line (cf., Sec. 11), and anthocyanin trails both above the band E3, and between the bands E3 and E1. Notably, no trails above the crystalline anthocyanin band E3 were detected in the 1962 chromatogram. The trails must represent partial hydrolysis undergone by the crystalline sample during storage. It is notable that the R_f value of the cyanidin spot obtained from the hydrolysis of the anthocyanin E3-138 (Fig. 15.3, Chromatogram No. II), as expected, is slightly higher than the cyanidin bands E1. However, it gave the cyanidin pink fluorescence, immediately after development and drying.

Similarly, the anthocyanin 5D isolated originally in 1962 (Table 19.VIII.5) upon rechromatography in HAc-HCl in 1965, shows a conspicuous anthocyanin trail extending approximately to R_f 0.90. The

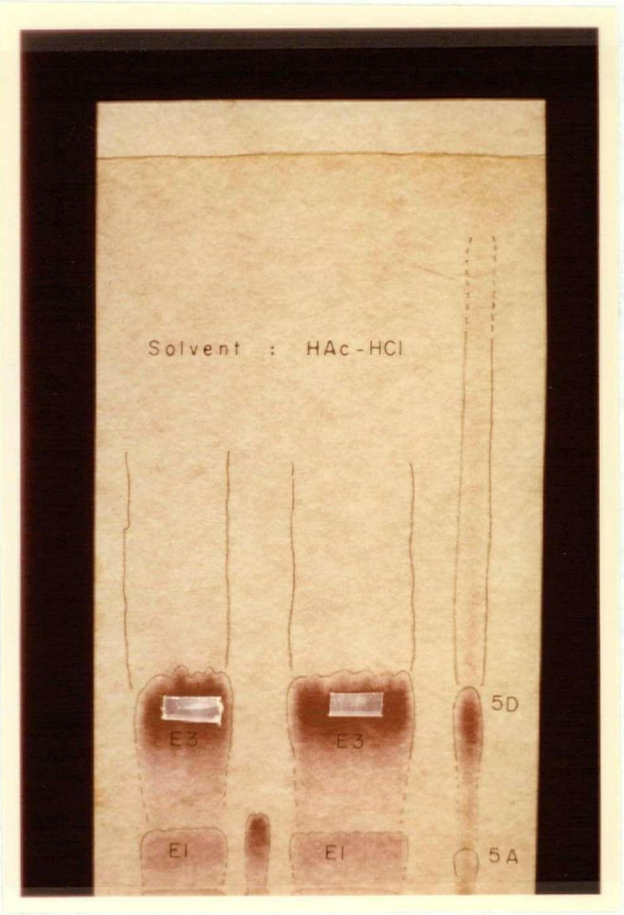

Fig. 15.3
Showing the appreciable hydrolysis of anthocyanin E3 and 5D during three years in the crystalline state. E3 was isolated from leaf sheaths and 5D, identical to E3, was isolated from caryopses. II is cyanidin. I was chromatospotted from 0.01% HCl-MeOH, II, III and IV from 1% HCl-MeOH.

anthocyanin also shows the other features shown by the crystalline anthocyanin E3-138.

Since pronounced hydrolysis occurs upon storage of the crystalline and semi-crystalline samples, it was decided to find out whether or not pronounced hydrolysis also occurred on paper during storage. Thus, a segment of the anthocyanin spot E3, which had been chromatographed in 1962 from the crystalline sample (Fig. 15.1, Chromatogram No. IV) was chromatographed in the HAc-HCl solvent by clamping (Fig. 15.4, Chromatograph No. I) in July, 1965. The results show that noteworthy breakdown occurs on paper during storage. It will

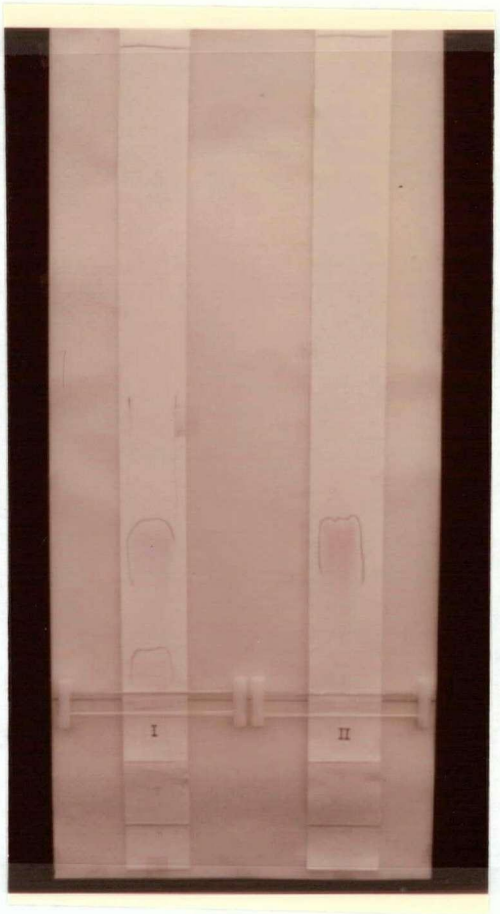

Fig. 15.4
Showing that appreciable hydrolysis of anthocyanin E3 occurs during three years storage on chromatopaper (I). Freshly isolated E3 (II) is not hydrolyzed. Chromatography by clamping obviated elution and, hence, any hydrolysis which would have occurred from this cause.

be further noted that whereas as the color of the stationary materials at the starting line in the 1962 chromatogram is barely visible, it is pronounced, as a reddish brown band, in the 1965 chromatogram.

It was, however, noted that if the segments excised from the band E3 (Fig. 15.3, Chromatogram No. III), either before, or two days after the solvent had dried, were rechromatographed in the HAc-HCl solvent by clamping, the breakdown did not occur as shown in Fig. 15.4, Chromatogram No. II.

Another important observation was made on the colors of the anthocyanins and anthocyanidins upon storage. The color of the antho-

cyanin E3 was dull bluish purple in ultraviolet light immediately after development. It became brighter magenta after 3-year storage. The brighter color is obviously due to the gradual *in situ* hydrolysis of the dull anthocyanin to the brighter aglycone. That the anthocyanins change color so markedly in storage was appreciated belatedly. Not only anthocyanins, but also anthocyanidins change color in storage, e.g., the anthocyanidin E1 fluoresces pink immediately after chromatography, it becomes bright mauve as it remains on the paper during a 3-year period. The color modifications in storage are minimal if several sheets are stored in a pile wrapped tightly with packing paper.

15.3 DISCUSSION

In the earlier stages of this work it was believed that the component E1-138 was an anthocyanin. The component could not be eluted successfully and it showed extensive breakdown to yellowish products (Sec. 11). The realization that the components known to be adsorbed irreversibly (Harborne, 1959a) must be similar to the component E1-138, was made possible by the concentration and stitching techniques. Although, the component E1-138 did show fluorescence (Sec. 11), characteristic of anthocyanidins, it was considered initially, the fluorescence could have been due to 5-glycosides or some other type of bright or fluorescing anthocyanin molecule. This is because the R_f values of anthocyanidins in the HAc-HCl, and the Aq-HCl (Table 11.I) solvents were not then known. The component broke down in the BAW solvent as shown elsewhere (Fig. 11.3). The HAc-HCl and the Aq-HCl solvents were earlier used exclusively for anthocyanins (*cf.*, Harborne, 1959a). Had the R_f values of the antho-

cyanidins been available in the two solvents, splitting of the anthocyanin E3-138, as being due to glycosidic hydrolysis would have been recognized earlier (See Sec. 11, and 13). It was the ease with which the partial hydrolysis of anthocyanins could be detected, as discussed in Section 17, that the HAc-HCl, the Aq-HCl and the 3:1:8 solvents were used routinely in the chromatography of both anthocyanins and anthocyanidins (Sec. 19).

The component E1 was identified as cyanidin later in 1962, when the crystalline components became available. That the crystalline component E1 was similar to the components adsorbed irreversibly was first suggested both by the insolubility of the crystalline sample in 1% aqueous HCl at room temperature, and by the immobility of the sample in the Aq-HCl concentrant. However, the finding that partial hydrolysis does occur on paper was established too late to be of any practical value in the work reported in the thesis. In the identification work reported in Section 19, and also elsewhere in this thesis, the elution was carried out from chromatograms sometimes as old as several months and sometimes immediately after development. No serious account was taken of the period the chromatograms remained in storage. Thus sometimes the components adsorbed irreversibly were encountered in substantial amounts and on other occasions only in negligible amounts. In fact, not being aware that the irreversible adsorption occurs due to hydrolysis every effort was made in the early phases of the development of the concentration and purification technique (Sec. 16) to effect total elution of the pigments from the paper. It was in the course of anthocyanin purification by the concentration technique that the equivalence of the 'tardy eluates' to cyanidin, peonidin and pelargonidin was established (See Sec. 19). Following these observations, the components that remained adsorbed

irreversibly were not eluted.

Although it was established by 1962 that glycosidic hydrolysis does occur on paper, it was not clear how, when and where the hydrolysis occurred. Does it occur due to high acidity that develops on the paper when the chromatographic solvents dry? Does it occur over a period of time during storage? Does it occur in solution as the anthocyanin is dissolved? If not, then why did the crystalline anthocyanin E3-138 show partial hydrolysis even when it was chromatographed from 0.01% methanolic HCl, and the anthocyanin was spotted as soon as the solution was made? Does it occur owing to the high acidity that may develop when an acidic anthocyanin solution, applied on the starting line, dries? Answers to some of the questions were obtained in examination of chromatograms in 1965.

The presence of a large quantity of cyanidin (Fig. 15.4, Chromatogram No. II) from anthocyanin bands remaining on paper for several years, and the absence of cyanidin in bands from freshly developed, or from a two-day-old spot obtained by clamping (Fig. 15.4) establishes that progressive glycosidic hydrolysis occurs on paper during storage. It is also clear that practically no hydrolysis occurs on paper at least until two days following chromatographic development. The observation shows why anthocyanins do not display the irreversible adsorption when eluted within a few days of chromatography (cf., Harborne, 1959a).

It was shown by stitching chromatography in Section 13 that anthocyanins split into several components. It will be noted from Table 13.I, that the anthocyanins of the collection No. 138 showed little splitting. Probably, the anthocyanins were chromatographed in

different solvents immediately after the BAW preparatory chromatography. However, collection No. 130 (Table 13.II) does show substantial splitting by stitching chromatography. It is likely that the chromatography was undertaken from relatively old chromatograms. Since collections No. 130 and No. 138 are from Gopal basal leaf sheaths harvested at different dates, it might have been concluded that differences in stage of development were responsible for the differences in number of anthocyanins found (Tables 13.I and 13.II). An explanation which is just as likely is that the differences in number are attributable to differences in breakdown. It will be noted further, that some anthocyanins in Table 13.II do show a component around R_f 0.10 in the HAc-HCl solvent. Similarly, several such components, namely A1, C1, D1, E1 and F1 were recovered from the eluates of the collection No. 138 (Table 11.I) or collections of the series 400 (Tables 19.VIII.A to 19.VIII.H) in the HAc-HCl solvent. The components, following identification, turned out to be the aglycones of the corresponding anthocyanin bands (Sec. 19). As expected they either disappear or fade in the butanolic solvents (Sec. 11 and 13). Since anthocyanins split into several components (Sec. 13), some anthocyanins in the aqueous solvent move very quickly, others very slowly: the mobility of the slow moving anthocyanin is confused occasionally as being due to irreversible adsorption. It is, however, clear that unless precautions are taken, the eluate from barley anthocyanins will not consist of a single anthocyanin but a mixture of several partially hydrolysed anthocyanins.

Several workers believe that anthocyanins, in general, are not 'amenable' to two-way chromatography: they tend to fade and form diffuse spots during chromatography in the second direction(review: Harborne,

1959a). However, others have experienced no difficulty (see Clevenger, 1964b). The reason for the discrepancy is likely linked with the preceding observations. If chromatography in the second direction is carried out immediately after drying, the diffusion is not likely to occur unless the anthocyanins carry acid-labile groups. However, if chromatography in the second direction is delayed for some time, the partial glycosidic hydrolysis may occur which may give rise to several spots if the anthocyanin is complex, and thus cause diffusion and overlapping of spots or bands. On this basis, anthocyanidins, in general, should be amenable to two-way chromatography irrespective of the period of storage following chromatography. Although this view needs substantiation, two-way chromatography of anthocyanidins has been carried out successfully (Clevenger, 1964a).

It was shown in Section 11 that a substantial part of the anthocyanin degration in solution was represented by the stationary materials at the starting line. The color of the stationary materials varied from brownish yellow, through brownish red to red. It is noteworthy that whereas the crystalline anthocyanin E3-138 did not show the stationary materials in 1962 (Fig. 15.1), it did show large quantities of the red stationary materials at the starting line on chromatograms in 1965 (Fig. 15.4). Moreover the band segments obtained from the freshly developed chromatograms, upon clamping chromatography, do not show the presence of stationary materials, but the band segment obtained from the three year old chromatogram did show substantial amounts of reddish stationary materials. Thus, the degradation, with time, to the stationary materials in crystals and on the paper is similar to the degradation in solution. Reddish colors at the starting line were noted usually when

solutions showing little breakdown were spotted and brownish red or brownish yellow colors when solutions in which breakdown was more pronounced were applied. Although little is known of the nature of the stationary materials, the indications are that degradation to the stationary materials is similar in the three states of anthocyanins, viz., in solution, on paper and in crystalline state except that the 'rate' of degradation in the last two cases is extremely slow. Further observations on the degradation through the stationary materials will be presented in Section 19.3.3.

The splitting of anthocyanins in the 1962 studies became confusing when the crystalline anthocyanin E3, chromatographed from 0.01% methanolic HCl split into two components (Fig. 15.1, Chromatogram No. IV). The splitting or hydrolysis of the crystalline anthocyanin could have occurred either during drying or storage over phosphorus pentoxide (see Secs. 19.2.2, and 19.3.1), or during the simultaneous crystallization of the two components inasmuch as the eluates of the anthocyanin E3-138 after three purifications in the HAc-HCl solvent still showed splitting. The possibility of hydrolysis being due to drying over phosphorus pentoxide in this particular case because the plugged sample (Sec. 15.1) was dried only for a short time, is remote. This is because the crystalline sample, after drying over phosphorus pentoxide, remained in storage, at room temperature, for a few months before it was chromatographed (in 1962) to determine if it would also show splitting as did the eluates of the thrice purified anthocyanin E3-138. The weak aglycone band in the crystalline sample (Chromatogram No. IV, Fig. 15.1) is most likely due to storage at room temperature. The view is supported fully by the 1965 studies, which provide clear cut evidence that the splitting is due to the hydrolysis

that ensues in storage both in the crystalline state (Fig. 15.3) and on the paper (Fig. 15.4).

The total evidence shows that the hydrolysis on the paper, and in the crystalline state upon storage is progressive. The crystalline samples and so also the chromatograms for further processing, or spectral analysis on paper (cf., Sec. 14), should have been stored preferably in a freezer to minimize hydrolytic breakdown. Moreover, the anthocyanin colors in ultraviolet light should have been noted immediately after chromatographic development. That the colors do change upon storage was not appreciated throughout.

Since hydrolysis on the paper was established from the 1965 studies, the observations on the anthocyanin-splitting studies obtained by the stitching technique (Sec. 13) were again examined. The splitting must be due to some type of hydrolysis that occurs during storage, because the anthocyanin spots travel to their respective R_f values largely as distinct entities (without splitting). If an anthocyanin is structurally complex, several anthocyanin spots, in addition to the corresponding anthocyanidin, may appear on the paper upon rechromatography owing to partial hydrolysis. Although in several cases a large number of 'nin spots and also some 'din spots were recovered (Fig. 13.8), in other cases only 'nins and no 'dins were recovered (Fig. 11.7, spots A, B, etc.). For example, the anthocyanin A-130, upon stitching rechromatography with the HAc-HCl solvent (Fig. 11.7), shows three anthocyanin components at R_f values 0.26, 0.56 and 0.65 at a relative concentration ratio of 1:20:4, respectively but no anthocyanidin component. The varying concentrations of the 'component' anthocyanins and the absence of the corresponding aglycone suggests that the splitting of barley antho-

cyanins may not entirely be due to glycosidic hydrolysis. The anthocyanin A-130, and several other anthocyanins are highly labile due to the attachment of some unknown components. The situation cannot be explained on the known behaviour of acylated anthocyanins (see Secs. 13, 5, and 19).

That the eluates of the highly purified anthocyanin E3-138 show breakdown does not appear to be a safe basis to conclude that hydrolytic breakdown does occur in solution. At the time these studies were carried out, the writer was not aware of the hydrolytic breakdown on the paper. No records of the dates of the three elutions (or purifications) are immediately available, though to the best of the writer's recollection, the last two purifications were successive. Nonetheless, there is a room to doubt that the purifications might have been conducted over such intervals of time that hydrolysis on paper could have occurred prior to the elutions. Hence, the question whether hydrolysis occurs in solution still remained inconclusive. That the hydrolysis in solution does occur under the recommended conditions of handling was established (Sec. 23) at a later date before undertaking hybrid analysis.

Although it was not established by 1961 that hydrolysis in solution does occur, the key observation (Sec. 15.2.1) that upon concentration of anthocyanin eluates <u>in vacuo</u> that which remains in the flask is a constant boiling hydrochloric acid suggested that the acid could be detrimental to anthocyanin stability. This is because the rate of evaporation of the methanolic anthocyanin extract is relatively fast in early stages of flash evaporation but very slow in the final stages. Thus, the anthocyanins remain in the environment of high acid concentrations and high temperature in the final stage of flash evaporation, the stage at which the degradation is maximum. The hydrochloric acid at

such high concentrations and temperature could have caused hydrolytic breakdown. Although this question will be dealt with in greater detail elsewhere (Sec. 23), it is sufficient to indicate that it was primarily on the assumption that high acid concentrations were detrimental to the anthocyanin stability, that the level of anthocyanin concentrant and eluent was kept low in the purification and elution technique described in the next section.

15.4 SUMMARY

Evidence is presented to prove that the splitting of barley anthocyanins on paper (Sec. 13) and in crystalline state occurs due to glycosidic hydrolysis that ensues gradually in storage. Also, glycosidic hydrolysis is the principal cause of the irreversible adsorption of anthocyanins that occurs on the paper a few weeks after development, and that the component adsorbed irreversibly is the corresponding aglycone. The implication of the observations are of particular importance in spectral analysis because, contrary to expectations, what may be eluted from stored chromatograms of complex barley anthocyanins, is a mixture of partially hydrolysed anthocyanins and not a pure anthocyanin. It is also shown that colors of anthocyanins, particularly in ultraviolet, change in storage due to partial hydrolysis, hence they should be recorded soon after chromatography.

The observations indicate that the degradation of anthocyanins to the stationary materials (cf., Secs. 11 and 19) is similar in the three states, namely, in solution, on the paper and in crystalline state, except that the 'rate of degradation' in the latter two states is very

slow.

In addition is described a key observation on the increase in acid concentration that occurs when acidified methanolic extracts are flash evaporated to dryness. The observation, in effect, enabled the development of a satisfactory method for elution and purification of anthocyanins described in the next section.

METHODS FOR CHARACTERIZING ANTHOCYANINS

16. A TECHNIQUE FOR PURIFICATION AND ELUTION OF ANTHOCYANINS

Although hydrolytic degradation of anthocyanins occurs both on the paper (Sec. 15) and in the solution (Sec. 11 and 23), it is extremely small on paper. Thus, the method of elution and purification should be so designed that anthocyanins, during purification and isolation, are kept for maximum time on the paper and minimum time in the solution. The degradation in solution is time-dependent: the technique of elution must also provide for the elution of anthocyanins in a highly concentrated state so that the eluates require minimal handling for concentration and drying (see Secs. 15, and 23). Since anthocyanins, because of contaminants, require several chromatographic purifications, the technique should be such that chromatographic purifications may be carried out in different solvents with as few elutions as possible. The technique it would appear must satisfy the two counteracting pH-dependent

variables: acid causes glycosidic hydrolysis of anthocyanins but acid also "preserves" anthocyanins. The concentration of acid in the eluent therefore, should be such that it maintains anthocyanins in the stable flavylium salt state and yet causes only minimal hydrolysis. A technique that satisfies the above requirements was developed in this laboratory and will be presented in this section.

The purification and elution technique was used for all anthocyanins of basal leaf sheath and grain tissues described in Section 19.

16.1 MATERIALS AND METHODS

Four solvents, namely, BAW, diethyl ether, equal parts of methanol and water containing 0.06% HCl, and HAc-HCl were used for the purification of anthocyanins. Chromatography in the BAW, and the HAc-HCl solvents was carried out via the usual chromatographic equipment (Sec.10), that in diethyl ether and the acidified mixture of methanol and water via a 'Concentration Chamber' described in Fig. 16.2. Certain features of the preparative chromatography, as the necessity arose, had to be tailored to suit the requirements of the purification and elution technique as described later in Fig.16.4.

16.2. A TECHNIQUE FOR ANTHOCYANIN PURIFICATION

It was observed from earlier studies (Sec. 13) that when the anthocyanin bands (Table 13.III) were concentrated by ascent (Lederer and Lederer, 1957; Lesins and Lesins, 1958), large amounts of yellowish

materials* derived from Whatman Paper No. 3 moved to the tip of the paper bands containing the anthocyanins. Thus, as a preliminary to purification, it was decided to remove the yellowish contaminants from the paper prior to anthocyanin chromatography. Blank papers were irrigated, by ascent, with a mixture of methanol and water. The operation concentrated the 'yellows' at the solvent front in generous amounts. However, re-irrigation with the same solvent, when the paper had dried, yielded the yellowish materials again. It was later found that the yellowish materials were readily soluble in ether which removed them further in generous quantities. The yellowish materials thus obtained by several trials were cut out and extracted with ether. Upon evaporation of ether at room temperature a yellowish oily material was obtained. The spectrum in the visible region of this material in 0.01% methanolic HCl is featureless, except that it shows a continuous rising trend towards shorter wavelengths similar to those shown elsewhere for the yellow ends (Figs.18.2 to 18.4). The ultraviolet spectrum of the yellowish oily material (Fig. 16.1), however, gives a distinct peak at 275 mu and an inflection around 280 mu. The ultraviolet peak is notably different from that obtained from the paper-derived materials elsewhere (Fig. 18.5). The presence of the yellowish materials interferes with the spectral determination of the peaks around 360 mu (Figs. 12.1, 12.2, 12.3, and 15.2).

Thus, there was a three-fold problem in the purification of the anthocyanins from the BAW bands: (a), how to eliminate the yellow

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*Inquiry made to the manufacturers of Whatman Chromatographic paper elicited no information on the nature of these impurities.

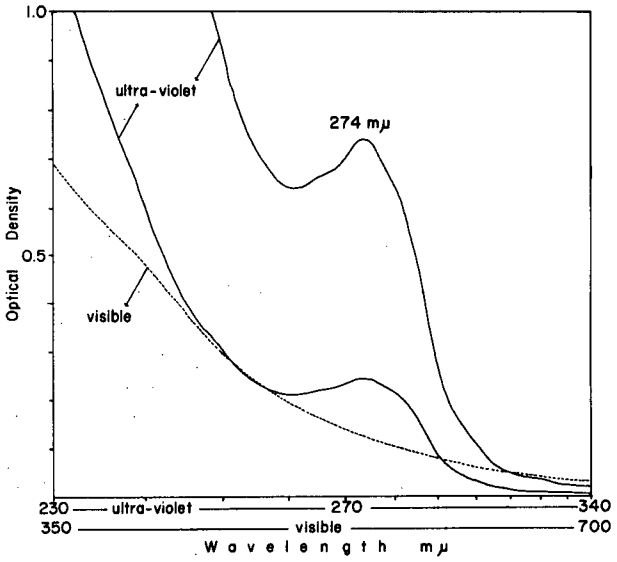

Fig. 16.1.
Absorption spectra in the visible and ultraviolet wavelengths of yellow contaminants removed from chromatopaper by ascending irrigation first with methanol-water and then with ether. Spectra were drawn from 0.01% methanolic HCl solutions.

contaminants from the paper bands, (b), how to purify anthocyanins on the band without elution, and (c), how to elute anthocyanins from the paper bands using the smallest volume and shortest time in the interest of anthocyanin stability. The problem was resolved with the development of a Concentration Chamber described below.

The Concentration Chamber, ideally, should consist of a glass jar, 23x23x23 cm, with rimmed border. However, any jar, for example Pyrex catalogue No. 6944, or even a beaker of suitable size may be used. In this study, however, battery cell jars, 30x20x14 cm, were used (Fig. 16.2). Although the jars are very sturdy and ideally constructed, they require larger quantities of solvent because of their height.

The jar is covered with a sturdy polyethylene sheet, which is secured by a pair of heavy-duty rubber bands, and a few strips of masking tape. The sheet should remain crinkle-free at the top under its own tension; it should never be over-stretched. Parallel incisions are then

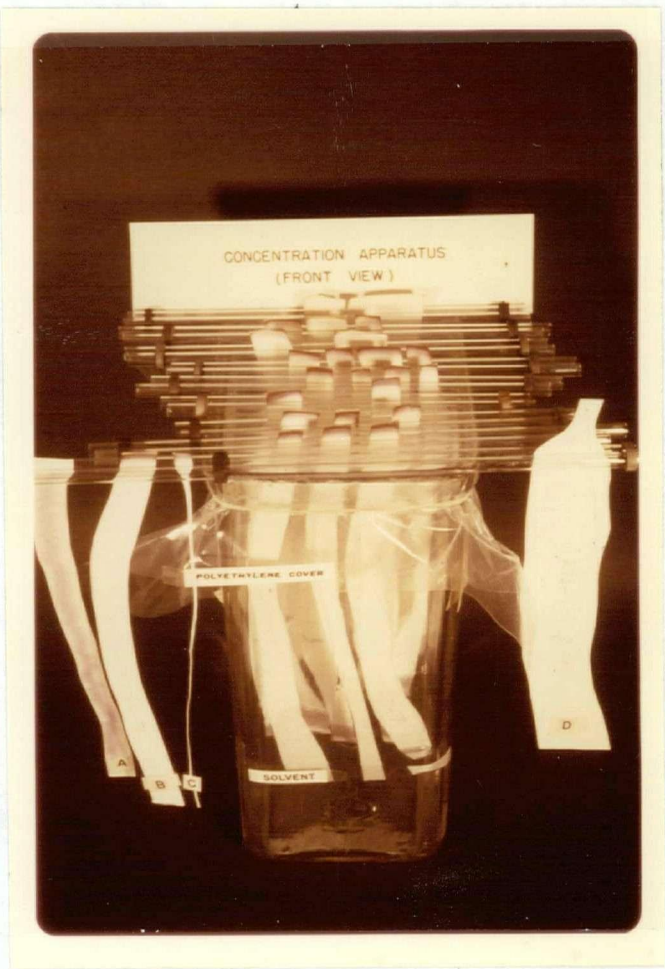

Fig. 16.2
Concentration chamber, front view, showing anthocyanins accumulating on paper tips. A, band before placing in chamber, B, after concentration and C, prepared for elution. D, shows band preparation for concentrating an anthocyanin from a diffuse or weak band. The totality of the anthocyanin movement is shown by B.

made on the polyethylene sheet across the width with a very sharp razor so that neither the sheet, nor the incised margins are stretched. The incision should remain away from the margins of the jar by 1 cm. The distance between the parallel incisions is governed by the width of the glass-rod suspension unit, which is made by fastening two glass tubes (each ca. 18 cm long) by rubber bands (see later). The anthocyanin bands are cut out, supported between the two glass rods of the suspension unit, and then inserted, through the incisions, in the Concentration Chamber. Sufficient quantity of the appropriate solvent (see later) is poured into the jar (actually, the solvent is poured

before mounting the polyethylene sheet) so that the lower end of the paper bands, which is anthocyanin-free, dips in the solvent.

In preliminary attempts, diethyl ether was used in the concentration chamber for the purification of anthocyanins. Ether was chosen because anthocyanins are insoluble and the yellow contaminants of the paper are soluble in this solvent. Should any ether-soluble admixture be present in the anthocyanin band, that could also be removed in the course of solvent ascent. Thus, within a few hours after anthocyanin bands were inserted in the concentration chamber containing ether, large amounts of yellow contaminants moved to the terminus of the bands. The yellow ends were snipped, and the suspended anthocyanin bands were transferred to another concentration chamber containing ethyl acetate, and the process was repeated, whence the suspended bands were finally transferred to another concentration chamber containing the anthocyanin 'concentrant', the acidified mixture of methanol and water. The yellow ends were finally trimmed off to concentrate the purified anthocyanins at the terminus for elution (see Secs. 18 and 19). Although ether, in particular, appeared to be an excellent purifying solvent, its potential could not be exploited for lack of appropriate facilities for handling volatile solvents. The anthocyanins bands were, therefore, purified and prepared for elution only in the anthocyanin concentrant. Some of the BAW anthocyanin bands, from basal leaf sheath and caryopsis anthocyanins (Sec. 19), thus purified and concentrated are shown in Fig. 16.2, which was taken during early phases of the development of this technique. In addition to the concentration apparatus Fig. 16.2 also shows four paper bands A, B, C, and D. These bands, however, are not a part of the concentration apparatus. The band B shows that anthocyanins as shown in the band A, may be

concentrated completely.* The band C will be referred to later. The band D was included to indicate that anthocyanins which may be very weak and diffused over a large area may be concentrated over a small area protruding above the suspension unit prior to elution. Fig. 16.3 shows the amenability of the technique to large scale isolation work.

Fig. 16.3.
Showing large scale concentration and purification of anthocyanins.

Several practical difficulties were encountered when the anthocyanins were purified and concentrated directly in the anthocyanin concentrant: (a) although the yellow contaminants move to the terminuses ahead of most anthocyanins, some contamination does occur because of the faster mobility

*The band B, (Fig. 16.2) in fact, was included to show that even anthocyanin components that remain adsorbed irreversibly (cf., Harborne, 1959a) may also be concentrated. Since it has now been demonstrated that the irreversible adsorption is due to hydrolysis (Sec. 15), and that the component remaining adsorbed on the paper is the corresponding anthocyanidin, the component should not have been concentrated.

of certain anthocyanin components, particularly, those which resolve around R_f above 0.65 in the HAc-HCl solvent, and (b) the terminuses where the anthocyanins concentrate as seen in Fig. 16.2 were rather too wide and required a larger amount of solvent for elution. Extra blank space near the upper terminus of the paper bands would resolve the difficulties mentioned in (a) and (b) above. Accordingly, a few features of chromatography were modified to suit the requirements of purification and elution (see Fig. 16.4).

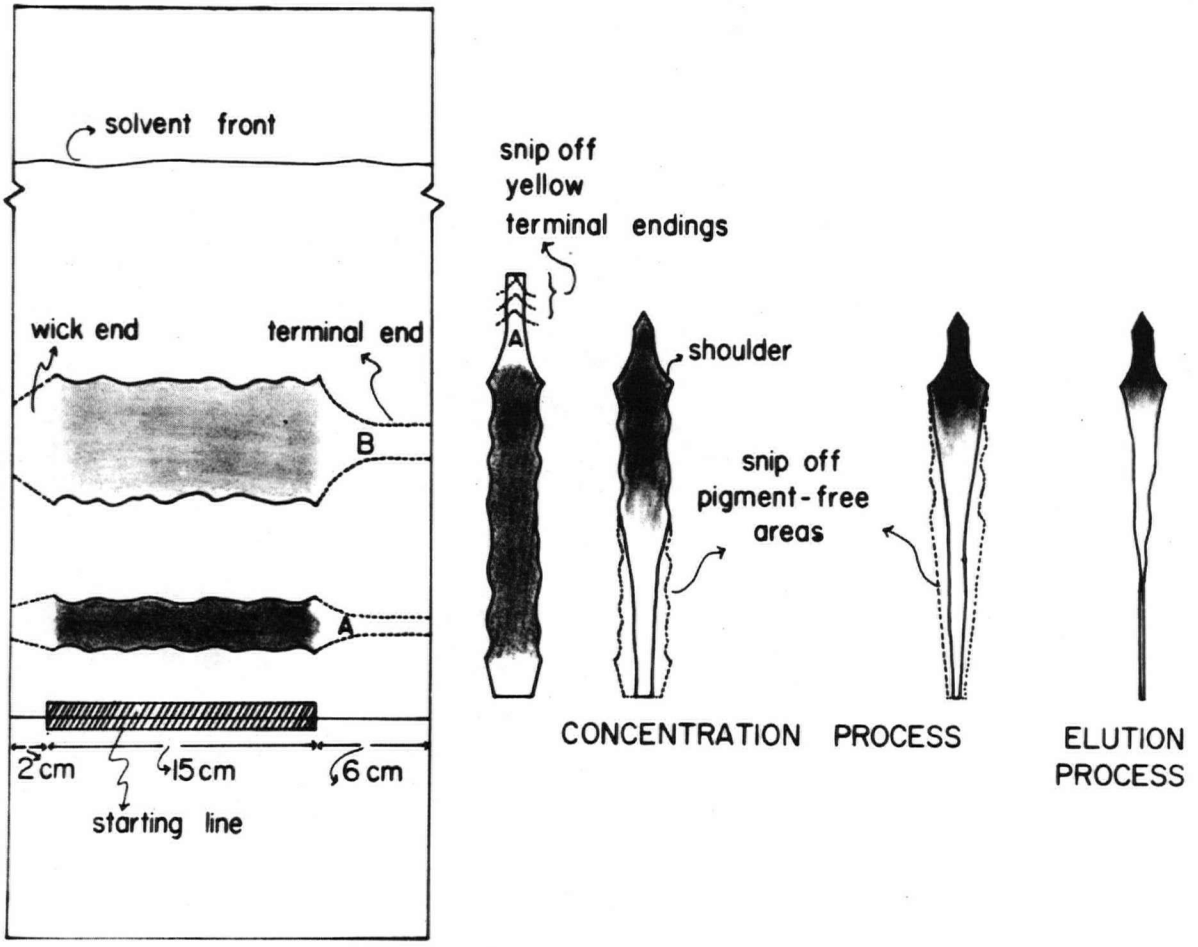

Fig. 16.4
Diagram of steps in concentrating, purifying and eluting anthocyanins from a chromatoband.

The standard size Whatman paper No. 3 (46x57 cm) is divided into halves along the length. Concentrated anthocyanin eluates (obtained from the BAW bands by the above procedure) were applied as a 15 cm long band on the starting line in such a way that 6 cm blank space is left on one side, and 2 cm blank space on the other side (Fig. 16.4). The chromatograms are developed in the HAc-HCl solvent for further purification. The HAc-HCl anthocyanin bands are marked, their designations are pencilled in the centre of the 6 cm blank area as shown in Fig. 16.4, and are cut out along the dotted lines. Note, the upper end is tapered: the width of the tapered end is usually kept to 0.7 cm, depending upon the width of the anthocyanin band.

The bands, as usual, were purified in the anthocyanin concentrant* for the second time. The following steps were taken further to reduce the contaminants derived from the paper to the minimum. The 6 cm blank terminus is kept above the surface of the polyethylene sheet. When the solvent had reached the end, a small table fan, placed a few feet from the chambers, was occasionally turned on to hasten ascent. About 5 hours later, the yellow substances, which collect in large amounts, are snipped off with a stainless steel scissors as shown in Fig. 16.4. The process can usually be repeated twice with slow-moving anthocyanins and, at best once, with fast-moving anthocyanins. Meanwhile the anthocyanins concentrate at the terminus, and lateral regions near the wick end become anthocyanin-free. Because ether could not be used, and because small amounts of yellow contaminants still moved to the terminus, the

*The ratio of the components of anthocyanin concentrant may be varied to suit the movement of admixtures, if any.

anthocyanin-free lateral portions of the paper band near the wick end are cut off as often as practicable to reduce the contaminants (Fig. 16.4). The width of the shoulder must be reduced as much as possible to suit the requirements of the elution technique (see later). Thus, as and when an opportunity permits, the shoulder should be slightly trimmed. When the bulk of anthocyanins reach the terminus, the suspension rods are pulled out and the bands prepared for elution (Sec. 16.3).

The time taken for anthocyanins to reach the ends varies from 6 to 24 hours. Thus, in order to facilitate the snipping operation (removal of the yellow terminuses and the anthocyanin-free laterals, the fast-moving anthocyanin bands and the slow-moving anthocyanin bands are put in separate chambers. This is easily determined because the anthocyanins which move faster in the HAc-HCl solvent, also move faster in the concentration solvent and vice versa. If occasionally the impurities could not be snipped off at least twice during working hours, the ascent was discontinued until the following morning by placing glass rods of suitable diameter between the surface of polyethylene sheet and the glass rod suspension units so as to break the contact of the paper bands with the concentration solvent.

The solvent requirement for concentration by ascent, in the concentration chamber, is minimal. Only 3 ml of the concentrant evaporated over a period of 24 hours from a strip of Whatman paper No. 3, which was 9" long, and 1¼" wide and which was exposed by 1½" above the surface of the polyethylene sheet.

16.3 AN ELUTION TECHNIQUE

Preparation of the paper bands for elution is undertaken as soon as the bands are removed from the concentration chamber. The terminuses containing anthocyanins are not allowed to dry. Therefore, the bands from the concentration chamber are removed at intervals. The regions of the bands containing either no anthocyanins or anthocyanins adsorbed irreversibly (Harborne, loc. cit.) are liberally cut off so that a narrow strip as illustrated in Fig. 16.2.C, and Fig. 16.4. is obtained. The width of the strip determines the rate of eluent flow. In order to collect anthocyanins in a minimum amount of the eluent, at least 9 cm length from the wick end of the strip is kept less than 1.5 mm wide. It is convenient in large scale elution work to return the prepared bands to the concentration chamber so that the upper terminuses remain moist. The anthocyanins elute faster, in a few drops of the elution solvent per strip, if the terminuses remain moist throughout handling. When sufficient strips for a given size of elution cabinet have been prepared, the elution is commenced.

A plexiglass elution cabinet was constructed to hold standard two-way chromatography glass troughs on the stainless steel brackets (Shandon No. 2124). The brackets were mounted so that the troughs were 25 cm high from the cabinet base. Two windows (15x15 cm) were provided on each side of the cabinet, at a height of 12 cm from the cabinet base, for easy access to the elution assembly. The windows were provided with air-tight lids. An ideal apparatus for the purpose is the one distributed by Kensington Scientific Corporation, California; however, the cost is high.

The elution solvent for anthocyanins consists of 0.03% methanolic

HCl. The solvent is poured into the elution troughs. The wick ends of the prepared strips, using surgical gloves, are set up in the troughs for elution by descent. Anti-siphon rods are not used, because when mounted at the usual distances* from the troughs, they retard the rate of solvent flow: the anthocyanin terminuses in the meanwhile dry and require a larger quantity of the solvent for elution. The anthocyanins by the above procedure are eluted only with a few drops per strip.

Occasionally, the anthocyanin ends may break away from the strip. The broken ends are moistened with a squirt of the eluent and then stuck to the end of an anthocyanin strip which is already eluting: the broken terminus does not fall off so long as it remains wet.

Complete elution of anthocyanins was achieved using the above techniques. When partial hydrolysis of anthocyanins was suspected (due to extremely slow mobility of the aglycone), the aglycones were neither concentrated nor eluted completely. The slow mobility, however, may not always be due to the aglycones: several anthocyanins move very slowly in the anthocyanin concentrant, hence, complete concentration and elution in such cases is desirable.

Further processing of the eluates was undertaken as described in Section 19.2.2.

16.4 DISCUSSION

The development of the Concentration Chamber, which is based on

*Probably, the anti-siphon rods, mounted immediately above the edge of the glass trough, may give superior elution to the one obtained without the use of anti-siphon rods.

the well known technique of concentration by ascent (Lederer and Lederer, loc. cit.; Lesins and Lesins, loc. cit.) has greatly advanced the objectives of this work. Through the development of the Concentration Chamber were obtained the clues that the irreversible adsorption of anthocyanins (review: Harborne, 1959a) may be due to the hydrolysis of anthocyanins on paper (Sec. 15), that the eluates of barley anthocyanins eluted from a purified anthocyanin band may contain a mixture of several anthocyanins (Sec. 15), and that the eluates may contain paper derived yellow materials and a large number of sugars (Sec. 19). The value of the Concentration Chamber in anthocyanin purification is adequately demonstrated in this section. The Chamber, in fact, is used as equipment for repeated chromatographic purification of the same anthocyanin band in several solvents without the intervention of elution. In simple chromatography, the contaminants, at best, are only separated, whereas via the Concentration Chamber they are concentrated, separated and removed in a single operation, and above all, the anthocyanins are also purified and concentrated at the same time for elution in a few drops of solvent at the same time. The anthocyanins purified by this method show brilliant colors and are relatively pure compared to those obtained by direct elution. If a large quantity of the anthocyanin is present in the band, crystallization occurs at the terminus in the course of continuous ascent.

Although specific solvents may be used for eliminating admixtures of compounds that may occur with anthocyanins, the use of ether is very advantageous because the yellow contaminants in the paper are readily soluble in the solvent. Although ether could not be used regularly under our conditions, a few precautions are essential while using the solvent: (a) the polyethylene sheets are mounted under their

own tension so that when the parallel incisions are made, the incised edges remain intact and are not pulled apart; (b) the upper ends of the paper bands, which normally are kept above the surface of the polyethylene sheet, are pushed all the way into the chamber as far as possible. The apparatus is then enclosed in an air-tight container* to hasten ascent of the yellow materials to the terminuses. The potentiality of ethyl acetate requires further experimentation.

The anthocyanin concentrant was chosen by trial and error. In the aqueous solvents (Sec. 13) several anthocyanins moved rather slowly, in alcohol they moved faster so that several anthocyanins reached the terminuses almost as quickly as the yellow contaminants. A mixture of alcohol and water gave good results. The acid concentration was kept low both in the eluent and the concentrant to keep hydrolysis of the anthocyanins to the minimum. Concentrations of acid below the 0.03% HCl were not tried because of the belief that low acid concentrations may cause some other degradative transformations or modifications. Also it was felt then that the time-induced degradations to yellow products were probably faster in solvents containing 0.01% conc. HCl (Sec. 12). In order, therefore, to complete the elution process quickly, only the acidified methanol (and no water) was used in the anthocyanin eluent.

Concentration and purification by this technique is 'mild' not only because of the low concentration of acid (0.06% HCl) used in the concentrant, but also because at no stage is the concentration of acid allowed to increase. Total amount of evaporation of the concentrant is less than 3 ml per strip per 24 hours. Because the amount of hydrochloric

*In our work, chromatocabinets and chromatography jars were used.

acid present in the 3 ml concentrant is negligible, and because throughout the process of concentration and elution, the anthocyanin terminuses are not allowed to dry, it is doubtful that under such mild chromatographic conditions, high acidity could have developed to cause any significant or detectable glycosidic hydrolysis.

Apart from the contamination, the yellow terminuses were snipped off originally because of a suspicion that they cause the increasing absorption in the wavelength range 300-400 mu (Fig. 16.1; also see Figs. 18.2, 18.3, and 18.4) and thus, interfere with the spectral analysis (Figs. 12.1, and 15.2). The realization that the yellow terminuses contain several types of sugars derived from chromatography paper (Sec. 18), chronologically came when the identification of the sugar components of the large number of anthocyanins purified by the above techniques was undertaken. Accordingly, it may be pointed out that when slow-moving anthocyanins were concentrated (R_f 0.5 in the HAc-HCl solvent) yellow contaminants were snipped off at least twice, and sometimes thrice before the anthocyanins reached the ends. The ends with the fast-moving anthocyanins (R_f 0.65, in the HAc-HCl solvent) could be trimmed only once. Thus, a possibility exists that such anthocyanin eluates may contain traces of paper-derived sugars. Although, the 6 cm blank terminus provides enough area for snipping away the contaminants, the area may be kept, in future work, at 8-10 cm in the case of the fast-moving anthocyanins, or else the proportions of solvents in the concentrant may be changed to secure the differential rate of movement of the contaminants and the anthocyanins. In any event, the above observations clearly reveal the advantages of purification *via* the concentration chamber. The sugars, which can be the potential cause of anthocyanin instability (Sec. 18.3) are probably

largely eliminated by the simple snipping operation. Because the paper contains large quantities of sugars, the lateral trimming of the bands during the concentration process, and the low acid concentrations used in the solvents further reduce the quantities of contaminants eluted from the paper.

The advantages of the lateral trimming of the bands for preparation of elution are two-fold: (a) the anthocyanins are eluted in a small quantity of the solvent, and (b) the small size of the trimmed bands conserves spaces in the elution cabinet. 10-15 bands of the same anthocyanin are mounted on either side of the trough, and collected in a single container. This, undoubtedly, increases the amenability of the technique for large scale elution work.

Recently it has been shown that the rate of anthocyanin destruction is pH dependent only in the presence of oxygen, and that pH has no effect on the rate of destruction in the presence of nitrogen (Lukton, Chichester and Mackinney, 1956). It, therefore, appears that the periodic evaporation of the solvent from the paper band strips, protruding above the level of the polyethylene sheet, should not have been achieved by blowing air with a table fan. Instead the concentration chamber should have been enclosed and a slow stream of nitrogen should have been used for evaporation to facilitate ascent of anthocyanins to the tip.

Although, the requirements for insuring anthocyanin stability, outlined in the introductory paragraphs, are fully satisfied via the purification and elution technique, and although, anthocyanins were obtained in a stable state, primarily, by exploiting their relative stability on paper as compared to solution, the techniques of purification and elution presented in this section must be provisional means for

working with barley anthocyanins until a deeper understanding of the real causes of anthocyanin instability is gained.

16.5 SUMMARY

The purification of anthocyanins, from the contaminants originating from extracts, in several solvent systems, without repeating elution and thus avoiding flash evaporation and rechromatography, was carried out using a 'Concentration Chamber'. Depending upon the solvents used in the concentration chamber, the contaminants originating from extracts or from the paper per se (such as sugars: the potential causes of anthocyanin instability) are removed merely by snipping the ends of paper bands where they are made to concentrate. During the purification, the anthocyanins are kept for maximum time on the paper and minimum time in solution because they are relatively stable both in a dry or wet state on the paper.

The two counteracting pH-dependent variables: acid causes glycosidic hydrolysis but acid also "preserves" anthocyanins, and also the time-dependent instability of an anthocyanin solution were provided for by eluting anthocyanins in a highly concentrated state in a few drops of 0.03% methanolic HCl, thus avoiding the time-consuming and hydrolysing effects (Sec. 23) of flash evaporation.

The techniques of purification and elution are amenable to large scale isolation work.

METHODS FOR CHARACTERIZING ANTHOCYANIDINS

17. SOLVENTS DEVELOPED AND USED FOR ANTHOCYANIDIN CHROMATOGRAPHY

There are two kinds of acidic solvents used in anthocyanidin chromatography: the organic solvents (generally, butanolic and phenolic) and the aqueous solvents containing a mixture of mineral and organic acids. Although, certain butanolic and phenolic solvents give a good 'spread' between R_f values of certain anthocyanidins, the spread cannot be exploited in identification because anthocyanidins often fade in the organic solvents. As a result, small amounts of anthocyanidins may not be detected.

The anthocyanidins are generally stable in aqueous acidic solvents, but there are only a few such solvents that are satisfactory for anthocyanidin identification. For instance, in Forestal solvent, it is very difficult to differentiate peonidin from pelargonidin, petunidin from cyanidin, and occasionally, cyanidin from malvidin. The formic

acid solvent of Harborne (1959a) (a modification of the solvent originally proposed by Endo, 1954), although useful, gives a poor spread between cyanidin and malvidin, malvidin and peonidin thereby making the identifications based on R_f values indefinite. The acetic acid-hydrochloric acid-water (5:1:5) solvent of Abe and Hayashi (1956) is not satisfactory because the R_f value ranges of anthocyanins and anthocyanidins overlap. This is an undesirable feature in the differentiation and identification of partially hydrolyzed products of anthocyanins.

Keeping in view the inadequacies of these solvents and the difficulties mentioned already, some new solvents were developed. The composition of these and some standard solvents is listed in Table 17.I. The range of R_f value variations, and average R_f values of standard anthocyanidins in the solvents used are listed in Table 17.II. The first line in each column indicates the fluctuation range in R_f values that were obtained on the basis of several runs. The number of such runs is indicated in parenthesis in the table. For instance, it will be seen that in the first left hand top column, the R_f values for pelargonidin in Forestal solvent on the basis of 18 determinations fluctuate from 0.62 to 0.74. The average R_f value in this case is 0.67. There was a contaminant present in a specific peonidin sample, and its R_f values are listed in the column alongside that for peonidin. For instance, peonidin in Harborne's formic acid solvent shows a pink fluorescent contaminant at R_f 0.05; it appears to be an anthocyanidin of unknown nature.

The Iso-PrOH solvent of Abe and Hayashi (1956) is excellent for differentiating peonidin from pelargonidin. The anthocyanidins, in this solvent, however, fade quite rapidly as is usually the case with alcoholic solvents (see component E1: Figs. 11.3 and 11.4). This

TABLE 17.1
COMPOSITION OF THE SOLVENTS USED FOR ANTHOCYANIDIN CHROMATOGRAPHY[1]

Abbreviation	Composition	Period of Development Hours
Forestal	HAc-HCl-H_2O:(30:3:10), S	16
FA-Harborne	HCOOH-HCl-H_2O:(5:2:3), S	7½
FA-4NHCl	HCOOH-4N HCl:(2:1), S	7½
HAc-HCl[2]	HAc-HCl-H_2O:(15:3:82), S	6
Propionic	PA[3]-HCOOH-HCl-H_2O:(2:5:1:6), U	10
3:1:8	HAc-HCl-H_2O:(3:1:8), S	8
5:1:5	HAc-HCl-H_2O:(5:1:5), S	11
Iso-PrOH[4]	i-PrOH-5% HCl:(55:45), S	20
Aq-HCl[2]	HCl-H_2O:(3:97), S	4
BAW	n-BuOH-HAc-H_2O:(4:1:5), U	13

[1]Chromatography was carried out at 20°C ± 1°C.

[2]Anthocyanin solvent of Harborne (1959a) extended to anthocyanidins.

[3]PA = Propionic acid.

[4]Iso-Propyl alcohol solvent of Hayashi (1957), Abe and Hayashi (1956).

S Single phase.

U Upper phase.

TABLE 17.II

RANGE OF R_f VALUE VARIATION AND AVERAGE R_f VALUES OF STANDARD ANTHOCYANIDINS IN THE SOLVENTS LISTED IN TABLE 17.I[a,b]

	R_f x 100								
Solvents	Pelargonidin[c] 1 OH	Peonidin[d] 1 OH, 1 OCH_3	Contaminant[f]	Malvidin[d-g] 1 OH, 2 OCH_3	Cyanidin[c] 2 OH	Petunidin[d,g] 2 OH, 1 OCH_3	Delphinidin[d] 3 OH	Apigeninidin[d] 1 OH	Luteolinidin[d] 2 OH[e]
Forestal	62-74	60-69		56-64	45-55	42-50	34-36	81-83	65-70
	(18)	(13)		(11)	(14)	(12)	(4)	(3)	(4)
	67			60	48	45	35	82	67
FA-Harborne	32-40	25-33		24-30	22-27	18-23	13-15	60-62	44
	(17)	(12)		(11)	(15)	(11)	(13)	(2)	(2)
	36	29	5	26	24	20	14	61	44
FA-4N HCl	47-55	44-51		42-64	33-38	30-35			
	(10)	(10)		(10)	(12)	(12)			
	51	48	10	44	37	33	21	69	56
HAc-HCl	15-19	12-13		8-10	10-12	7-8.5		27-30	18-21
	(15)	(5)		(10)	(13)	(10)		(3)	(3)
	18	12		9	11.5	8	7	29	20
3:1:8	25-28	19-21	3	13-16	15-18	11-13	9		
	(12)	(7)		(11)	(15)	(10)	(3)		
	26	20	3	15	16	12	9	38	27

TABLE 17.II continued

Solvents	R_f x 100								
	Pelargonidin[c] 1 OH	Peonidin[d] 1 OH, 1 OCH_3	Contaminant[f]	Malvidin[d-g] 1 OH, 2 OCH_3	Cyanidin[c] 2 OH	Petunidin[d,g] 2 OH, 1 OCH_3	Delphinidin[d] 3 OH	Apigeninidin[d] 1 OH	Luteolinidin[d] 2 OH
Propionic	45-50 (14) 46	39-43 (8) 40	8	33-38 (9) 35	28-32 (13) 30	24-29 (12) 26	20 (3) 20	65-67 (3) 65	51-53 (3) 52
IsoPrOH	54-73 (13) 68	45-52 (6) 50		h 33	45-42 (12) 48	h 33			
5:1:5	49			38	38	31			
Aq-HCl	4.5-5 (2) 5	2.5-3 (20) 3		2-2.25 (3) 2	2.5-3 (5) 2.5	2-2.5 3 2	1.5[i]	7	4.25
BAW	80-82 (2) 64	61-68 (2) 64			52	60	46		

[a] In general, the R_f values of each anthocyanidin in each solvent are listed in three rows: first row shows the range of R_f value fluctuations obtained in several runs. The number of such runs is shown in

parenthesis in the second row. The average R_f value is shown in the third row.

[b] The chromatography was carried out at 20°C ± 1°C.

[c] Provided by Dr. R. M. Acheson, University of Oxford, England.

[d] Provided by Dr. J. B. Harborne, John Innes Horticultural Institution, Hertfordshire, England (see g, h, and i).

[e] OH at 3-position absent.

[f] R_f values of the fluorescent pink contaminant present in the peonidin sample.

[g] The malvidin and petunidin were made available as a mixture.

[h] Malvidin and petunidin do not separate in the Iso-PrOH solvent.

[i] Delphinidin specimen showed a fluorescent pink contaminant at R_f 0.04 in Aq-HCl solvent.

solvent cannot be used as a general solvent for anthocyanidin chromatography because malvidin and petunidin and also cyanidin and peonidin resolve at about the same chromatographic loci and therefore cannot be distinguished.

The formic acid-4N HCl (2:1) solvent developed in this laboratory is a good one for routine use and has a greater spread in R_f values than the formic acid solvent of Harborne (loc. cit.).

The use of the HAc-HCl solvent originally proposed by Harborne (1959a) for anthocyanins was extended to anthocyanidins in this laboratory (see Sec. 15). In certain cases, for instance studies on the stability of anthocyanins and partial hydrolysis, it is essential to determine R_f values of anthocyanins and anthocyanidins in the same solvent. The HAc-HCl solvent possesses excellent potential for this purpose and its use was extensively exploited: the R_f values of anthocyanins in the solvent are generally above 0.20 and those of anthocyanidins below 0.20. The solvent gives sharp resolution and minimal variation in R_f values of anthocyanidins. It is interesting to note from Table 17.II that the fluctuation in the R_f value range of Forestal solvent as compared to the HAc-HCl solvent is rather high, though both of these solvents basically have the same components. The HAc-HCl solvent, like the Iso-PrOH solvent, has a merit in differentiating pelargonidin from peonidin.

It is known that R_f values of anthocyanidins in a given solvent are predictable on the basis of their structural features. It is worthy of note that although the basic constituents of the HAc-HCl solvent are the same as those of Forestal solvent, the order of R_f values of malvidin and cyanidin, as found in Forestal solvent is reversed in the HAc-HCl solvent. This additional fact can be used in the identification

of malvidin.

The 3:1:8 solvent of Abe and Hayashi (1956) gives quite good results. Its behaviour is quite similar to the HAc-HCl solvent discussed above. The solvent, although useful in the characterization of anthocyanins, is quite acidic and was not used in the purification of anthocyanins owing to the likely hazards of partial hydrolysis (Sec. 15). Again, like the HAc-HCl solvent, the order of R_f values for malvidin and cyanidin, as found in Forestal solvent, is reversed in this solvent. The fluctuation in the R_f value range of anthocyanidins in this solvent, as compared to Forestal solvent, is minimal.

The Propionic acid solvent developed in this laboratory also appears to be an excellent solvent of identification. There is a uniform spread of approximately 0.05 R_f value units between the anthocyanidins studied. The R_f values of most anthocyanidins are multiples of 5.

The 1% aqueous HCl solvent (Harborne, 1959a) is excellent for purification and characterization of anthocyanins. The R_f values for anthocyanidins are very low. Nonetheless, the R_f values of anthocyanidins are included in Table 17.II because of their value in assessment of the products of partial hydrolysis of anthocyanins. Of all the solvents listed, the colors of anthocyanins and anthocyanidins, both in visible and ultraviolet lights, are sharp and well-defined in the Aq-HCl solvent.

17.1 SUMMARY

The range of R_f value variation and the average R_f values of eight authentic anthocyanidins in a total of ten solvents, which were used for anthocyanidin chromatography, are given. Of the ten solvents,

two, namely FA-4NHCl and Propionic, were developed in this laboratory. Another two, namely HAc-HCl and Aq-HCl, which are generally used for anthocyanins, were extended to anthocyanidins. The ability of each solvent to differentiate the hard-to-differentiate anthocyanidins is discussed briefly. Although all other anthocyanidins can be characterized dependably on the basis of R_f values alone in the ten solvent systems, the need for a solvent to differentiate petunidin from cyanidin still remains.

METHODS FOR CHARACTERIZING ANTHOCYANINS

18. CHROMATOPAPER AS A PRODUCER OF ANOMALIES IN GLYCOSIDIC AND SPECTRAL ANALYSIS OF ANTHOCYANIN ELUATES

It has been recently reported that arabinose arises from chromatography paper as an artefact in anthocyanin eluates and that its presence can be misleading. (Harborne, 1959a). Preparatory to undertaking sugar analysis of the large number of anthocyanins (Secs. 19.3.5 and 19.3.7) isolated by the techniques described in Section 16, the anthocyanin DE3-400 (Table 19.VIII.DE) series was analysed for sugars by the method described in Section 19.2.4. Since no arabinose was detected from the anthocyanin DE (Table 19.VI) other anthocyanin isolates were hydrolyzed for sugar analysis. In several instances, however, arabinose was found in addition to glucose, galactose and xylose (see Table 19.VI). The source of arabinose, in view of Harborne's finding became a matter of

concern. Accordingly, blank chromatography papers were processed according to our technique of anthocyanin purification and elution to determine if glycosidic artefacts could be derived.

18.1 MATERIALS AND METHODS

18.1.1. Preparation of Anthocyanin, Anthocyanidin and Sugar Blanks

The analysis of paper-derived materials was carried out on the eluates obtained from blank papergrams developed by descent in the HAc-HCl (Sec. 16). The R_f value distribution of anthocyanins in this solvent was between the R_f value range 0.1 to 0.85. Twenty blank Whatman papers No. 3 developed in this solvent, accordingly, were cut into 7 sets of blank paper strips (each set consisting of 20 strips) representing the R_f value ranges as shown below:

Set #	R_f Value Range	Set #	R_f Value Range
I	0.1 to 0.2	V	0.5 to 0.6
II	0.2 to 0.3	VI	0.6 to 0.7
III	0.3 to 0.4	VII	0.7 to 0.85
IV	0.4 to 0.5		

The blank strips from each set were placed in the concentration chamber (see Fig. 16.2) and processed exactly in the manner described for the paper strips containing anthocyanin bands. As yellow contaminants moved to the end of the blank paper strips (usually it took a period of 6-8 hours), the ends were snipped off from paper bands and consolidated. A portion was extracted with water-methanol (1:1) and another portion with the elution solvent for anthocyanins, namely, 0.03% HCl-MeOH (Sec. 16). The eluates obtained with the anthocyanin eluent will, hereafter, be referred to as 'anthocyanin blank'. An aliquot of anthocyanin blank obtained from the yellow ends, following the usual deacidification and

drying (Sec. 19.2.4), was spotted on Whatman paper No. 1 for sugar analysis in the pyridine solvent (Sec. 19.2.4). The remainder of the anthocyanin blank was hydrolysed to obtain 'anthocyanidin blank' and 'sugar blank'. The anthocyanidin blank refers to materials extracted in isoamyl alcohol from the hydrolysates of anthocyanin blank, and the sugar blank refers to the materials remaining in the aqueous portion of the hydrolysates after extraction with isoamyl alcohol. The sugar blank of the yellow ends was chromatographed for the presence of sugars; the anthocyanidin blank was used for spectral analysis.

The blank paper strips from which the yellow ends had been snipped were allowed a further period of 10 hours for ascent in the Concentration Chamber (Sec. 16). The anthocyanin, anthocyanidin and sugar blanks were obtained by the procedure mentioned above, except that the elution was carried out by trimming sides of the paper strip as was done for the strips containing anthocyanin bands. The anthocyanin, anthocyanidin and sugar blanks were obtained from all the sets of paper bands representing different R_f value ranges, separately. All blanks were obtained from equal numbers of paper strips under identical conditions. The anthocyanin and anthocyanidin blanks were dried directly in a vacuum oven at room temperature; the sugar blanks, however, were neutralised as described in Section 19.2.4 before drying. The sugar and anthocyanin blank materials were dissolved in 5 drops of water, spotted on Whatman paper No. 1 giving five applications with a capillary (O.D. 0.8 mm) for sugar chromatography (see Sec. 19.2.4). The remaining solutions were dried in vacuum oven.

18.1.2. Spectral Measurements

The spectra of the appropriate blanks were drawn on a Beckman DK-2 Ratio Recording Spectrophotometer in 1-ml silica cuvettes. The blanks were dissolved in methanol containing 0.01% HCl (Harborne, 1958) to determine their qualitative spectra in visible and ultraviolet light.

18.1.3. Removal of Sugar Artefacts from Anthocyanin Eluates

The procedure to remove the paper-derived sugars from anthocyanins, both of which are readily soluble in water, was based on the following observations: (a) the adsorption of anthocyanins on a column of nylon powder* in an aqueous system, acidified weakly, was so strong that the absorbed ring of anthocyanins scarcely moved down the column even after hours of continuous washing, (b) aqueous solutions of sugars passed through the nylon column without adsorption, and (c) the anthocyanin ring which remained tenaciously adsorbed on the column in the aqueous medium could be eluted readily with acidified methanol. The procedure, thus, is based on the differential adsorptivity of anthocyanins on nylon powder in aqueous and alcoholic media.

About 40 grams of nylon powder was made into a slurry with elution solvent (0.03% HCl-MeOH) and packed into a column. The column was

*Kindly supplied by Dr. W.O.S.Meredith, Grain Exchange Laboratory, Winnipeg, Manitoba. Nylon powder was originally used by Harris and Ricketts (1959) for concentrating anthocyanogens of beer, and later extended to the separation of anthocyanins from other flavonoid aglycones by Chandler and Swain (1959). Recently another polyamide "Polyclar-AT" (Antara Chemicals, New York, N.Y.) has been introduced. This may be far superior to nylon powder.

washed with about 100 ml of the elution solvent followed by 0.05% aqueous HCl under suction. A concentrated anthocyanin eluate was poured into the column, and washing of the column with 0.05% aqueous HCl under suction was started. The column effluent* in 10-ml portions was continuously analysed for the presence of sugars by the Molisch test. It usually took 5-7 minutes when substances positive to Molisch test (sugars) began to appear in the effluent. The bulk of the sugar contaminants are eluted within 10 minutes. The washing, however, was continued, to be sure, for about an hour. The rate of movement of the anthocyanin ring, when the column was washed continuously with the aqueous medium under suction, was about 1 cm/hour. The anthocyanins were finally eluted with the solvent, hydrolysed and chromatographed for the identification of sugars by the usual methods (Sec. 19.2.4).

18.2. RESULTS

18.2.1. Sugars from the Paper Blanks

The results of sugars derived from anthocyanin and sugar blanks are shown in Fig. 18.1. An estimate of relative concentration of different sugars within a particular eluate are noted in the spots by figures (Fig. 18.1). Weak spots are shown by dotted lines. The sugars were identified by cochromatography with known sugars in the pyridine solvent. The average R_g values for xylose, arabinose and galactose in

*If desired, the effluent may be concentrated and chromatographed to determine the nature of the contaminant sugars.

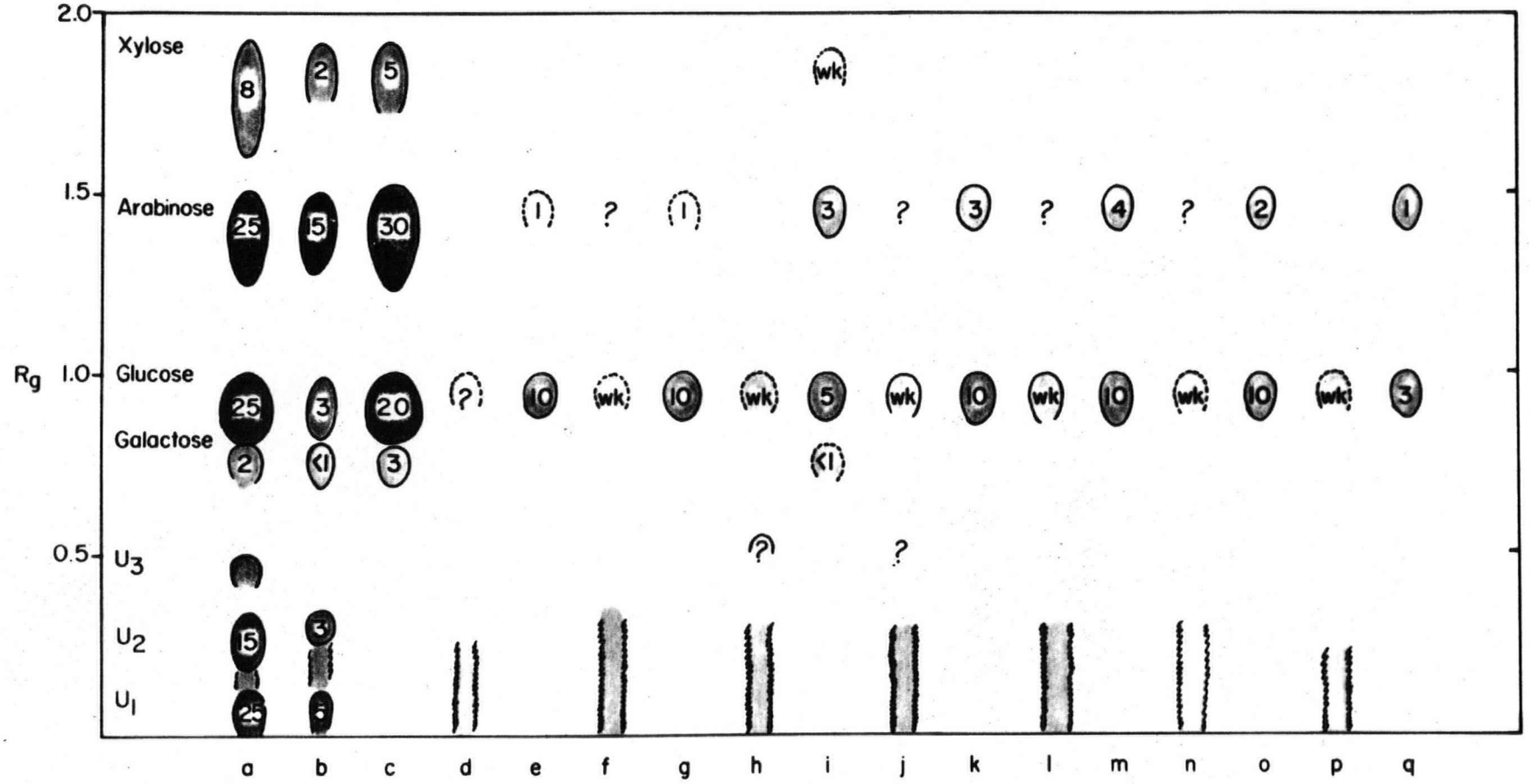

Fig. 18.1.
Sugars and related products derived from chromatopaper: a-c chromatograms of yellow materials accumulating at band ends during the first 6-8 hours of concentration, d-z, chromatograms of materials concentrating at band ends after 8 hours; bands taken from several regions of HAc-HCl papergrams treated as if anthocyanins were present. Eluants: a-unacidified MeOH-H_2O, b-q, .03% HCl-MeOH, the anthocyanin eluant. Hydrolysis, as for anthocyanins, using aquaeous layer following scrubbing with isoamyl alcohol, (sugar blanks) c, e, g, i, k, m, o, q. Not hydrolyzed, chromatographed directly (anthocyanin blanks) b, d, f, h, j, l, n, p. Pyridine solvent used throughout. U= unknown.

the pyridine solvent were 1.90, 1.50 and 0.82 respectively.

The eluates from the yellow ends were obtained primarily to determine the sugars and other substances eluted, if any, from the blank papers in the first 6-8 hours, the usual period of time taken for elution by the methods commonly recommended in the literature (Harborne, 1959a). It will be noted that the yellow ends were discarded in our method of anthocyanin purification and elution (Sec. 16). In one instance, the yellow terminuses were extracted with the mixture of water-methanol to determine the influence of water in elution solvents: the sugars being more soluble in water than methanol.

The water-methanol extract from the yellow ends gave 6-7 spots of sugars, namely, xylose, arabinose, glucose, galactose and three unknown spots (Fig. 18.1, a). The unknown spot No. 1 gave brown (greenish fluorescence) and the unknown spot No. 2 red (reddish fluorescence) colors with aniline phosphate (Sec. 19.2.4). The unknown spot No. 3 was most probably reddish. The R_g values and colors of these spots suggest they may be low molecular weight polymers of hexose and pentose type sugars. The presence of galactose is shown by dotted lines, not because it was weak, but because it failed to separate as a distinct spot from the glucose spot in the usual 24 hour period of development in the pyridine solvent.

The anthocyanin blank of the yellow ends, which was extracted with the acidified methanol showed two unknown sugars present in small amounts and also the four known sugars. (Fig. 18.1, b). It is to be noted that the relative concentration of sugars with water-methanol extraction and acidified methanol extraction vary widely: in the water-methanol the ratio of glucose to arabinose is almost 1:1, in the acidic methanol it is 1:5. Similarly, the unknown sugars were present in

larger quantities in the H_2O-MeOH extract than in the acidified methanol extract. However, when the acidified methanolic extract i.e., the anthocyanin blank of the yellow ends was hydrolysed to obtain sugar blank (see Fig. 18.1, c), the unknown sugars disappeared. Note the relative concentrations of the known sugars upon hydrolysis change conspicuously (compare anthocyanin blank and sugar blank of the yellow terminuses).

The anthocyanin blanks eluted from the paper strips from which the yellow ends had been snipped off, upon chromatography, showed the presence of glucose and a weak trail beginning from the starting line, and in some cases, traces of xylose arabinose and some unknown sugars (see anthocyanin blanks obtained from different R_f value ranges in Fig. 18.1, d, f, h, j, l, n, p). However, when these anthocyanin blanks were hydrolysed the unknown sugars disappeared (see sugar blanks in Fig. 18.1, e, g, i, k, m, o, q). Thus, the results of the hydrolysis are comparable to those obtained from the hydrolysis of the anthocyanin blanks of the yellow ends. Also, arabinose appears distinctively in the hydrolysates as revealed by a comparison of the relative concentration ratios of glucose and arabinose in the anthocyanin and corresponding sugar blanks. Fig. 18.1, also, shows that galactose is largely eliminated, from the paper, in the yellow ends.

18.2.2. Visible and Ultraviolet Spectra of the Blanks

The spectra of the anthocyanidin blank of the yellow ends from wavelength range 350 to 700 mu are shown in Fig. 18.2. The spectra were drawn from a series of dilutions with methanol containing 0.01% HCl, the solvent used for the spectral studies of anthocyanidins. Note the blank does not give any spectral peak when the concentration is increased or

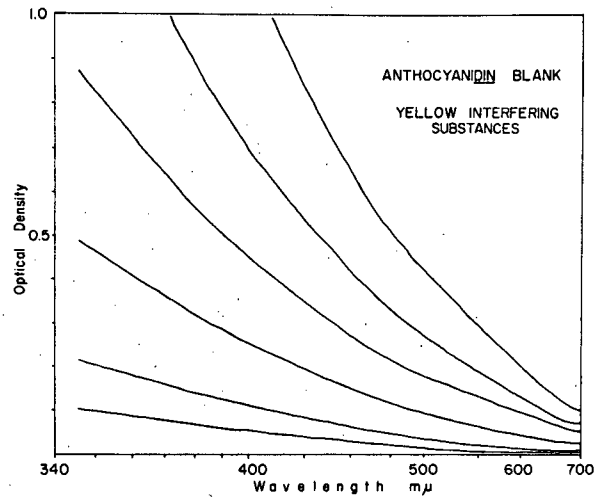

Fig. 18.2
Spectra of isoamyl alcohol soluble materials (anthocyanidin blanks) partitioned from hydrolyzed eluates, which were obtained from the HAc-HCl blank chromatobands with the yellow ends intact. Dilution series with 0.01% methanolic HCl; peak at ca. 360 mu, characteristic of anthocyanin and anthocyanidin degradation, is not observed.

decreased. However, the blank does show a peak around 255 mu in ultraviolet similar to those shown in Fig. 18.5. Visible spectra of the anthocyanidin blanks obtained from each of the 7 sets (Sec. 18.1.1) of the blank paper bands, the yellowed ends of which had been snipped off are shown in Fig. 18.3 and are identical to those of the yellow ends shown in Fig. 18.2.

Visible spectra of the anthocyanin blanks obtained from each of the 7 sets (18.1.1) of blank paper bands, the yellowed ends of which had been snipped off are shown in Fig. 18.4. In addition, the figure also includes the spectrum of the anthocyanin blank obtained from the H_2O-MeOH eluate. Note all spectra are identical: they, however, differ from the spectra of the anthocyanidin blanks (Figs. 18.2 and 18.3), inasmuch as they show a distinct shoulder between 360-370 mu.

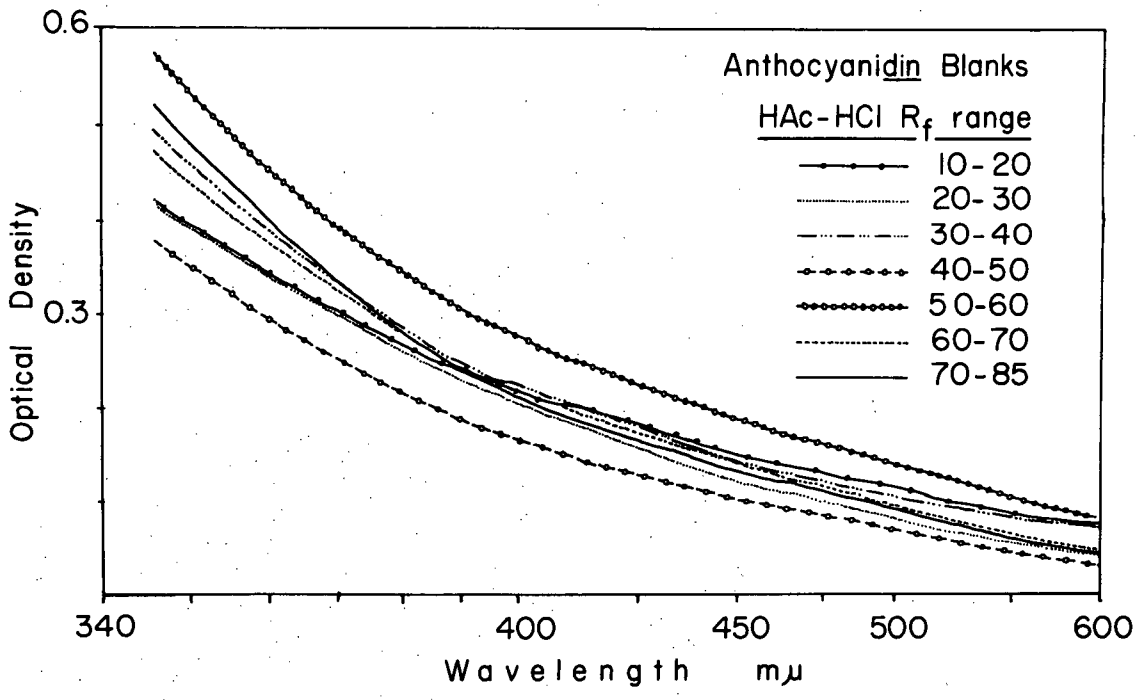

Fig. 18.3
Spectra of isoamyl alcohol soluble materials (anthocyanidin blanks) partitioned from hydrolyzed eluates, which were obtained from the HAc-HCl blank chromatobands after yellow ends had been removed. Curves are similar to those of Fig. 18.2, in that degradation peaks do not appear.

Ultraviolet spectra of all the anthocyanin blanks of Fig. 18.4 is shown in Fig. 18.5. All blanks show a peak at 255 mu and an inflection around 250 mu.

18.2.3. Removal of Sugar Artefacts from Anthocyanin Eluates

The nylon method (Sec. 18.1.3) was developed belatedly after most of the available anthocyanin isolates had been hydrolysed for sugar determinations (Sec. 19.3.5) by the standard method (Sec. 19.2.4).

The details of the method were established by working with the eluates of several anthocyanins which had undergone partial or complete

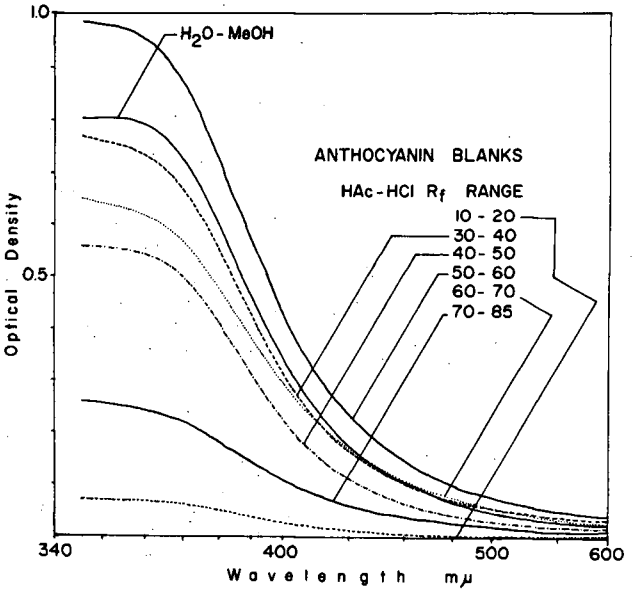

Fig. 18.4
Spectra of materials obtained from the HAc-HCl blank chromatobands from which yellow contaminants had first been removed (anthocyanin blanks). Similar curves are given when unacidified MeOH-H_2O and HCl-MeOH are used as eluants. A distinct shoulder distinguishes these curves from those of hydrolyzed contaminants.

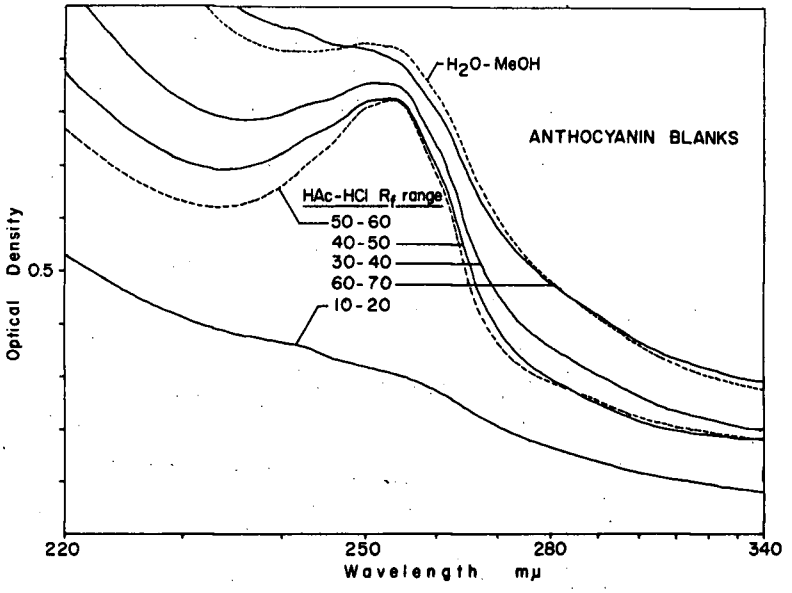

Fig. 18.5
U.V. spectra of non-hydrolyzed contaminants from paperbands (anthocyanin blanks) show a characteristic peak at 255 mu.

breakdown in the course of processing. It was observed that within a few minutes of the adsorption of the eluates and the washings with 0.05% aqueous HCl, the effluent showed large amounts of sugars, which were eluted completely within about 10 minutes.

Owing to the scarcity of materials, only two anthocyanins, 5D and IB5GH (Table 19.VI) were available to compare the results of sugar analysis by the nylon method, and the standard method. The anthocyanin 5D has been completely characterized but the anthocyanin IB5GH has only been partially characterized (Table 19.VIII.5) The anthocyanin IB5GH gave glucose, arabinose, and xylose by the standard method. The relative concentration ratios of the sugars, as estimated visually, were 30:12:8. However, by the nylon method, only glucose and arabinose in about equimolecular proportions were recovered. The anthocyanin 5D largely gave glucose by the standard method. Although it also gave glucose only by the nylon method, the effluent did show the presence of sugars detectable by the Molisch test.

The nylon treatment was also applied to a few other anthocyanins, namely DE5, DE6, and IB5FG (Table 19.VI). These anthocyanins were not hydrolysed originally by the standard method because they were available in small quantity. The effluents obtained from the three anthocyanins were positive to Molisch test. The color of the anthocyanin eluates, however, had disappeared during hydrolysis. Nonetheless, the hydrolysates of the anthocyanin DE5 showed glucose and some arabinose and that of DE6 and IB5FG almost equimolecular proportions of glucose and arabinose.

18.3 DISCUSSION

The chromatography paper as a producer of arabinose artefacts in the glycosidic analysis of anthocyanin eluates, was first demonstrated by Harborne and Sherrat (1957). Later on, in a review on the chromatographic identification of anthocyanins, Harborne (1959a) pointed out that Nordstrom (1956) and Asen, Siegelman and Stewart (1957) had also encountered similar difficulties. The investigation reported in this section demonstrates that the blank paper bands, besides arabinose, also contain other monosaccharides, such as, glucose, xylose and galactose, and several unknown 'di- or tri-saccharides' of pentose and hexose sugars.

It is clear from the results presented in Fig. 18.1, a and b, that all the paper-derived monosaccharides, and unknown oligosaccharides, noted above, concentrate in the paper-strip ends, in the first few hours of the concentration process. Because about the same period of time is taken for eluting anthocyanins and because the nature of several sugars derived from the paper *per se* and those derived from anthocyanins are identical, the potential for error in both qualitative as well as quantitative studies is self-evident.

The results in Fig. 18.1 demonstrate that the 'slow-moving' unknown oligosaccharides, which are present in all anthocyanin blanks (a, b, d, f, h, j, l, n, p), disappear following hydrolyses, i.e., from all sugar blanks (c, e, g, i, k, m, o, q). The results in Fig. 18.1 further indicate that not only the unknowns disappear but also the relative concentration ratio of the monosaccharides undergoes significant alterations following hydrolysis. For example, it will be noted that whereas the relative concentration ratio of glucose to arabinose is low

in the anthocyanin blanks of the yellow ends (Fig. 18.1,b), it increases markedly when the blank is hydrolysed (Fig. 18.1,c). Notwithstanding that the study was basically of a qualitative nature, the experiences indicate that following hydrolysis, the concentration of the monosaccharides increases. These observations, therefore, strengthen the tentative identification of the 'slow-moving' sugars, on the basis of R_f values and reactions with aniline phosphate and p-anisidine chloride, as di- or tri-saccharides. The increase in the concentration of monosaccharides following hydrolysis may not be due to the di- or tri-saccharides alone. It was observed that the eluates from the paper contain micro fibrils, presumably of cellulose or cell wall debris consisting of cellulose and pectic substances, which upon hydrolysis, could also have given rise to glucose, and traces of arabinose and xylose. The purification of anthocyanin eluates on the nylon column eliminates the microfibrils.

It is also clear that in contrast to the number of sugars obtained from the yellow ends (Fig. 18.1, a, b, and c), relatively a small number is obtained from the paper strips, the ends of which had been snipped off (Fig. 18.1, d, e, f, g, h, i, j, k, l, m, n, o, p, q). The results therefore show that snipping off the yellow ends in this anthocyanin purification technique (Sec. 16) was an advantageous operation for eliminating most of the sugar artefacts, particularly, when anthocyanins are known to be labile in the presence of sugars and their degradation products (see later).

Harborne (1959a) indicates that sugar artefacts can be eliminated if the mineral acid used in the elution solvent is substituted with acetic acid. Although this is an undoubted improvement, results show clearly that the water-methanol eluent (a, Fig. 18.1) without the

presence of any acid, in fact, eluted the maximum number and also quantity (as revealed by their relative concentration ratios) of sugars. It is noteworthy that eluents containing water-methanol (Harborne, 1958) are generally used for the elution of anthocyanins. It is also considered advisable to wash the chromatography paper before use with dilute acetic acid, to remove soluble impurities (Harborne, 1959a). However, since most anthocyanin solvents contain mineral acids, the production of some sugar artefacts in the paper as indicated by Harborne (1959a), should occur again following chromatography. Thus, the washing, also, does not appear to offer full protection against the production of sugar artefacts.

The nylon procedure eliminates the sugar artefacts completely. The appearance of large quantities of Molisch test positive substance in the effluent from the nylon column on which anthocyanin eluates are adsorbed and also the presence of sugars in the anthocyanin blanks (Fig.18.1) substantiates the presence of paper-derived sugars in the anthocyanin eluates. However, the presence of arabinose and glucose in the anthocyanins hydrolysed after nylon purification indicates that arabinose likely exists as a glycosidic component of some barley anthocyanins.

It is not only the appearance of sugars in the column effluents but also the chromatographic disappearance of trace quantities of xylose, arabinose, galactose, from the anthocyanin eluates following the nylon treatment (Sec. 18.2.3) that indicates clearly the suitability of this method in the glycosidic analysis of chromatographically purified anthocyanins. The anthocyanins obtained by nylon purification are also freed of the yellow materials. The paper-derived yellow materials may influence the accuracy of studies on anthocyanin breakdown products because they mask the spectral absorptions of the anthocyanin breakdown

products around 360 mu as shown elsewhere (Figs. 12 and 12.3). Moreover, the spectral absorption of the yellow compound at 440 mu interferes with the E_{440}/E_{max} ratio which has been used (Harborne, 1958) for differentiating 3-glycosides from 3, 5-diglycosides.

The presence of large quantities of several pentose and hexose type mono- and oligo-saccharides derived from chromatography paper may shed some further light on the problem of anthocyanin instability. Considerable work on the factors concerning anthocyanin instability both in food products and model systems has been done in the last decade in the field of food technology. Meschter (1953) showed that a strawberry anthocyanin, pelargonidin-3-glucoside, degrades at a faster rate in the presence of labile sugars such as arabinose, levulose and sorbose than in the presence of the stable sugar maltose and sugar alcohol sorbitol. Tinsley and Bockian (1960) showed that the degradation is considerably increased in presence of fructose and glucuronic acid as compared to gluconic acid. The degradation products of sugars are even more important 'catalysts' of anthocyanin degradation than sugars per se. For instance, anthocyanin instability is greatly increased in the presence of furfuraldehydes and also 5-hydroxymethylfurfural (Markakis et al., 1957; Tinsley et al., 1960). It is known that the rate of degradation of glucose and fructose to 5-hydroxymethylfurfural increases as the pH decreases (Singh, et al., 1949). The pentoses and glucuronic acid, in general, are more labile and break down to more readily corresponding furfurals under relatively dilute acid conditions than do hexoses.

Tinsley and Bockian also found that the rate of pelargonidin-3-glucoside degradation increased more in the presence of oxygen than in nitrogen. There was, however, a marked increase in the rate of antho-

cyanin degradation in presence of sugars, etc., and oxygen. Since both furfural and 5-(hydroxymethyl)furfural are quite labile in presence of oxygen and acid, possibly some oxidative product might be the reactive compound in anthocyanin degradation; the mechanism of this breakdown reaction in the presence of sugars is not known.

The addition of amino acids to the anthocyanin-sugar solution enhances the rate of anthocyanin breakdown - thus indicating that sugars are active in the form of their degradation products in affecting the rate of pigment degradation, and that amino acids act by accelerating the production of these breakdown products (Mackinney, Lukton and Chichester, 1955; Tinsley and Bockian, 1960). The involvement of ascorbic acid in the breakdown of anthocyanins under aerobic and anaerobic conditions is well known (Pratt et al., 1954; Meschter, 1953; Sondheimer and Kertesz, 1953). It appears to be quite possible that the effect of ascorbic acid could be due in part to its breakdown to furfural (Tinsley and Bockian, 1960).

In view of the involvement of sugars and their breakdown products with the instability of anthocyanins, the importance of snipping off the yellow terminuses, which also contain sugars, in the purification and concentration process (Sec. 16.2) is self-evident. It is entirely possible that during the process of volume reduction of anthocyanin eluates on a flash evaporator, the paper-derived sugars in presence of constant boiling hydrochloric acid and continuous agitation of the flask in an atmosphere of oxygen (even though, it be quite low) could have broken down to corresponding furfural derivatives. This then is another factor that could have, conceivably, caused irreversible degradation of anthocyanins to yellow compounds during volume reduction. The situation with the chromatographically purified anthocyanins is rather confounded.

There seem to be at least three counteracting pH-dependent variables involved in the instability of these anthocyanins: low pH is believed to increase their stability but low pH also promotes their hydrolysis (Sec. 23) and production of sugar degradation products.

Nothing can be stated at this time with respect to the influence of the yellow compounds derived from paper in the process of elution. Since the degradation was most pronounced in chromatographically purified anthocyanins and anthocyanidins, it appears desirable that the nature and influence of the yellow compounds on anthocyanin stability should be a subject of another investigation.

Apart from the probable degradation of anthocyanins due to paper-derived sugars and their oxidized breakdown products, the degradation could also have occurred due to the presence of peroxide groups in chromatopaper. It has been demonstrated recently that the peroxide groups present in the paper cause partial oxidation of xylose and arabinose to corresponding aldonic acids during chromatography (Kleinert, Kielmann, and Marraccini, 1961). The authors also found that the oxidative breakdown of sugars and other easily oxidizable substances, such as anthocyanins, can be prevented by washing the paper with $NaBH_4$.

18.4 SUMMARY

The glycosidic analysis of a large number of anthocyanin isolates revealed the presence of arabinose in addition to glucose, galactose and xylose (see Sec. 19.3.5). In order to establish the origin of arabinose, which is known to arise as an artefact from chromatopaper, the conditions of anthocyanin elution and processing had to be scrutinized

using blank chromatopaper strips. The scrutiny establishes that, in the course of elution, glucose, galactose, xylose and at least three di- or polymeric sugars of pentose and hexose type are eluted from the blank strips, besides arabinose. When the eluates are hydrolysed, the unknown polymers disappear and only the monosaccharides remain on chromatograms. A method for eliminating the paper-derived sugars by adsorption of anthocyanin eluates on nylon powder is presented. The probable influence of the paper-derived sugar artefacts and the peroxide groups present in chromatopaper on anthocyanin stability is discussed.

Along with the sugar artefacts are also eluted large amounts of yellow compound(s) from the blank strips. The spectra of the yellow compounds, before and after hydrolysis, are presented, and it is shown that they interfere with (a) the detection of the degradative peaks (of anthocyanins) around 360 mu (see Sec. 12), and (b) the dependability of the ratio E_{440}/E_{max} for differentiating 3-monoglycosides from 3, 5-diglycosides.

CHARACTERIZATION OF ANTHOCYANINS

19. CHARACTERIZATION OF BASAL LEAF SHEATH AND CARYOPSIS ANTHOCYANINS

The distribution of anthocyanic and melanic colors in many parts of the barley plant and in many barley varieties has been reported in earlier pages. Histological examination of color was undertaken in barley caryopses. Standard methods for the extraction and purification of anthocyanins proved to be inadequate for barley pigments and new or modified methods were developed. Although some inadequacies in methods remained, it was decided to proceed towards the realization of the objectives of the study viz., to relate and collate the genetics and biochemistry of the barley anthocyanic pigments. Characterization of the anthocyanins therefore was next approached. The undertaking proved to be formidable and of necessity has been restricted to a few tissues and varieties.

19.1. MATERIALS

19.1.1. Basal Leaf Sheaths

The basal leaf sheaths extracts of the purple varieties obtained in 1960 (Sec. 10) were exhausted in the course of investigations described in Sections 10 to 17. Accordingly the purple variety, Gopal, and Black Hulless along with several other varieties, isolines and their hybrids were planted on August 24, 1961 in the University Greenhouses. The basal leaf sheaths of the varieties, Gopal, and Black Hulless were harvested on the dates indicated below:

Collection No.	Variety	Date of Tissue Collection
400	Gopal	November 7, 1961
404	Gopal	November 16, 1961
405	Gopal	November 27, 1961
402	Black Hulless	November 7, 1961
407	Black Hulless	December 10, 1961
408	Black Hulless	December 10, 1961

The Greenhouse collection 400 showed a good development of anthocyanins and was approximately equivalent to the Field Collection 130. The collections 404 and 405 also showed good development of anthocyanins. The Black Hulless Collections 402 and 407 were made from tissues showing good development of anthocyanins. However, Collection 408 was made from the sheaths showing less color development.

19.1.2. Caryopses (Inact Grains without Hulls)

The characterization of anthocyanins of the caryopses will be described only for the purple variety, Black Hulless. The materials came from the stocks planted on May 13, 1960 as described in Section 10. Although the caryopses tissues were collected at nine stages of development, the results of only those collections which were made around the stage of

maximum pigment development will be described. The collection 217 was made on August 15, 1960, close to the period of normal crop harvesting, on the basis of color development in hulls. The hulls of Collection 270 were deep dark purple, those of Collection 272 were purple with yellowish patches, those of Collection 274 were deep purple in lemma veins but weak yellowish purple in interveins, and those of Collection 278 were almost entirely yellow. The kernels of Collection 270 were close to hard dough stage, those of Collection 278 close to early flinty stage, and those of the other collections at an 'inbetween' stage near maturation. The caryopses of the Collection 270 showed maximum color, those of the Collection 278 relatively less color and the caryopses of the other collections showed color between 270 and 278.

19.2. METHODS

19.2.1. Extraction and Processing

Robinson and Robinson (1933) believed that 1% HCl extracts and preserves anthocyanins. Harborne (1958, 1959a, 1960) recommended and used about the same acid concentration (methanol-HCl, 97:3, v/v) for extraction and processing of anthocyanin extracts for chromatography. A possibility existed that the instability of anthocyanins encountered thus far could have been due to the use of low acid concentration in our extraction solvent, namely 1% conc. HCl. Accordingly, the basal leaf sheath (included in Sec. 19.1.1) were extracted with 1% methanolic HCl to bring the extraction procedure in line with those of Harborne (ibid.). The extracts were concentrated in vacuo, however, at 30°C rather than 50°C as used by Harborne (ibid.). The volume of the extracts was reduced to ca. 15 ml per

gram dry weight of the tissues following the preliminary extraction (Sec. 10.2.1).

The intact caryopsis (seeds without hulls) were extracted with methanol containing 1% conc. HCl as described in Section 6.3.5.c. The extraction and processing was carried out along with the other 300 extracts as described in Sections 10.1 and 10.2.1.

19.2.2. Purification of Anthocyanins

The preparative chromatography of the anthocyanin extracts of both the basal leaf sheaths and the caryopses was carried out in the BAW solvent as described in Section 10.2.2. The eluates of the BAW anthocyanin bands obtained via the concentration and elution techniques (Sec. 16) were transferred to smaller beakers (10 ml to 50 ml size) from the larger beakers in which they were eluted originally. The larger beakers were washed with a few squirts of unacidified methanol and the washings were poured into small beakers. The beakers were placed immediately in a vacuum oven for drying the eluates at room temperature in the dark. If the eluates were obtained from a large number of paper bands, they were divided and placed into two to three beakers primarily to avoid probable 'polymerization' (see later), and also to achieve rapid drying in the oven. When the volume of the eluates was large, it was first reduced substantially in a flash evaporator* before drying in the oven. The isolation of the many BAW anthocyanin bands from the many tissue collections

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*In a few cases the eluates dried inadvertently during flash evaporation and yet there was no detectable visual degradation to yellow products.

at different stages of development of the basal leaf sheath and the caryopsis tissues took over two months. All isolates immediately after oven drying were kept in vacuum desiccators containing phosphorus pentoxide at room temperature in the dark. Although the length of time the beakers remained over phosphorus pentoxide was not recorded, a matter which later assumed importance, the beakers, in general remained in the desiccator for several weeks; in some cases, however, they remained for a few days, while in others they remained for a few months. After removal from the desiccators, the beakers were covered with polyethylene sheets. A few of the caryopsis isolates, which were obtained from the lower R_f value regions in the BAW solvent, showed the presence of gummy hygroscopic substances. Such isolates after drying over phosphorus pentoxide were kept in a freezer away from light.

The dried BAW eluates, which had been purified once *via* the concentration chamber (Sec. 16), were chromatographed in the HAc-HCl solvent for a second purification. The HAc-HCl eluates were processed again *via* the purification and elution techniques (Sec. 16). Drying of the eluates over phosphorus pentoxide was discontinued (see later). Drying in a vacuum oven was undertaken only after the spotting was completed. The eluates, immediately after elution, were either placed in a freezer (-15°C), or chromatographed directly for characterization. The chromatographic characterization was carried out on Whatman paper No. 1 in at least five solvents, namely, BAW, Bu-HCl, Aq-HCl, HAc-HCl and 3:1:8 (see Table 10.II). Synthetic cyanidin-3-glucoside, and pelargonidin-3-glucoside were run as controls on each sheet.

19.2.3. Preparation of Pure Anthocyanidins

Since anthocyanidins are usually less stable than anthocyanins, the purification of anthocyanidins by chromatography, elution and flash evaporation (see Harborne, 1958) was given up. Instead, anthocyanins after purification in at least three solvents, as described in Sections 16 and 19.2.2, were hydrolysed to the corresponding anthocyanidins largely by the method of Harborne (1960) with slight modifications. Dry anthocyanins were dissolved in 8-10 ml of N HCl and hydrolysed for 1-1/2 hours(instead of 1 hour recommended by Harborne, 1960) in an atmosphere of nitrogen under half light conditions. During this period, anthocyanins were completely hydrolysed.

In some cases where the quantity of anthocyanins for hydrolysis was very small, the hydrolysis was conducted on a boiling water bath for a little over 2 hours (Abe and Hayashi, 1956). This was done because a small quantity of anthocyanins 'chars' owing to super heating in the region above the liquid level in the pear-shaped three-neck flask when a bunsen burner is used.

The partial hydrolysis was conducted under the same conditions using an interval of 5', 20', and 40 minutes. The hydrolysates are spotted without extraction with isoamyl alcohol.

An anthocyanidin, after extraction in isoamyl alcohol (instead of hexanol used by Harborne (ibid)), was dried in a small beaker in a vacuum oven. A few flakes of the dried anthocyanidin were dissolved in hot 1% aqueous HCl (not in acidified methanol) and the solution spotted on several sheets of Whatman Paper No. 1 to obtain R_f values in several solvents (Table 17.I). It is noteworthy that the solution of dried

anthocyanidins in hot aqueous HCl leaves behind possibly paper-derived methanol-soluble, water-insoluble contaminants. Further, the relative insolubility of anthocyanidins in water and the greater affinity of cellulose for water makes possible a spot of restricted area on chromotography paper at the time of application. The ease of obtaining a concentrated spot in a restricted area and relative freedom from contaminants make for better chromatographic resolution, economy of time and precise R_f values.

The anthocyanidins were identified by cochromatography with authentic compounds in seven solvents, namely, Forestal, FA Harborne, FA-4NHCl, HAc-HCl, 3:18, Propionic, and Iso-PrOH solvents described in Section 17, Tables 17.I and 17.II. Whenever practicable, the chromatographic identifications were supplemented with spectral measurements with and without the addition of aluminum chloride (Harborne, 1958). Spectra were drawn on Beckman Dk2 Ratio Recording Spectrophotometer.

19.2.4. Sugar Analysis

After extraction of the aglycone with iso-amyl alcohol (Sec. 19.2.3), the remaining aqueous hydrolysate is processed for sugar analysis largely by the method described by Harborne (1960). The hydrolysates are neutralized either with a tertiary amine or ion exchange resins. The hydrolysate, which may be slightly red, owing to the presence of unhydrolyzed anthocyanins, is washed with a 10% solution of di-n-octylmethylamine in chloroform. Usually, two such washings with 5 ml portions at a time are sufficient to 'neutralize' the acidity. The 'neutral' point is indicated by the disappearance of red anthocyanin coloration. The deacidified hydrolysates are washed further with 10 ml

portions of chloroform twice.

The deacidification and purification of hydrolysates is better achieved by treatment with a mixed bed ion exchange resin, Amberlite MB-1 (Pratt and Wender, 1961). It was later found that Amberlite MB-3 was more economical and efficient. An appropriate quantity of the resin is added to a beaker containing the hydrolysate. After at least 15 minutes, the contents of the beaker are poured into a column and the eluate tested for acidity. When deacidification is complete, the column is washed with at least 50 ml distilled water and the total eluate is reduced in volume in vacuo at 30°C, and finally dried in a small vial in a vacuum oven at room temperature.

The dried sugar in the vial is dissolved in one drop of water and spotted repeatedly with a fine capillary (ca. 0.8 mm O.D.) on chromatography paper. In general, five such applications are sufficient to resolve the sugars present in the unknown mixture. Of several solvents tried for sugar chromatography, the top layer of pyridine-ethylacetate-water (2:8:2) and the BAW solvents were found satisfactory. Of these two, the pyridine solvent was the better for resolving hard-to-separate mixtures of sugars such as glucose and galactose. The chromatograms were developed for 24 hours at 20°C $\pm$ 1°C in this solvent. In general, glucose and galactose did separate from one another in a period of 24 hours. Better separations were obtained by extending the period of development to 30 hours.

The sugars were developed with aniline phosphate spray reagent (Frahn and Mills, 1959). This reagent is economical and was found superior to p-anisidine hydrochloride and aniline hydrogen phthalate. The aniline phosphate reagent was prepared by mixing 11.5 ml freshly distilled aniline

and 20.2 ml orthophosphoric acid (H_3PO_4) in 1968.3 ml n-butanol containing 12% water. This reagent can be stored indefinitely in a refrigerator. However, it must be warmed to ca. 40°C to redissolve the crystallized aniline phosphate prior to spraying. The chromatograms are liberally sprayed with the reagent and developed in an oven, provided with a glass window, at around 110°C for a few minutes until characteristic colors of the sugars appear.

It is useful to state that our early attempts to resolve glucose and galactose were not successful. Trials were therefore made to study the spectral characteristics of the colored products of glucose and galactose obtained from the 'unsulfonated resorcinol reaction' (Devor et al., 1958). This reaction has been used by Harborne (1960) in characterizing sugar eluates. This method worked very satisfactorily with a known specimen of glucose and galactose but it would not be readily used if sugars are eluted from the paper. Elution may only be undertaken successfully if a relatively large quantity of anthocyanin pigments are available for initial hydrolysis. Since glucose and galactose can be effectively resolved by the pyridine solvent, no further attempts were made to study this method in detail.

Whenever possible, the anthocyanin eluates, in a very few cases, were purified first by a nylon treatment (Section 18.1.3) before undertaking hydrolysis for sugar analysis.

19.3. RESULTS

19.3.1. Preparatory Chromatography, Purification and Isolation: Basal Leaf Sheath Anthocyanins

The results of the preparatory chromatography of the basal leaf sheath extracts of Gopal (Greenhouse Collections 400, 404, and 405), and

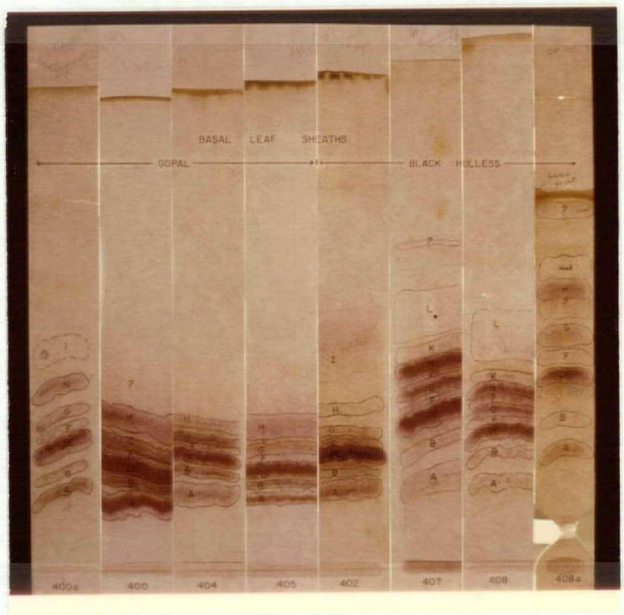

Fig. 19.1
Preparatory chromatography of extracts from greenhouse grown Gopal (Collections 400, 404, 405) and Black Hulless (Collections 402, 407, 408) basal leaf sheaths. Solvent BAW; by ascent. Note the occurrence of weak orange pelargonidin derivatives (band C) in collection 402 and the absence in all others. Chromatostrips 400a and 408a are attempts to obtain maximum band resolution and to show, if present, masked pelargonidin derivatives.

Black Hulless (Greenhouse Collections 402, 407, and 408), in the BAW solvent by ascent, are shown in Fig. 19.1, chromatograms 400a, 400, 404, 405, 402, 407, 408 and 408a.

The results of the Gopal basal leaf sheath extracts obtained from the 1961-62 (Winter) Greenhouse Collections (Fig. 19.1) and the 1960 (Summer) Field Collections (Fig. 10.3), in the BAW run, were largely identical except for at least one major difference. It was shown in Section 13, that the orange red band G-130 or G-138 was a mixture of cyanidin (see Table 13.II). Although the pelargonidin band did separate from the cyanidin band in the BAW solvent, the separation was generally not clear-cut and therefore the band was eluted as a mixture. In contrast, the orange red pelargonidin band was not detected in the Gopal leaf sheaths obtained from the Greenhouse Collections (Fig. 19.1). Several Gopal leaf sheath chromatograms were developed using different quantities of the extracts, and using longer periods of chromatographic development in the BAW solvent to obtain better resolution (for instance, see Chromatogram 400a). The orange red pelargonidin band did not separate in the 'greenhouse'

chromatograms. The orange red band G-402, although weak, was present in the basal leaf sheath Collection 402 (Black Hulless), but absent from the same greenhouse plants, the basal leaf sheaths of which were collected about a month later (cf., Sec. 19.1.1) e.g., Collections 407 and 408. In order to be sure, special strip chromatography* (Fig. 19.1, Chromatogram 408a) to obtain better resolution was undertaken, but in no case did the orange red pelargonidin band occur.

In addition to the differences in the band G, the BAW bands F and H (Fig. 19.1) showed quantitative variations within different collections of the same variety. Whereas the band F is barely visible in the Greenhouse Collections 404, 405, 407, and 408 (Fig. 19.1), it was one of the major bands in the Field Collections 130, and 138 (Fig. 10.3). The Black Hulless extracts 407 and 408 showed the presence of additional anthocyanin bands K and L.

Several bands of each collection were eluted separately after the usual purification in the anthocyanin concentrant as described in Sec. 19.2.2. An aliquot of each eluate, immediately after elution (without drying) was chromatographed, by banding, on Whatman paper No. 1, by descent, in four anthocyanin solvents listed in Table 10.II. The banding in the four solvents, namely BAW, Bu-HCl, Aq-HCl and HAc-HCl was undertaken to establish the correspondence of the anthocyanins of Gopal and Black Hulless basal leaf sheaths, and also to determine the correspondence of the Gopal leaf sheath anthocyanins obtained under field conditions (Secs. 10 and 13) and under greenhouse conditions (Fig. 19.1).

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*The special strip chromatography in principle is similar to that developed for amino acids by Matthias (1954).

A skeleton of the chromatographic results obtained in only two of the four solvents, namely, BAW and HAc-HCl is included in Table 19.I. Despite the mild conditions of elution and processing, the splitting of anthocyanins into several components occurred as usual (cf., Secs. 11 and 13). The corresponding R_f values of the split anthocyanin components in the two solvents are arranged on the basis of relative concentration ratios (estimated values only) and on the basis of their visible and ultraviolet color characteristics. Although the anthocyanins split into several components, the production of the corresponding aglycones occurred in only trace amounts in some eluates. The aglycones, whenever they occurred, were not detectable in the BAW and Bu-HCl solvents because they fade, as is well known, in the butanolic solvents (cf., Harborne, 1959a); they were detected only in the Aq-HCl and the HAc-HCl solvents (see Sec. 17). The components around R_f 0.10 in the HAc-HCl solvent (Table 19.1) are due to the corresponding aglycones.

The anthocyanin band A in all cases splits into two components and yields no aglycone. The anthocyanin band B splits into three major anthocyanins in all cases. However, in a few cases, some additional minor spots and also traces of the aglycone were detected (Table 19.I). The presence of additional spots is most likely due to variable hydrolysis during handling. The band C separated only in the case of Black Hulless Collection 402. The pigment was obtained in small amounts and could not be analysed further. However, its R_f values in the BAW solvent suggest that it may be a component of band B. The band B it will be shown later is a mixture of two anthocyanins derived from cyanidin and peonidin. As noted elsewhere (Sec. 13.2.3), and it will also be gathered from Fig. 19.1 that the bands D and E, generally resolve as a single band. However, in Chromatograms 405, and 408, they split into their component parts. Upon

TABLE 19.I.
CHROMATOGRAPHIC DATA* OF THE BAW BANDS FROM GOPAL AND BLACK HULLESS BASAL LEAF SHEATHS (COLLECTIONS 400, 404, 405, & 407), IMMEDIATELY AFTER ELUTION (WITHOUT DRYING), IN BAW AND HAc-HCl SOLVENTS

	R_f x 100								
	BAW				HAc-HCl				
BAW Band No.	GC 400	GC 404	GC 405	GC 402	GC 400	GC 404	GC 405	GC 402	Average ** Rel. Conc.
A	11	17	15	14	58	60	60	59	(5)
	22	26	25	24	26	29	29	28	(1)
B		6				77			(t)
	9	10	10	10	56	59	56	57	(10)
			12						(t)
	14	15	16	15	65	66	63	64?	(2)
	21	22	21	21	29	29	29	29	(8)
	25?	30	?		35	36			(1)
					9	9			(t)
C				21					(1)
				25					10
				14					(1)
D			14				60		(1)
			24				32		(1)
							21		
							11		(t)
E			26				34		(15)
			?				21		?
							11		(1)
DE	12	14	14	?	58	60	60	?	(1)
	24	26	26	24	34	34	34	34	(10)
					?	22	?	?	(2)?
					10	11	11	?	(t)
F(4)	27	30	29	30	38	38	38	39	***
(3)	23	25	25	25	29	31	31	31	
					18	21			(t)

TABLE 9.I continued

BAW Band No.	R_f x 100								
	BAW				HAc-HCl				
	GC 400	GC 404	GC 405	GC 402	GC 400	GC 404	GC 405	GC 402	Average** Rel. Conc.
G				32				38	(1)
	30	30?	?		40	40			(1)
	26	26	26	26	30	30	30	29	(10)
		21	?						(1)
		15				60			(t)
					16	17		18	(1)
					9	9			(t)
H	28	29	29	?		35	36		(1)
	24	25	25	24	30	30	30	30	(20)
					16	16	?		(2)
					9	9	?		(t)
I	26				25				10
	F			55	12			12	10

GC = Greenhouse Collection No.
F = Fades
t = trace

*Chromatography was carried out by banding. The R_f values are arranged on the basis of color comparison and relative pigment concentration ratios.

**Estimated values are given to differentiate the major bands.

***The relative concentration ratios of the spots which ultimately were designated as F3 and F4 (HAc-HCl R_f components 0.31 and 0.38, respectively) varied considerably. For example, the F(3):F(4) ratios for the extracts No. 400, 404, 405 and 402 were 1:1, 2:1, 4:1, and 1:3 respectively.

rechromatography (see Table 19.I), the band D-405 splits into two major components and the band E-405 into one major component. The eluates of the bands D and E, however, gave only two major components in all cases. The HAc-HCl component at R_f 0.22 is difficult to demarcate because the component at R_f 0.34, usually trails to R_f 0.15.

The band F splits into two major components in all cases. In general, the HAc-HCl component at R_f 0.38, which is a peonidin derivative, decreases towards the later stages of development (see footnote (***) in Table 19.I). It is clear cut that the decrease in the ratios is due to development rather than to partial hydrolysis in this case because the other HAc-HCl component (R_f 0.31) is a cyanidin derivative. Moreover, the glycosidic hydrolysis was not detected in any of the eluates either in the Aq-HCl or the HAc-HCl chromatography. It may, however, be noted that the band A also showed substantial variations in the concentration ratios of the two spots shown in Table 19.I. The variations in the band A are, in fact, due to differential hydrolysis and not due to variation in development because, the HAc-HCl component at R_f 0.59 upon rechromatography again splits into the two components as noted elsewhere in Section 11. Similarly the relative concentration ratios of the other split components included in Table 19.I varied between the collections. It is in the interest of brevity that only the average relative concentration ratios of the split components are given in the table primarily to differentiate the major from the minor components.

It was noted early in this Section that the orange red pelargonidin component was not present in the BAW bands G obtained from the greenhouse materials. Because of this important observation the results of chromatography of all eluates of the band G in different solvents were

compared carefully to determine if the orange red band was present as a mixture in the band G. The orange red HAc-HCl component (R_f 0.38) of the band G-402 (Table 19.I) gave R_f values 0.32, 0.38, and 0.12 in the BAW, Bu-HCl and the Aq-HCl solvents respectively. Although the R_f values did not match strictly with the component G-130 or G-138 (Tables 13.I, 13.III, 13.IV), they are nonetheless quite comparable. Based on the comparability and the distinctive orange red color, the component appears to be similar to the orange red component of the band G-130. The component was available only in small amounts: no further studies could, therefore, be carried out. A similar HAc-HCl component R_f ca. 0.40 was also noted upon chromatography of the band G-400 (see Table 19.I). The component was very weak, and its color could not be differentiated with certainty in all the four solvents. However, the HAc-HCl component, R_f 0.40, of the band G-404 was distinctly bluish red, and so also were the HAc-HCl components, R_f 0.38, of the band G-407 and G-408. No orange red component from the bands G-404, G-405, G-407 and G-408 was detected in any of the three other solvents.

The band H in all collections of Gopal showed two to three anthocyanins and one aglycone (Table 19.I). The Black Hulless Collection No. 402, however, showed only the major component and no aglycone. The band I-400 gave one anthocyanin and cyanidin (Table 19.I).

The BAW eluates of all anthocyanin bands shown in Fig. 19.1 remaining after the above chromatography, were dried in a vacuum oven, and then placed for desiccation over phosphorus pentoxide (Sec. 19.2.2). A portion of each of the desiccated eluates was chromatographed in the five anthocyanin solvents that were used extensively in the characterization work (See sec. 19.3.7.3). It was noted invariably that all the desiccated eluates showed substantial quantities of the corresponding aglycones (the

lowermost components in the HAc-HCl, Aq-HCl, and 3:1:8 solvents) due to glycosidic hydrolyses. In addition, each of the desiccated eluates gave greater numbers of anthocyanins than were recovered from the corresponding eluates without drying and desiccation as shown in Table 19.I. The glycosidic hydrolysis and the increase in the number of anthocyanins following desiccation was also observed consistently with the eluates of the caryopses anthocyanins (Sec. 19.3.2). It was noted from the results presented in Table 19.I that most of the eluates prior to drying and desiccation, either did not show the glycosidic hydrolysis, or at best showed the aglycones in barely detectable amounts on the chromatograms. Although it was established that pronounced hydrolysis of anthocyanins occurs upon drying the 1% methanolic HCl extracts (Sec. 23), drying alone cannot account for the substantial hydrolysis because the anthocyanin eluent contained only a small amount of acid (0.03% HCl), and the quantity of the eluent used for elution was very small by our techniques (Sec. 16). The hydrolysis could have occurred in the presence of the acidic phosphorus pentoxide,* and was enhanced by the long periods of desiccation over this material. The view is strengthened because those eluates, which were dried but not desiccated over phosphorus pentoxide did not show significant hydrolysis.

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*Being unaware of the hydrolytic influence of phosphorus pentoxide, suitable precautions could not be taken during vacuum desiccation. One of the prime factors that could have contributed to hydrolysis appears to be the release of vacuum whenever a fresh batch of the dried anthocyanin isolates was introduced in the desiccator. Moisture-free air should have been introduced to release the vacuum. Moisture in the air, each time the vacuum was released to introduce more isolates, accumulated as a wet film or layer over phosphorus pentoxide.

The chromatograms of the desiccated eluates of the six basal leaf sheath collections, namely, 400, 404, 405, 402, 407 and 408, in the five solvents were carefully compared with those obtained from the undesiccated eluates (e.g., Table 19.I). Although the band A showed only two spots in the collections prior to desiccation, it showed seven components in the HAc-HCl solvent (see later Fig. 19.2, and Table 19.II) in all collections following the desiccation. The behaviour of other isolates was also identical. On the basis of detailed comparisons in different solvent systems, it was established that all major components of each band of each collection were identical in both Black Hulless and Gopal basal leaf sheaths. It is certain that no orange red spot indicative of pelargonidin

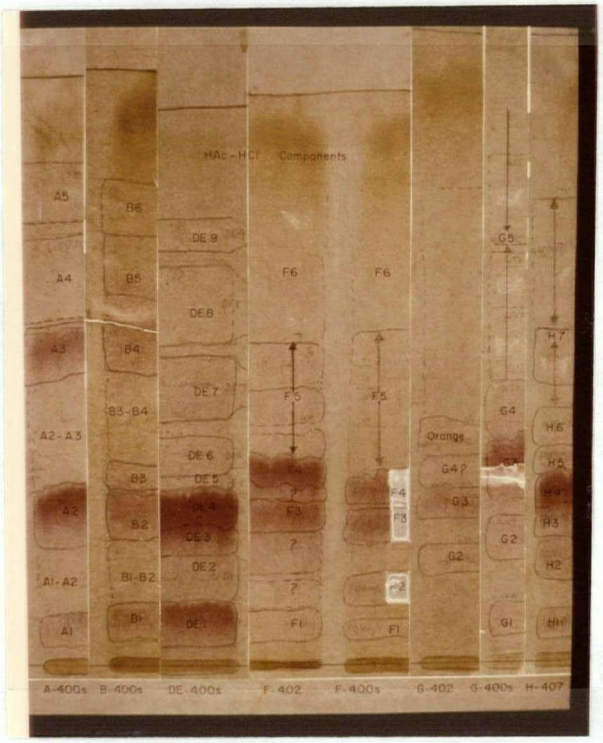

Fig. 19.2
Components obtained, using HAc-HCl solvent, from eluted BAW chromatobands of Fig. 19.1. Anthocyanins originated in basal leaf sheaths of Gopal and Black Hulless.

derivatives was present in the Collections 404, 405, 407 and 408. It is also certain that the orange red spot was present in the Collection 402, and that its presence in the Collection 400 is doubtful. The Collections 407 and 408 contain two additional, relatively fast moving bands K and L which were not present in any other collection. A few of the bands of different collections did show additionally some weak spots for instance as that shown in Table 19.I, but these were regarded to be due to differential degree of partial hydrolysis.

All corresponding BAW isolates of different collections, which showed identical results on the basis of above comparison were mixed and chromatographed in the HAc-HCl solvent for further purification. The results are shown in Fig. 19.2. The chromatographic data of the HAc-HCl purification is summarised in Table 19.II.

The designation of anthocyanin bands is based on the system described in Section 11.2.3. For the ease of reference, the mixed anthocyanin bands of the several collections shown in Fig. 19.1 will be referred to collectively as those belonging to the greenhouse collections of the 400 series (s). Thus, for example, the band A (Fig. 19.2) because of its chromatographic identity in the Collections 400, 404, 405, 402, 407, and 408 (Table 19.I) will be designated as band A-400s. The suffix 's' denotes the 'series'.

The BAW band A splits into seven HAc-HCl components in all cases as shown in Fig. 19.2, and Table 19.II. The BAW band B splits into eight HAc-HCl components in all collections except No. 402. Although the BAW band B-402 and C-402, when combined, gave about the same number of HAc-HCl components as given by the band B of the other five collections, the colors of a few components appeared to be different and therefore,

TABLE 19.II
CHROMATOGRAPHIC DATA FROM GOPAL AND BLACK HULLESS BASAL LEAF SHEATH ANTHOCYANINS FOLLOWING PURIFICATION IN HAc-HCl

HAc-HCl compon-ents	R_f x 100	Rel. Conc.***	Colors	
			Visible	UV
A1	12	100	Bluish red	RPk f
A1-A2	12-22	3	Bluish purple	wk
A2	31	150	Redder than A1	B1P
A2-A3	32-45	1	weak	wk
A3	58	125	Redder than A2	B1P to M
A4	73	5	Redder than A3	wk
A5	82	1	weak	wk
B1	11	100	Magenta or Bluish red	RPk f
B1-B2	12-22	2	weak red	wk
B2	29	150	Redder than B1	DM
B3	35	15	Pinkish	b Pk
B3-B4	36-50	5	Reddish	wk
B4	59	100	Redder than B2	DM
B5	60-72	15	Reddish	?
B6	72-82	1	weak	wk
DE1	10-12	30	Magenta	RPk f
DE2	21	3	weak bluish purple	Mv
DE3*	26	5	Purplish	wk
DE4	33	100	Rose red	B1P
DE5	36	2	Reddish	wk
DE6	38-43	3	Reddish	?
DE7	45-58	6	slightly bluish red	?
DE8	58-71	2	Reddish	?
DE9	71-80	1	Reddish	?
F1	10	5	Bluish red	?
F2	17	15	Bluish purple	B1P-VP
F3	29	100	Bluish red	B1P
F4	35	100	Pinkish	bPk**
F5	36-58	5	Reddish	wk
F6	58-75	1	Reddish	wk

TABLE 9.II continued

HAc-HCl components	R_f x 100	Rel. Conc.***	Colors	
			Visible	UV
G1	10	2	Reddish	wk
G2	19	5	Bluish purple	wk
G3	30	100	Bluish red	BlR
G4	39	5	Bluish red	wk
G5	40-75	5	Reddish	wk
H1	10	20	Mauve or Bluish red	Mv
H2	19	10	Reddish violet	Vio
H3**	28	20	Rose red	bR
H4	32	80	Rose red	bR
H5	36	3	Bluish purple	wk
H6	42	2	Reddish	wk
H7	45-75	2	Reddish	wk

*Estimated value

**Lower portion of the major component

the bands B-402 and C-402 were not mixed with the other collections. No new bands were detected when the region between the BAW bands B and D (Fig. 19.1) was eluted and chromatographed. The BAW band D and E separated only on a few chromatograms of the Collections 405 and 408. The chromatographic distribution of their major components in the HAc-HCl purification was identical to that shown for the band DE in Table 19.I except that several other weak components did appear as a consequence of partial hydrolysis following the desiccation. Because the bands D and E in general did not resolve, as indicated already, they were eluted as a mixture. The mixture of the bands D-E resolved into 14 to 15 components (similar to anthocyanin 5 -- see later) but in some chromatograms the resolution was not clear cut. Therefore, only nine components which

could be easily demarcated were eluted. Accordingly, some of the components shown in Fig. 19.2 must be a mixture of two or more anthocyanins.

The band F gave six HAc-HCl components. The Component F2 did not appear in all collections: it was present conspicuously only in the Collections No. 400, 402, and 404. There are also reasons to believe that there may still be another anthocyanin component between the components F3 and F4. However, the pigment from the region F3-F4 could not be collected in sufficient amounts for further studies. It also appears probable that the Collection 402 contains a component immediately 'above' the component F4 which was not present in other collections. However, no work could be done on the component owing to the scarcity of material. The components F5 and F6, each resolved into 2 to 3 anthocyanin bands but they were rather weak and were, therefore, not eluted. Thus, only three bands, namely F2, F3, and F4 which were the major bands of both Gopal and Black Hulless were eluted.

The band G from the Collections 404, 405, 407 and 408 gave up to 8 HAc-HCl components (Fig. 19.2, chromatogram G-400s). The band G from the Collection 400 was, however, lost. The band G-402 did show all the HAc-HCl components as were shown by the above four collections, except one significant difference: the relative concentration ratio of the major component G3-400s (R_f 0.30) to the component G4-400s (R_f 0.39) is 20:1,* and that the color of the two component G4 is bluish red (Fig. 19.2, and Table 19.II). The band G-402 (Fig. 19.2) gives the major component G3 (R_f 0.29) (and probably G4), but also an orange red component at R_f 0.39. The relative concentration ratio of the major to the orange red component was

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*estimated value only

5:1*. The hydrolysis of the orange red component gave pelargonidin** (Table 19.V). It will be noted from Table 19.II, that G3 is the major component, and G2, G4, are the minor components of the band G. Although at least four spots, R_f values 0.46, 0.54, 0.65 and ca. 0.75 were detected, they were, however, very weak and were eluted as one band designated as G5.

The band H gave up to 9 HAc-HCl components. The component H3 is the lower portion of the major component. H3 was eluted separately to determine if it were a contaminant of H4. The component H7 (R_f range 0.45 to ca. 0.75), in fact, consisted of three bands at R_f values, 0.54, 0.68, and 0.74. The bands were, however, weak and diffuse and therefore eluted as a mixture.

The desiccated isolates K and L of the Collections 407 and 408 were available in small quantity and therefore could not be purified in the HAc-HCl solvent. The results of their chromatography in the five anthocyanin solvents are shown in Table 19.VIII, K and L.

It is evident from the results presented above that all basal leaf sheath anthocyanins of barley split into several components. All anthocyanins yield weak components which give very high R_f values in the HAc-HCl solvent. As far as practicable, most of the HAc-HCl components were eluted after the usual purification in the anthocyanin concentrant Sec. 19.2.2). An aliquot of each eluate was chromatographed directly after elution on Whatman paper No. 1 by descent in the BAW, Bu-HCl, Aq-HCl, HAc-HCl, and 3:1:8 solvents. It may be noted that the eluates were neither

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* estimated value only

** Owing to the scarcity of the material no further work on the orange red component of G-402 could be carried out.

dried in vacuo, nor desiccated over phosphorus pentoxide. Whenever necessary, and the necessity arose only in a few cases, the eluates at best were concentrated in a vacuum oven prior to chromatography. The eluates were hydrolysed for the determination of sugars (Sec. 19.2.4) and the correspondong aglycones (Sec. 19.2.3). It will be observed that a large number of the HAc-HCl components (see Fig. 19.2) were rather weak. The eluates from the weak bands were chromatographed only for anthocyanins and not for anthocyanidins and sugars owing to the scarcity of the material.

Before presenting the results of characterization, the preparative chromatography and the details of isolation of the caryopsis anthocyanins will be described next.

19.3.2 Preparative Chromatography, Purification and Isolation: Caryopsis Anthocyanins

The results of the preparative chromatography of the Black Hulless caryopses extracts (Field Collections 217, 270, 274, and 278, in BAW are shown in Fig. 19.3. The results of the Field Collection 272 are similar and are shown elsewhere in Section 6 (Fig. 6.2). The caryopses were extracted in August 1960; the processing and standardization of the extracts, along with the other 300 extracts, was completed in February 1961 (Sec. 10.2.1). The chromatogram 270-a (Fig. 19.3) was developed in March 1961. All concentrated extracts of the above collections remained in storage as described elsewhere (Sec. 10) until February 1962, when preparative chromatography of the extracts, chromatogrammed in Fig. 19.3, was undertaken. About 20 to 60 chromatograms were prepared from each of the extracts. A comparison the chromatogram 270-a and 270-c, developed in

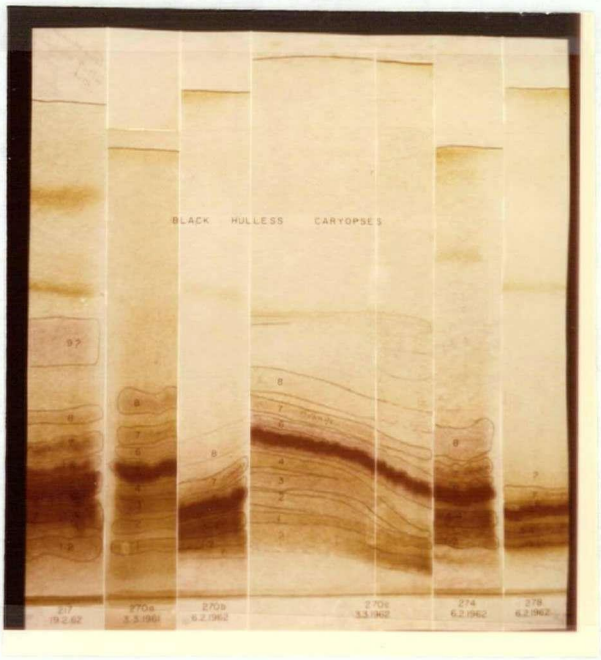

Fig. 19.3
Preparatory chromatography of caryopsis extracts from Field Collections 217, 270, 274, 278 of Black Hulless. Solvent BAW; by ascent. Collections made in one day but increasing maturity is represented from 217 to 278. Chromatostrip 270a was developed in 1961, 270b and c, one year later; all show essentially the same patterns but most bands are weakened as a result of extract storage. Bands 1-4, in general, resolved poorly and often appeared as 1 or 2 consolidations. Bands of materials visible under u.v. light after NH_3 treatment are shown weakly at 270c right.

March 1961 and March 1962 respectively, shows that the only change that occurred in the extract in a year's storage was the significant reduction in the intensity of band 8, and increase in the intensity of band 5. Of course, the overall intensity of all the bands, in general, decreased. It may be noted that the resolution of bands 5, 6, and 7, in general, is clear-cut in all chromatograms. The bands 1 to 4, however, do not resolve distinctly in most of the chromatograms (see Fig. 19.3); chromatograms 217, 270-b, 274, and 278. In some chromatograms, for instance, chromatogram 272 (Fig. 6.2) and chromatogram 270-a, the resolution of the bands 1 to 4 is clear-cut. However, in chromatogram 270b, the anthocyanins 1 to 4 do not resolve, and in chromatogram 270-c, the resolution on the right hand side is clear-cut but confounded on the left hand side of the chromatogram. The results from other chromatograms indicate that the region between the bands 1-4 may, in fact, have five bands instead of four

bands. The delineation of the boundaries of each band is very exacting. It is very probable that the band 4, may in fact be a mixture of two bands. It separated only rarely into two components. The difficulties in resolution are primarily due to brown pigments, which are extracted in large amounts from the caryopses, and which resolve below the major anthocyanin band 5. No such pigments were encountered in the leaf tissues (Fig. 19.1). the 'co-pigments' not only interfere with the chromatographic resolution but also, owing to their superimposition, the detection of anthocyanins. The anthocyanins 1 to 4 occur in small amounts as compared to the other anthocyanins; in the chromatograms shown in Fig. 19.3, they appear as strong bands in the photograph only because of the superimposed brownish pigments. It may be added that the bands 1 to 4 showed generally a great deal of variation; e.g., the bands 1 and 2 appear to be absent from the Collection 278, the collection which was made from nearly flinty kernels.

It was pointed out in the preceding section (19.3.1) that the orange red band, a pelargonidin derivative, which was conspicuous in the Gopal leaf sheaths in the 1960 summer plantings (field conditions) (Fig. 10.3) was absent in the 1961 Winter plantings (greenhouse conditions) (Fig. 19.1). In the case of the caryopses tissues of Black Hulless, a conspicuous orange red band 7 (Fig. 19.3) was present in the 1960 summer plantings (field conditions) but absent in the 1961 winter plantings (greenhouse conditions)(Fig. 20.3). The results of the 1961 winter planting, which show several other basic differences are not reported. However, an extraction of the spermoderm tissues of Black Hulless (shown in Fig. 20.3) was also undertaken on the materials grown in the 1965 summer (see Fig. 22.1). Although an orange red band, equivalent to the band 7 was not detected, nonetheless, the 1965 extracts did show the

probable presence of trace amounts of pelargonidin (Fig. 22.2).

The relative concentration of the band 6 which is equivalent of the band F (Fig. 19.1) also, like the leaf sheaths, decreases towards maturity (Fig. 19.3). The bands 8 and 9 are relatively labile in extended storage and also probably, in the course of preparative chromatography in BAW. The clear-cut resolution of these bands as shown in Fig. 19.3 was obtained rarely; in general, the bands were diffuse, weak and discontinuous on the paper which made difficult the delineation of the corresponding bands even from chromatograms developed at the same time.

The BAW bands 1-4 were eluted via the usual purification through the anthocyanin concentrant. Owing to their poor resolution, they were eluted separately only from a few chromatograms of the Collections 217 and 272, which showed distinct resolution. The eluates, immediately after elution (without drying in vacuo, or desiccation over phosphorus pentoxide), were chromatographed* by banding for the HAc-HCl purification. The results of the HAc-HCl purification are shown in Fig. 19.4. The band 4 shown in Fig. 19.4 is probably an equivalent of the BAW band D of basal leaf sheaths (Sec. 19.3.1). Because of the scarcity of the materials further characterization of only a few of the HAc-HCl components was undertaken. As indicated already, the BAW bands, 1-2, resolved as a single band, and the BAW bands, 3-and a portion of 4, resolved as another single band. The rest of the band 4, which usually occurred as a mixture of two bands, usually did not separate from the band 5. The HAc-HCl chromatography

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*Aliquots of the eluates from the bands 1, 2, and 3 were also chromatographed in the four anthocyanin solvents. The results are presented elsewhere in Section 19.3.7.3 (Table 19.VIII.1.2.3).

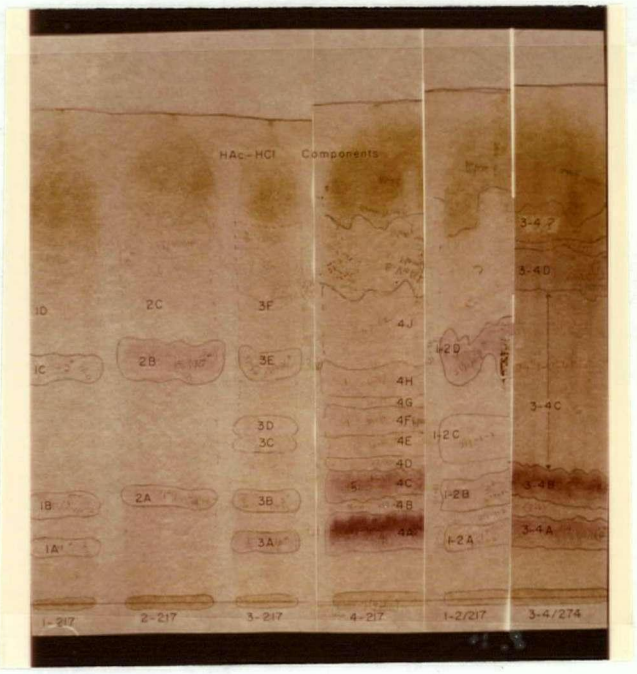

Fig. 19.4
Components obtained, using HAc-HCl solvent, from eluted BAW chromatobands 1-4 of Fig. 19.3. Anthocyanins originated in caryopses of Black Hulless.

of the bands 1-2 and 3-4 is also shown in Fig. 19.4 and the results of the bands 1 to 4 are summarized in Table 19.III. Almost all collections gave similar results except that towards maturity of the tissues, the relative concentration ratios of the bands decreased significantly. For instance, the BAW chromatogram of the Collection 278 (Fig. 19.3) shows that the BAW bands 1-2 were missing. The region of the bands 1 to 4 was eluted as a mixture and rechromatographed in the HAc-HCl to ascertain if the bands 1-2 were indeed absent. The component, R_f 0.56 (characteristic of the BAW bands 1 and 2) was barely detectable.

Chronologically, the characterization of the BAW anthocyanins 1 to 4 was undertaken first. The occasions and reasons for splitting of the anthocyanins into several components were not clear then. Accordingly, the HAc-HCl eluates were repurified in several solvents but the splitting to several components still occurred. In the process most of the eluates were used up, and detailed characterization of the anthocyanins 1-4 could

TABLE 19.III
CHROMATOGRAPHIC DATA FROM ANTHOCYANIN BANDS, 1-4, FROM BLACK HULLESS CARYOPSES FOLLOWING PURIFICATION IN HAc-HCl.

HAc-HCl components	R_f x 100	Rel. Conc.	Colors	
			Visible	UV
217/1A	13	1	Bluish red	wk
217/1B	24	5	Bluish red	wk
217/1C	51	2	Bluish red	wk
217/1D	ca.70	t	weak trail	
217/2A	25	1	Bluish red	dB1P
217/2B	55	10	Bluish red	dB1P
217/2C	ca.70	t	weak trail	wk
217/3A	16	10	Purple	DVioP
217/3B	24	3	Purple to mauve	dB1P
217/3C	36	1	Purplish	wk
217/3D	40	1	Purplish	wk
217/3E	54	1	Purplish	wk
217/3F	ca.80	t	Purplish	wk
1-2A	19	1	Purplish	wk
1-2B	26	3	Magenta	dM
1-2C	36	t	Reddish	wk
1-2D	56	10	Redder than 1-2B	dB1R
3-4A	16	2	Purple	dVioP
3-4B	27	5	Magenta	dM
3-4C	28-60	1	weak Purple trail	wk
3-4D	ca.70	1	Purple	dB1P
217/4A	18	100	Purple	dVioP
217/4B	21	5	Reddish trail	wk
217/4C	27	50	Magenta	dM
217/4D	30	5	Reddish	wk
217/4E	34	3	Reddish trail	wk
217/4F	39	10	Mauve	?
217/4G	41	3	Reddish trail	wk
217/4H	47	10	Mauve	?
217/4J	47-62	5	Reddish trail	wk

not be carried out. Nonetheless, pertinent chromatographic data of several HAc-HCl components in several solvents will be included elsewhere (Tables 19.VIII.1.2.3. and 4). It was primarily because of the problem of anthocyanin splitting that further purification of BAW anthocyanin eluates 5 to 9 (caryopsis tissues), and the BAW eluates of the basal leaf sheath anthocyanins (Sec. 19.3.1), which was undertaken concurrently, was withheld. The eluates of the anthocyanins 5 to 9, as also the eluates of the basal leaf sheath anthocyanins (Sec. 19.3.1) were dried in a vacuum oven, and desiccated over phosphorus pentoxide. The drying was undertaken primarily to ensure convenient handling of the isolates until the establishment of suitable methodology for further processing.

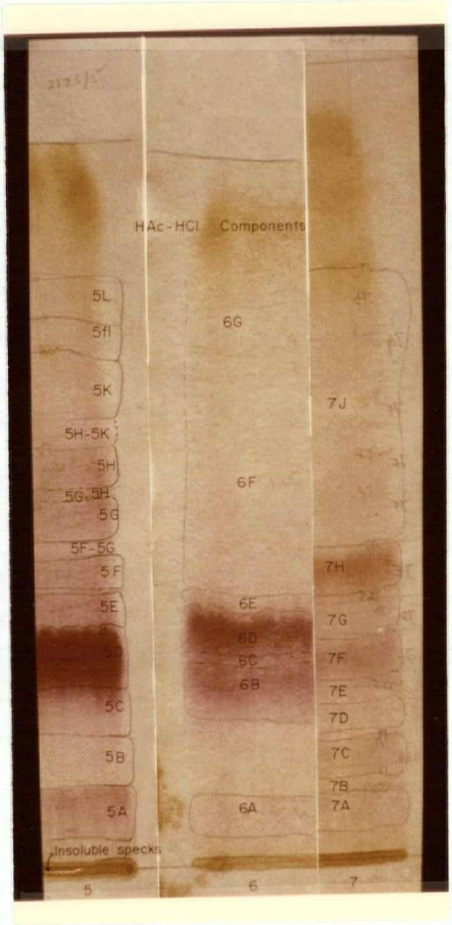

Fig. 19.5
Components obtained, using HAc-HCl solvent, from eluted BAW chromatobands 5-7 of Fig. 19.3. Anthocyanins originated in caryopses of Black Hulless. Note the bright orange pelargonidin derivatives of 7H.

An aliquot of each of the desiccated BAW eluates 5 to 9, of all collections, was chromatographed in the five anthocyanin solvents exactly as described for the basal leaf sheath anthocyanins (Sec. 19.3.1). The corresponding anthocyanin bands of different collections were mixed on the basis of chromatographic identity and chromatographed in the HAc-HCl solvent for further purification. The results of the HAc-HCl purification are shown in Fig. 19.5, and summarized in Table 19.IV. The designation of the anthocyanin bands is based on the system described in Section 11.2.3.

It is clear from the comparison of the results presented in Fig. 19.4 and Fig. 19.5 that when the anthocyanins are chromatographed without drying or desiccation, the hydrolysis, if any, is not detectable e.g., Fig. 19.4. However, when the anthocyanins are chromatographed after drying and desiccation (Fig. 19.5), a great deal of glycosidic hydrolysis occurs. It was noted generally, and it is revealed clearly on comparison of the results shown in Fig. 19.4 and Fig. 19.5 that the formation of 'polymerized' stationary materials, noted earlier in Sections 11 and 15 (see also next section), was minimal when the anthocyanin eluates were not dried than when they were dried and desiccated.

The BAW anthocyanin band 5, which is equivalent of the anthocyanin DE-400s, splits into a least thirteen components (see Table 19.IV). The component 5C was marked arbitrarily because it was slighly bluer than its parent component 5D, the major component. The component 5G was most prominent in the Collection 274 and the component 5H in the Collections 272 and 217. The components 5F-5G constituted the trail between the components 5F and 5G. Since the materials from band No. 5 were available in large quantity, all the HAc-HCl components of the band were eluted for

TABLE 19.IV

CHROMATOGRAPHIC DATA OF ANTHOCYANIN BANDS, 5-9, FROM BLACK HULLESS CARYOPSES FOLLOWING PURIFICATION IN HAc-HCl

HAc-HCl components	R_f x 100	Rel. Conc.	Colors	
			Visible	UV
5A	12	10	Pink	pink fluorescence
5B	18	wk	Reddish (wk)	
5C*	26	5	Bluish red	dull bluish purple
5D	34	100	Rose red	dull bluish purple
5E	38	7	Reddish mauve	deeper mauve
5F	43	7	Reddish mauve	mauvish
5F-5G	46	wk	Mauvish	
5G	51	5	Reddish mauve	mauvish
5G-5H	53	wk	Bluish tinge	
5H	59	10	Bluish purple	dull bluish purple
5H-5K	62	wk	Reddish tinge	
5K	73	1	Reddish tinge	
5f1**	76	wk	Reddish tinge	
5L	82	wk	Reddish tinge	
6A	10	2	Bluish red	weak fluorescence
6B	29	50	Mauve	dull bluish purple
6C	31	5	Mauve	dull ?
6D	37	100	Pink	bright pink
6E	39	1	Orange Red	weak
6F	39-64	2	Reddish trail	
6G	64-ca.85	wk	Reddish trail	
7A	10	10	Bluish red	pinkish fluorescence
7C	16	15	Red	red fluorescence
7D	20	1	?	yellowish red, fluorescence
7E	24	wk	Reddish	
7F	29	50	Purple	dull bluish purple
7G	35	5	Reddish	?
7H	41	100	Orange Red	Red
7J	41-73	4	Orange shade	
8A	8	20	Pink	Mauve fluorescence
8B	14	1	wk	
8C	29	50	Purple to mauve	dull bluish purple
8D	32	3	wk	
8E	38	10	Red to mauve	dull mauve
8F	42	wk	weak	
8G	52	1	weak	
8H	61	1	weak	
8K	64-80	wk	weak trail	

TABLE 19.IV continued

HAc-HCl components	Rf x 100	Rel. Conc.	Colors	
			Visible	UV
8-9A	9	10	Mauve	Mauve fluorescence
8-9B	15	wk	wk	
8-9C	29	30	Purple to mauve	dull bluish purple
8-9D	33	1	weak	
8-9E	39	5	Orange red	Red
8-9F	40-70	2	weak trail	
8-9K	80	wk	weak	

*Lower portion the major band

**The trail designated 5f1 shows a fluorescent contaminant

further characterization.

The BAW band 6 which is broadly equivalent of the band F-400s, gave at least seven HAc-HCl components The band 6B and 6D were the major components of the band. The major components of the band 7 are the orange red band 7H and the purple band 7F. The bands 7D and 7E were observed only in the Collections 272 and 274. The trail (R_f 0.41 to 0.73) above the component 7H was weak and could not be resolved into clear-cut components. The trail was eluted as a single component designated 7J.

The BAW bands 8 and 9, as already mentioned, are highly labile and did not resolve properly. Therefore, only in a few cases, the eluates of the band 8 were obtained separately. In general, the bands 8 and 9 were eluted together. The results of the band 8 and the bands 8 and 9 are summarised in Table 19.IV; it will be noted that the major components of the bands gave identical R_f values and colors in the

in the HAc-HCl solvent. Because the pigments of the bands 8 and 9 were available only in small quantity, the corresponding eluates from the HAc-HCl bands were generally mixed.

The HAc-HCl anthocyanin components of all bands were eluted after the usual purification in the anthocyanin concentrant and processed further for the analysis of aglycones, sugars and anthocyanins as was done for the HAc-HCl components of the basal leaf sheaths. However, before presenting the characterization data, a few observations on the polymerization of anthocyanins made when the eluates were dried will be presented in the next section.

19.3.3. Polymerization of Anthocyanins During Drying

In a few instances, the following observations were made on the eluates of the bands 5 (Fig. 19.3) and DE (Fig. 19.1) during drying in a vacuum oven. The BAW bands DE and 5 are, otherwise, identical.

The band 5 or DE are the major bands and depending upon the number of such bands eluted simultaneously, the volume of the eluates varied from, say, 5 ml to 50 ml. It was noted that when the volume of the eluate was small and the relative concentration of anthocyanins in the eluate large, the anthocyanins dried without apparent modification. However, when the quantity of the eluate was large, because of elution from large number of bands, a major portion of the anthocyanins turned irreversibly to reddish brown to dark brown and finally to black colors. The dark brown product was sparingly soluble and the black product insoluble in 0.1% methanolic HCl, suggesting thereby 'polymerization' of anthocyanins. It was noted that when an eluate was dried in a flask with a substantial surface on a flash evaporator, the polymerization to slightly brownish red

material was minimal. When, however, the eluates were dried in a beaker of small surface in a vacuum oven, at room temperature, much polymerization occurred. The polymerization in the beaker, however, was not uniform: along the peripheral depression of the beaker, the anthocyanins became carbon black and insoluble in the acidified methanol, in some regions of the beaker they turned brownish, and in others they did not show any apparent modification. Polymerization in such cases could be minimized by tilting and rotating the beaker periodically to spread the anthocyanins over substantial surface of the beaker just when the anthocyanins were about to dry (see Sec. 19.2.2). Under such conditions, the anthocyanins did not become carbon black; at most a portion turned only to brownish red colors. Thus the transformation of anthocyanins to brownish colored products seems to be an intermediate step to eventual blackening.

The browning and blackening of anthocyanin isolates attracted our attention for several reasons, the principal among them being the sizeable loss of the pigment. It was also observed that the residual anthocyanins in tissues, which had been extracted once with acidified methanol (Sec. 10.2.1), turned black upon drying in an oven above 80°C. Thus, the blackening of anthocyanins, and the co-occurrence of anthocyanins and melanins in the black varieties warranted further scrutiny of the black brown and reddish brown products. A few chromatographic observations on the nature of the brownish products obtained from the isolates 5-274 and 5-217 will be described next.

It was observed that the eluates of the BAW anthocyanin bands 5-274 and 5-217, in a few cases, had partially turned brown upon drying in a vaccum oven. The browning was localised over certain areas of the containers (beakers). The isolates were desiccated over phosphorus pentox-

ide as described in Section 19.2.2. It was observed that the brownish material dissolved very slowly in 0.01% methanolic HCl and in the case of the isolate 5-217, a few dark specks remained insoluble. The isolates were banded on Whatman paper No. 3 for the usual purification of the anthocyanins in the HAc-HCl solvent. The insoluble specks of the isolate 5-217 were also deposited on the band during banding. The results of the HAc-HCl chromatography are shown in Fig. 19.6: Chromatograms 5-274 and 5-217. Both the chromatograms show the brownish materials, and also the insoluble dark specks (chromatogram 5-217) only at the starting line. The chromatograms also show the usual anthocyanins (cf., Fig. 19.5) that are obtained from the band 5; however, the components above the component 5D in Fig. 19.6 are

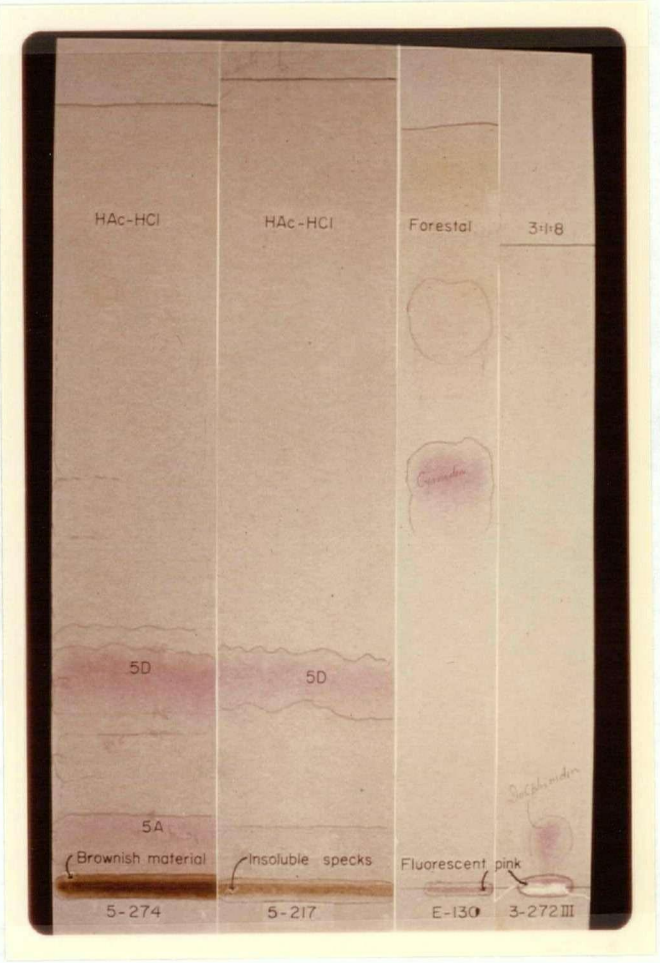

Fig. 19.6
Polymerization of anthocyanins and anthocyanidins as a result of drying. Note brown materials derived from anthocyanins immobile at the starting lines of chromatostrips 5-274 and 5-217. Note the pink anthocyanidins, derived from partial hydrolysis (E-130) and complete hydrolysis (3-272 III) of anthocyanins, immobile on the starting lines of the two chromatostrips on the right.

rather weak and have not been pencilled. The materials at the starting line, in colors, resemble exactly to the stationary materials which are encountered at the starting line whenever anthocyanin eluates are chromatographed (see Secs. 11 and 15). Similar brownish and brownish red materials are also present at the starting line in the case of eluates obtained from other bands though the eluates upon drying did not show any localized browning in the containers (see Figs. 19.2, 19.4 and 19.5). The only difference is that the ratio of the stationary materials to anthocyanins was minimal when no localised browning occurred in the containers during drying.

The color of the stationary materials varies depending upon the conditions. For example, when the BAW anthocyanin bands A, E, F, G of the collection 130 (see Sec. 10) were hydrolysed partially as described in Section 19.2.3, the color of the materials at the starting line was generally bright red or pink (fluorescent under UV). The results for the band E-130 are shown in Fig. 19.6. Similarly the fluorescent spot at the starting line was noted when the delphinidin derivative 272-III/3 (Sec. 6.4.2.c) was hydrolysed directly on paper by the method described in Sec. 20. The results of the hydrolysis are also included in Fig. 19.6. If, however, the hydrolysis was carried out to completion in solution, the colors of the stationary materials, in the case of hydrolysates from the purified anthocyanins (Sec. 19.3.4) were generally red and only occasionally brown. Similar variation in the colors of polymerized anthocyanins was noted throughout the course of this investigation. For example, when the anthocyanin eluates are chromatographed, the colors of the polymerized materials were sometimes weak red, generally brownish red, but at times brownish yellow (see Figs. 19.4 and 19.5). Occasionally, however, the color of the stationary materials as shown in Fig. 19.1, in the case of

the greenhouse Collection 407 was bluish purple, but in the case of the Collection 408, which was obtained on the same day from the same materials is weak light red. Similar variation in the colors was noted in several other cases. The polymerized materials obtained from anthocyanidins, at least under the conditions mentioned above, fluoresce and those obtained from anthocyanins show dull absorption. The above observations clearly indicate that there must be a link between the brown, red, and purple stationary materials, and that both anthocyanins and anthocyanidins undergo polymerization. The conditions under which the colors of the polymerized products vary are not understood, and therefore the necessity of further scrutiny of the techniques of processing anthocyanins in relationship to polymerization can be hardly overemphasized.

The brownish materials remained stationary in all anthocyanin solvents listed elsewhere (Table 10.II) including the Bu-HCl solvent. Inasmuch as the reddish brown precipitate of Lukton, Chichester and Mackinney (1956) were readily mobile in the Bu-HCl solvent and the brownish materials encountered in this study immobile, the latter may, therefore, be of different nature. Lukton et al., (ibid.) suggested that the insolubilization of their reddish brown precipitate could have been due either to polymerization or conversion of the pseudo base to the precipitate or due to the hydrolysis of the pseudo base to the aglucone with subsequent conversion to the precipitate. The polymerization of anthocyanins to the brownish red products encountered in this study cannot be due to the polymerization of pseudo bases because the conditions of our handling preclude pseudo base formation. Moreover, Lukton et al. (ibid.) have implicated the degradation through pseudo base formation on the assumption that it is the flavylium state which is more stable. The studies described

in Section 24 show that it is the pseudo base state which is more stable than the flavylium state.

It is clear from the above observations that depending upon the conditions, varying degrees of anthocyanin losses occur upon drying in the form of stationary materials due probably to polymerization. Elimination of such losses is very important particularly from the point of view of hybrid analysis which requires sound basis for quantification.

19.3.4. Identification of Anthocyanidins: Basal Leaf Sheaths and Caryopses

The identification data of the anthocyanidins of the HAc-HCl anthocyanin components, and, only in some cases, the BAW anthocyanin components of the basal leaf sheath (Sec. 19.3.1) and caryopsis (Sec. 19.3.2) anthocyanins are included in Table 19.V. The R_f values of the anthocyanidins were compared carefully with authentic anthocyanidins in seven solvent systems, two of which, namely, the Propionic acid solvent and the FA-4NHCl solvent (See Tables 17.I and 17.II) were developed in this laboratory. Unless stated otherwise, the R_f values of the identified aglycones were identical with the corresponding synthetic aglycones on the same sheet in at least three runs. Whenever the pigment was available in sufficient quantity, the chromatographic identification was supported by spectral data. Control spectra of the synthetic aglycones, in general, was also determined on the same day. The spectral measurements of the aluminum chloride shift were especially helpful in reinforcing the identifications.

In the next section are presented the characterization of sugars obtained from the hydrolysates of the anthocyanin components.

TABLE 19.V
R_f VALUES AND SPECTRAL CHARACTERISTICS OF THE ANTHOCYANIDINS FROM THE BASAL LEAF SHEATHS AND THE CARYOPSES

BASAL LEAF SHEATHS

Band No.	R_f x 100							λ_{max} mu			Aglycone[f]
	Forestal	FA Harborne	FA-4NHCl	HAc-HCl	3:1:8	Propionic	Iso-PrOH	Observed	Standard	$AlCl_3$ Shift	
A1	50	23	33	11	14	30	43				Cyanidin
A2	46	23	36	11	16	29	45	532	532	16	Cyanidin
A3	45	24	33	12	15	31	50	534	534	22	Cyanidin
A4	46	25	38			30	51	532	532	20	Cyanidin
A	48	27	37	12	15	30	50	534	534	26	Cyanidin
B1	50			11	14	30					Cyanidin
B2	50	22	36	12	14	31	48	534	534	22	Cyanidin
B3	62	29		12		40	50				Peonidin
	46	23				30					Cyanidin (wk)
B4	48	25	37			30	50				Cyanidin
	T60	30?	48T			40					Peonidin (wk)
B	50		37			30	50				Cyanidin (5)
	64		50			40					Peonidin (1)
G-402	46	22	36			30					Cyanidin (5)
	64	33	50a			41					Peonidin (1)
DE1	50	24	33	12	15	29	42				Cyanidin
DE2		15	28?	6[k]		20					Delphinidin[c] (1)
	46	23	36	10		29					Cyanidin (5)
DE3	46	22	36	11		26	43	534	534	21	Cyanidin
DE4	48	22	35	12		30	45	533	534	22	Cyanidin

TABLE 19.V continued

Band No.	R_f x 100							λ_{max} mu			Aglycone[f]
	Forestal	FA Harborne	FA-4NHCl	HAc-HCl	3:1:8	Propionic	Iso-PrOH	Observed	Standard	$AlCl_3$ Shift	
DE7	47	21	38	9	14	31	46	532	534	36	Cyanidin[b]
DE8						30					Cyanidin[c]
DE9		20	33			28					Probably Petunidin[c]
D-405	50	24	34	10		29	45				Cyanidin
E-405	48	22	36	12		28	45				Cyanidin
F2[d]	52 66				15 19						Petunidin[ce] (5) Peonidin or Malvidin[c]
F3	48	26	37	11		30	50				Cyanidin
F4	62	31	46	15		40	51				Peonidin
G3	46	21	33			30	50	534	534	13	Cyanidin
G-402[g]	68	38	51			47	60				Pelargonidin (see text)
H1	48 60		34 68	10 27		27 54					Cyanidin[c] (1) PPPH (2)
H2	46			10							Cyanidin or Petunidin: see also F2
H3	55				18						Cyanidin[c d h]
H4	48	26	36			32	53	532		30	Cyanidin
K	46 60	25 33	38 50			33 40					Cyanidin[c] (7) Peonidin (1)
L	46	25	40			30					Cyanidin[c]

TABLE 19.V continued

CARYOPSES

HAc-HCl component No.	R_f x 100 Forestal	FA Harborne	FA-4NHCl	HAc-HCl	3:1:8	Propionic	Iso-PrOH	λ_{max} mu Observed	Standard	$AlCl_3$ Shift	Aglycone[f]
3-4A	35	15	21	6	10	20					Delphinidin
4A	35	16	20	7	9	20	30				Delphinidin
4C	48	22	34	11	15	30	44				Cyanidin
5A	49 59	24	36			30	43				Cyanidin (15) ? (1)
5B	45	22	37	11		31	47				Cyanidin
5C	46	25	36	11	16	30	48	534	534	22	Cyanidin
5D	46	26	36	10	17	28	45	534	534	20	Cyanidin
5D	(nylon purified							534	534	21	Cyanidin
5E	46 63	22	36 46		16	29 40	43	530	532	10 30	Cyanidin (20) Peonidin (1)
5F	46	25	35		16	29	49	532	534	*	Cyanidin
5G	44	24	36			29	48	532	532	29	Cyanidin
5H	46	22 52	36 70			27 60	46	535	534	28	Cyanidin (5) PPH (1)
5K	47	24	36			30	48				Cyanidin
5f1	46 74	24 57	35 67			30 65	52 F				Cyanidin (10) PPH (1)
5L	46	23	36	9	14	30		*		*	Cyanidin

TABLE 19.V continued

HAc-HCl component No.	R_f x 100 Forestal	FA Harborne	FA-4NHCl	HAc-HCl	3:1:8	Propionic	Iso-PrOH	λ_{max} mu Observed	Standard	$AlCl_3$ Shift	Aglycone[f]
IB5GH		23	36								Cyanidin (10)
IB5KH	47 74	23 55	37 67			30 64	50 F				Cyanidin (25) PPH (1)
6B	48 64 (wk)	26 30	37 47	11		30 41	50	532		16	Cyanidin (10) Peonidin (3)
6C	46 62	24 32	35 48			29 40	51				Cyanidin (1) Peonidin (3)
6D	62	31	46	15	18	40	51	530	530	0	Peonidin
7A	51	23	32				44				Cyanidin[c]
7C				16							Pelargonidin
7F	48	24	35	10		29	48				Cyanidin
7G[d]					16* 25		37* 52				Cyanidin[c] (1) Pelargonidin[c] (2)
7H	64	38	50	17		47	71	518	518	0	Pelargonidin
7J	45 64	24 37	33 48	11 17[j]		30 47	51 69	516			Cyanidin (1) Pelargonidin (4)
8A						28					Petunidin or Cyanidin
8&9C	47	24	37	11		31	51				Cyanidin
8D	46	24	35	10		30	47				Cyanidin
8&9E	65	36	52	16[j]		46	68				Pelargonidin
8FGH	50 67	24 36	36 50			29 43	48 66				Cyanidin (5)* Pelargonidin (1)
8&9K	45	23									Cyanidin

TABLE 19.V continued

F = fades
PPPH = probably partial products of hydrolysis
a = R_f values identical with synthetic peonidin on the same sheet
b = although R_f values in some solvents (and also colors) resemble closely petunidin, the pigment is identified as cyanidin primarily on the basis of spectral characteristics
c = provisional identification
d = R_f values under standard conditions could not be determined
e = R_f values and colors do not tally with synthetic cyanidin on the same sheet
f = an estimate of relative concentration of the two aglycones is included in parenthesis
g = the orange red HAc-HCl component of the band G-402 (cf., sec. 19.3.1)
h = R_f values 'tally with' cyanidin on the same sheet
j = pelargonidin on the same sheet gave an R_f value 0.21
k = see Table 19.VII.
* = degrading during spectral analysis; the specimen did show the aluminum chloride shift
T = trail
wk = weak

19.3.5. Characterization of Sugars: Basal Leaf Sheath and the Caryopsis Anthocyanins

The sugars obtained from the hydrolysates of the HAc-HCl anthocyanin components of the basal leaf sheaths and the caryopses are included in Table 19.VI. The sugars were identified by cochromatography with known compounds in the pyridine solvent and BAW solvent (Sec. 19.2.4). It will be noted that the sugars obtained from the hydrolysis of both the anthocyanin eluates (Table 19.VI), and the anthocyanin blanks (Fig. 18.1) are identical, namely, glucose, galactose, arabinose xylose. The investigations on the glycosidic components of the sugar blanks (i.e., the blanks obtained from the hydrolysis of anthocyanin blanks: see Sec. 18.1.1) were carried out only at the conclusion of the sugar determinations of all HAc-HCl anthocyanin eluates reported in Table 19.V. Since all anthocyanin isolates had been used up in the determinations of aglycones, sugars,

TABLE 19.VI
SUGARS OBTAINED FROM THE HYDROLYSATES OF THE HAc-HCl ANTHOCYANIN COMPONENTS OF THE BASAL LEAF SHEATHS AND THE CARYOPSES

HAc-HCl Component	Sugars**
A2	glu. glu.
A3	glu.(20): ara (1): xyl (1) glu
A4	glu glu
A5	glu: ara (t): xyl (t)
B4	glu (5): ara (1) glu
DE2	Ul: glu glu
DE3	glu glu
DE5	glu: ara (wk)*
DE6	glu (1): ara (1): xyl (t)*
DE7	glu: ara (t): xyl (t) glu
DE8	glu (20): ara (1): xyl (1) glu (10): xyl (1)
DE9	glu glu
G3	glu (10): ara (1): xyl (t) glu
H2	glu glu
H4	glu glu

TABLE 19.VI continued

HAc-HCl Component	Sugars**
5B	glu (30): ara (5): xyl (2) glu
5C	glu (20): ara (3): xyl (3) glu U2: glu*
5E	glu (20): ara (4): xyl (2)
5F	glu (10): ara (2): xyl (1) glu
IB5FG	glu (1): ara (1)*
5G	glu (20): ara (5): xyl (4): U5 (BAW only) glu (5): ara (1): xyl (1)
IB5GH	glu (10): ara (3): xyl (2) glu (5): ara (1): xyl (1) glu (1): ara (1)*
5H	U3: U4: U5(BAW): gal (1): glu (15): ara (3): xyl (2) U3: U4: U5(BAW): glu (10):ara (3) xyl (3)
IB5KH	glu (20): ara (8): xyl (5): U5 (BAW) glu (10): ara (5): xyl (4)
5K	U3: U4: U5: U6: gal (2): glu (20): ara (8): xyl (5)
5L	U5: U6: gal (5): glu (20): ara (10): xyl (5)
5f1	glu-gal (30): ara (3): xyl (3)
6B	glu: gal (t): ara (t): xyl (t) glu
6C	glu
6D	glu
6E	glu: ara (t) glu

TABLE 19.VI continued

HAc-HCl Component	Sugars**
7F	glu: ara (t)
7H	glu
7J	glu (30): ara (1): xyl (2) glu
8-9C	glucose
8DE	gal (1): glu (10): xyl (1)
8-9DE	glucose
8FGH	gal (1): glu (20): ara (2): xyl (1) glu (10): xyl (1)
8-9K	gal (1): glu (20): ara (1): xyl (1) glu

First row: original results
Second row: results after correcting for blank values (see text)
* results obtained by nylon treatment
U = unknown
Pyr = pyridine solvent
gal = galactose
glu = glucose
ara = arabinose
xyl = xylose
t = trace amounts
U1 = R_g0.34 (Pyr).
U2 = R_g 0.51 (Pyr).
U3 = R_g 0.06 (Pyr): pink fluorescence
U4 = R_g 0.25 (Pyr), 0.47 (BAW): dull yellow (UV): R_g values identical with gentio biose
U5 = R_g 1.52 (BAW): bluish green fluorescence
U6 = R_g 0.54 (BAW), 0.30 (Pyr): red fluorescence
** figures in parentheses indicate relative concentration ratios of the different components on the same sheet. The ratios were estimated visually.

and anthocyanins, redetermination of the glycosidic components of anthocyanins by the nylon method (cf., Sec. 18.1.3) could not be undertaken.

To secure a reasonable measure of confidence in the glycosidic analysis of anthocyanin eluates, the anthocyanin sugars were rechromatographed along with the sugar blanks obtained from the corresponding R_f regions (Sec. 18.1.1). As far as possible, the sugar blanks were prepared from the same number and same size of the HAc-HCl developed blank paper bands as were used for the elution of anthocyanins. The relative concentration ratios of the sugars obtained from the sugar blanks and those obtained from the anthocyanin hydrolysates were carefully compared. Although the comparison was not fully quantitative, by subtracting the blank ratios from the ratios of anthocyanin sugars, it could be determined whether the sugars like arabinose, and xylose were present as probable anthocyanin components or as mere artefacts derived from the paper. In Table 19.VI, the anthocyanin sugars are generally given in two rows and occasionally in three rows. The results given in the first row are the actual sugars that were obtained upon hydrolysis of the anthocyanin eluate. The results in the second row are those obtained after subtracting the blank ratios. The results marked with an asterisk, sometimes given in the third row, are the ones obtained by the nylon treatment. Because a densitometer was not available, only an estimate of the relative concentration ratios of sugars is stated in parenthesis in Table 19.VI.

It was pointed out elsewhere (Secs. 16 and 18) that a large quantity of sugar artefacts from the paper concentrate at the yellow endings i.e., the yellow terminuses. The paper blanks from which the yellow ends are snipped off show only small amounts of glucose and

arabinose (Fig. 18.1). Inasmuch as the yellow ends of the HAc-HCl anthocyanin components included in Tables 19.II, 19.III and 19.IV had been snipped off prior to elution (Sec. 16), the bulk of sugar artefacts were thus removed. In particular, the yellow ends of the slow-moving anthocyanins (HAc-HCl R_f below 0.5) were snipped off at least two to three times, because the anthocyanins reach the terminuses slowly in relation to the 'yellow' and the sugars (cf., Sec. 16). The eluates from slow-moving anthocyanins, therefore, must contain minimum of sugar artefacts. However, the snipping of the yellow terminuses became difficult in the case of fast-moving anthocyanins, which resolve above R_f 0.65 in the HAc-HCl solvent (see Sec. 16). The difficulty arises because the 'yellows' and the fast-moving anthocyanins concentrate at the paper ends at about the same time. At the time the anthocyanin eluates were prepared, the author was not aware of the presence of sugars artefacts at the yellow terminuses. Therefore, in several cases, the yellow ends were snipped off only once. The R_f values in the HAc-HCl solvent suggest that the fast-moving anthocyanins must be polyglycosylated. Moreover, the majority of the fast moving anthocyanins show the presence of sugars, such as, galactose, glucose, arabinose and xylose. These sugars also arise as artefacts from the paper, a fact which casts doubt if all of these sugars, in the observed quantities, exist as components of the anthocyanins. However, the presence of glucose, arabinose and xylose in some hydrolysates of the nylon purified anthocyanins is definite and is reliable evidence that these sugars may be the components of some anthocyanins.

It is amply clear from the results of the anthocyanin 5G-5H that the concentration ratios of sugars included in Table 19.VI are not

dependable. For example, the concentration ratio of glucose: arabinose: xylose without the nylon purification of the anthocyanin 5G-5H was 10:3:2. After nylon purification, xylose disappeared and the ratio of glucose: arabinose fell to 1:1. Thus, glucose is the major artefact remaining after snipping the yellow ends. This is further supported by the results of sugar blanks shown elsewhere (Fig. 18.1). The results, however, clearly indicate that arabinose is a definite component of the anthocyanin.

The BAW anthocyanin band 5 (caryopses) and the band DE (basal leaf sheaths) are similar and give a large number of components in HAc-HCl. The concentration ratios of the sugars obtained from the HAc-HCl components of the two BAW bands, however, do not tally. An observation, which further indicates the undependability of the glycosidic analysis reported in Table 19.VI. Thus the identification of most of the anthocyanins included in all Tables numbered 19.VIII must await further precision in the glycosidic analysis by the nylon method (see Sec. 18.1.3). On the basis of the results obtained by the nylon method, it appears that barley may possess anthocyanin series consisting largely of gluco-arabinosides in contrast to the gluco-rhamnosides discovered by Harborne (1960) in potatoes.

19.3.6. Partial Hydrolysis of Anthocyanins: Basal Leaf Sheaths and Caryopses

The results of partial hydrolysis of a few anthocyanins from the basal leaf sheaths and the caryopses are included in Table 19.VII. The identification of the products of partial hydrolyses, mainly, anthocyanins and anthocyanidins, is based on the R_f values and spectral data and is summarized elsewhere in Tables 19.V. and all Tables numbered 19.VIII.

TABLE 19,VII
PARTIAL HYDROLYSIS OF THE ANTHOCYANINS

HAc-HCl Component	R_f x 100				Identification[c]
	HAc-HCl		3:1:8		
	5'	20'	5'	20'	
A2	10	11	15	16	Cyanidin
	28	26	39	37	Cyanidin-3-glucoside
A3		11			Cyanidin
		26			Cyanidin-3-glucoside
B2	9	10	14	15	Cyanidin
	25	-	34		Cyanidin-3-glucoside
DE2		6			Delphinidin (1)
		10			Cyanidin (5)
		18			Delphinidin-3-glucoside[a]
		24			Cyanidin-3-glucoside[a]
DE3	10	12	14	16	Cyanidin
	27	26	37	wk	cyanidin-3-glucoside
DE7	9	9	13	15	Cyanidin*
	24	24	36	37	Cyanidin-3-glucoside[a]
	58		58?		
DE8		10			Cyanidin
		25			Cyanidin-3-glucoside
G3	11				Cyanidin
	26				Cyanidin-3-glucoside
H2		10			Cyanidin or Petunidin[b]
H4			12		Cyanidin
			26		Cyanidin-3-glucoside
5B		11			Cyanidin
		26			Cyanidin-3-glucoside
5C	10	10	16	16	Cyanidin
	27	26	37	37	Cyanidin-3-glucoside
5D	10	11	16	16	Cyanidin
	27	24	37	wk	Cyanidin-3-glucoside
5E	10	12	15		Cyanidin
	24	26	37		Cyanidin-3-glucoside
	33	wk	46		Peonidin-3-glucoside[a]

TABLE 19.VII continued

HAc-HCl Component	R_f x 100				Identification[c]
	HAc-HCl		3:1:8		
	5'	20'	5'	20'	
5F	10	11	15	16	Cyanidin
	27	26	39		Cyanidin-3-glucoside
	40	wk	53		Probably a 3,5-diglycoside
5G	9	10	14	16	Cyanidin
	26	24	37	38	Cyanidin-3-glucoside
	48		59		Cyanidin polyglycoside
5H	8	9	14	15	Cyanidin
	26	25	38	38	Cyanidin-3-glucoside
	48?				
	54		69		Cyanidin polyglycoside
5K	9		14		Cyanidin
	19		29		??
	26		36		Cyanidin-3-glucoside
5f1		11			Cyanidin
		21			???
		26			Cyanidin-3-glucoside
5G-5H	9	10	13	14	Cyanidin?
	26	24	37	39	
	52		66		
5K-5H		10			Cyanidin
		24			Cyanidin-3-glucoside[a]
6B		11			Cyanidin
		24			Cyanidin-3-glucoside
6D		12		18	Peonidin
		35		43	Peonidin-3-glucoside
7F		10			Cyanidin
7H		17		22	Pelargonidin
		37		47	Pelargonidin-3-glucoside
7J		11			Cyanidin (1)
		17			Pelargonidin (4)

TABLE 19.VII continued

c = the figures in parenthesis are estimates of the relative concentration ratios
* = see footnote for anthocyanidin DE7 in Table 19.V.
a = provisional identification
b = see Table 19.V.

It was observed that most of the anthocyanins even after 5 minutes of partial hydrolysis gave only the corresponding aglycone and its 3-glycoside, when, in fact, some of the anthocyanins, which are highly complex, were expected to yield several products of partial hydrolyses. For example, the anthocyanin 5D, on the basis of partial hydrolyses and comparability of R_f values in the five anthocyanin solvents, was initially identified as cyanidin-3-glucoside. It was however established later (Sec. 19.3.7) that the identification was erroneous because upon chromatography of the stored sample after 3 years (See Fig. 15.3), the anthocyanin 5D yielded almost all the components obtained from the desiccated anthocyanin isolate No. 5 (Fig. 19.5). Similarly, anthocyanin DE4 (Sec. 19.3.7), and several other anthocyanins (compare Tables 19.VII, and 19.VIII), upon partial hydrolysis for only five minutes, gave fewer components than were obtained following extended storage (see Fig. 15.3)* or after mild conditions of elution. These results suggest that the conditions of partial hydrolysis, employed in this investigation, which were found satisfactory for potato anthocyanins (Harborne, 1960), were too 'drastic' for barley anthocyanins. The partial hydrolysis, doubtlessly, should have been carried out under very mild conditions,

*The anthocyanin E3 shown in Fig. 15.3 is the same as the HAc-HCl component designated as DE4 in this section.

probably, comparable or even milder than those used by Abe and Hayashi (1956).

The anthocyanins of barley it may be recalled from earlier sections, are highly labile: they split into several components even upon rechromatography without elution (Sec 13) and following elution under very mild conditions (Table 19.I). The hydrolytic breakdown was very marked upon desiccation over phosphorus pentoxide (Sec. 19.3.1 and 19.3.2). Desiccation over phosphorus pentoxide as a drying procedure proved to be unsatisfactory because it resulted in partial hydrolysis of the anthocyanins. However, this misfortune became good fortune when it was seen that phosphorus pentoxide hydrolysis was less 'drastic' than the hydrolysis effected by the Harborne procedure for partial hydrolysis. Although the phosphorus pentoxide was considered as an undesirable drying agent, it proved to be an excellent mild hydrolytic agent and the chromatography of the 'HAc-HCl components', partially hydrolyzed in drying, yielded a great deal of information on the nature of units entering into the complex anthocyanin molecules *in vivo*. This information is presented in the next section.

19.3.7. Chromatographic Characterization of Anthocyanins

19.3.7.1 General Comments

The chromatographic results from the HAc-HCl components of the basal leaf sheath anthocyanins A to H, and caryopses anthocyanins 1-2, and 4 to 9 in the five anthocyanin solvents (see Table 10.II) are summarized in Tables 19.VIII.A to 19.VIII.H and 19.VIII.1 to 19.VIII.9. The leaf sheath anthocyanins K and L, and caryopsis anthocyanins, 1, 2, and 3 were available in such small amounts that they could not be purified

in the HAc-HCl solvent. The BAW eluates of these anthocyanins, after the usual purification in the anthocyanin concentrant (Sec. 16) were chromatographed directly in the five solvents. The results are summarized in Tables 19.VIII.K, 19.VIII.L, and 19.VIII.1.2.3.

It was indicated early that the HAc-HCl components represented the products of partial hydrolysis, because the BAW eluates unless otherwise stated were desiccated over phosphorus pentoxide. In order, therefore, to understand the complexity of each BAW anthocyanin band, even the weakest of the HAc-HCl components, whenever practicable, were eluted. Sometimes, weak components were present as unresolved trails between two well resolved bands. Such components were also eluted, and are designated with a hyphen between the names of the two components between which the unresolved or trailing component was present. For example, the designation A1-A2 means the trailing or unresolved component between the HAc-HCl components A1 and A2. The system of designating anthocyanins is that described elsewhere in Section 11.2.3. Briefly the anthocyanins of basal leaf sheaths are designated with latters of English alphabet and those of caryopses with Arabic numerals.

The results shown in Tables 19.VIII.A to 19.VIII.H and 19.VIII.1 to 19.VIII.9, clearly indicate that most of the HAc-HCl components split again into several sub-components. It is notable that none of the eluates showed their aglycones primarily because of the mildness of the purification and elution techniques (Sec. 16). The HAc-HCl components, which did not split, or which split only into two to three sub-components did not cause any difficulty in determining their R_f values in the different solvent system. In general, corresponding R_f values were determined on the basis of the relative concentration ratios and colors

in ultraviolet light. The determination of the corresponding R_f values was particularly difficult when the anthocyanins were either labile or showed extensive trailing or when two or more sub-components resolved as a single spot or a trail. In several cases, as shown in the tables, trails were differentiated by ultraviolet light. It will be observed from perusal of Table 19.V. that BAW anthocyanin bands B,F,G and H, and bands 4, 6, 7 and 8 are mixtures of anthocyanins derived from different combinations of aglycones such as pelargonidin and cyanidin, delphinidin and cyanidin, or peonidin and cyanidin. Although it was difficult to differentiate cyanidin from peonidin derivatives on the basis of visible colors, the differentiation of the R_f value of cyanidin from those of pelargonidin, or those of cyanidin from those of delphinidin derivatives was greatly aided by the characteristic visible colors of these anthocyanins.

It is appropriate to add a few words about the colors of anthocyanins. It is clear from Table 19.V that most of the anthocyanins of barley are derived from cyanidin. The cyanidin and cyanidin derivatives, in general, give bluish red (magenta: Harborne, 1959a) colors in visible light. It was, however, noted that the visible colors of cyanidin derivatives differed from one another. The kind of differences involved will be illustrated from the colors of the HAc-HCl components of the BAW band A noted in Table 19.II. All the components are cyanidin derivatives (Table 19.V), and their identification is detailed in Table 19.VIII.A. The component A1 is cyanidin and its color is bluish red (Table 19.II). The component A2, a monoglycoside of cyanidin is bluish red, but distinctly redder than the bluish red color of cyanidin. The complex component A3, the parent of the component A2, is in turn bluish red but is otherwise

distinctly redder than the A2 bluish red. The component A4, which is also a polyglycoside of cyanidin, in turn is redder than the component A3. Only when all the components are on one sheet that the differences in shades are easily discernible by human eye. The designation of the differences in the shades or tones of each component by comparison with Standard Color Charts is not very fruitful. Although the cyanidin components, in general, are bluish red, some of the bluish red shades could hardly be differentiated from the pinkish shades of the peonidin components. The designation of anthocyanin colors is trying, particularly when dealing with a large number of spots.

In addition to the difficulties of differentiating and designating the shades of anthocyanin colors dependably, the differentiation of colors in this particular investigation was complicated by color changes occurring in storage. It was shown in Section 15 that the dull bluish purple (UV) color of the anthocyanin E3-138 changed to brighter magenta (UV) color upon storage. The change occurred due to the _in situ_ partial hydrolysis of the anthocyanin (dull in UV) to anthocyanidin (fluorescent in UV). It was also shown that the fluorescence of anthocyanidins becomes duller upon storage. The fluorescence, dullness and brightness of anthocyanins in ultraviolet light, however, provide characteristic information as to the state of glycosidation of an anthocyanin molecule (Hayashi and Abe, 1952; Harborne, 1959a). The realization that colors of anthocyanins could have undergone modifications during storage came regretably late in the study. Although the chromatography of anthocyanins included in all Tables numbered 19.VIII was completed in 1962, a critical evaluation of the diagnostic features of anthocyanin colors in visible and ultraviolet light was undertaken only in 1965. Thus in

light of the above observations, the colors determined in 1965 could not be relied upon. Accordingly, only such anthocyanin color differentiations, which seem dependable and useful in characterization are included in all Tables number 19.VIII.

Cyanidin-3-glucoside, and pelargonidin-3-glucoside were the only available synthetic anthocyanins, which were cochromatographed as controls, on every sheet in all the five solvents.

It was shown in Section 19.3.5 that glucose, arabinose, xylose, and in some cases galactose were the principal simple sugar components of several anthocyanins included in all Tables numbered 19.VIII. The belated realization that the same principal sugars, namely, galactose, glucose, arabinose and xylose could have been eluted along with anthocyanins as artefacts from chromatography paper (see Sec. 18) has made the characterization of the glycosidic component of each anthocyanin uncertain. Nonetheless on the basis of chromatographic data included in Tables 19.V, and 19.VII and 19.VIII, a good deal of valuable information on the complexity and novelty of barley anthocyanins has been obtained and is presented next. It will be noted from the Tables 19.VIII that, of the five anthocyanin solvents, the first four are the solvents used by Harborne (1959a) and fifth solvent is that used by Hayashi (1957). These solvents were selected because chromatographic data of over 55 natural anthocyanins is available in these solvents.

19.3.7.2. Characterization of Basal Leaf Sheath Anthocyanins

BAW Band A-400s: HAc-HCl Components

BAW band A, when it was chromatographed immediately after elution (without drying or desiccation), gave only two components and no

aglycone (Table 19.I). However, following the partial hydrolysis over phosphorus pentoxide, it split into 5 discrete anthocyanin components A1, A2, A3, A4, A5, and two trails A1-A2, and A2-A3 during purification in the HAc-HCl solvent (Table 19.II). The HAc-HCl components A, and A3, which are the major anthocyanin components (A1, is also a major component but it is cyanidin: see Table 19.V) are, in all probability, the two components (Table 19.I), which were obtained prior to the desiccation of the eluate. The BAW band A, and four of its HAc-HCl components are derivatives of cyanidin (Table 19.V). The major anthocyanin components A2 and A3, each yielded cyanidin-3-glucoside on partial hydrolysis (Table 19.VII). With the above information regarding the band A consolidated from different Tables, we shall now consider the chromatographic results of the band A included in Table 19.VIII.A. A list of footnotes for Tables 19.VIII.A to 19.VIII.K.L and 19.VIII.1 to 19.VIII.9 is shown on page 397.

The component A2 upon chromatography in the five solvents did not split, and gave R_f values identical to cyanidin-3-glucoside. The component A3, even under the mild conditions of elution (Sec. 16), and also under chromatography without elution (see Figs. 13.4, and 13.7), splits into two sub-components i.e., the major component A3 and the minor component A2, the cyanidin-3-glucoside (see the estimated relative concentration ratios of the two components in Table 19.VIII.A and Table 19.II). Inasmuch as the splitting of the component A3 was noted consistently, it is clear that the component A3 consists of cyanidin-3-glucoside and an unknown substituent(s), which is highly labile. None of anthocyanin components included in Table 19.VIII.A showed hydrolytic breakdown to the corresponding aglycones, particularly in the Aq-HCl

TABLE 19.VIII.A
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND A OF GOPAL AND BLACK HULLESS LEAF SHEATHS

HAc-HCl component	R_f x 100					Rel. Conc.	Components or sub component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
A1	F	68	2.26	11	14		A1	Cyanidin
A2	32	23	8	27	37		A2	Cyanidin-3-glucoside
A3	20	5	25	56	63	(5)	A3	Cyanidin-3-gluco-(?)-glycoside. A new cyanidin derivative
	31	22	7	25	39	(1)	A2	Cyanidin-3-glucoside
A4	10-15	7*	46*	68	75	(3)	A4	A new Cyanidin-3-glucosidic derivative
	T*	12	T*	T*	T*		A2-A3	?
	21	5	25	56	64	(2)	A3	Cyanidin-3-gluco-(?) glycoside
	32	25	7	26	39	(1)	A2	Cyanidin-3-glucoside
A5cd		7	50	76	80		A5	A new anthocyanin: highly glycosylated & very labile
		T*	T*	T*	T*			
A1-A2	32	24	8	26	38	(2)	A2	Cyanidin-3-glycoside
	T	29	4	19	30	(1)	A1-A2	New bluish Purple anthocyanin: cf., H2 & DE2b
A2-A3	21	5	26	55	64	(t)	A3	Trail of A3
	28	13	16*	40	52		A2-A3	New anthocyanin: probably a 3,5-diglycoside cf., DE6a, 5Fi
	33	22	7	25	41	(t)	A2	Same as A2; arising as a result of splitting of component A3

FOOTNOTES FOR TABLE 19.VIII.A TO 19.VIII.K.L

AND 19.VIII.1.2.3 TO 19.VIII.8.9

P-3-g = Pelargonidin-3-glucoside

r = rose

t = traces

T* = resolves as a long trail which makes for the difficulties of determining the number and the exact R_f values of the component anthocyanins

* = the R_f values are not clearcut because of trailing or resolution of two components around the same R_f region

b = difficult to determine the corresponding R_f values of the component(s) in the different solvent.

c = the component was highly labile

d = Isolate was available in small quantity. Therefore no further characterization could be undertaken

e = R_f values are arranged in ascending order in each solvent because of the difficulties outlined in b above.

f = the component was desiccated over phosphorus pentoxide prior to chromatography

g = pure component: was eluted without contaminants

h = in other chromatograms, the following R_f values of the component 7H and synthetic pelargonidin-3-glucoside were observed on the same sheet.

	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8
7J	41	50, 44, 42	15	39	49, 47
P-3-g	40	47, 40, 38	12	37	47, 44

j = R_f values determined in "RSCO" Two-way chromato-cabinet at room temperature on Whatman paper No. 3.

solvent in which they are detected readily. The absence of aglycones is principally due to the mild conditions of the purification and elution techniques (Sec. 16). The aglycones were, however, present when the

the usual techniques of elution were used (see Sec. 11.3. Fig. 11.1).

The HAc-HCl component A4, although discrete is otherwise a minor component (see Table 19.II). The component splits into three discrete, or probably four sub-components in the five solvents. It is interesting that one of the discrete sub-components is cyanidin-3-glucoside (A2), the second discrete sub-component is A3 and the third, the major component is A4 itself. In addition it gives a trail probably similar to the component A2-A3. It is thus clear that the anthocyanin A4 consists of the anthocyanin A3, A2, A2-A3 and an unknown labile component. It is also clear that the basic structural unit of the component A4 is cyanidin-3-glucoside as was also the case with the component A3.

The fastest moving HAc-HCl component A5, otherwise a very minor component (see Table 19.II), is extremely labile and shows continuous trailing in the five solvents. It could not be isolated in sufficient quantity even for the determination of R_f values. It may be noted that it gives very high R_f values in the aqueous solvents and very low values in the organic solvents: this suggests that component A5 is highly glycosylated.

The trail between the components A1-A2 yields two discrete anthocyanin components, one of which, as expected, is the trail of A2. The second component, designated as A1-A2, however, is distinctly bluish purple and differs from the visible and ultraviolet colors of all other components of the band A. The R_f values of the component A1-A2 are comparable to the components DE2 and H2 but they are not comparable in the five solvents with any of the known anthocyanins. The anthocyanin A1-A2 is, therefore, a new anthocyanin. Owing to its small quantity, no further characterization data is available. The detection of a new anthocyanin

component from the weak trailing region emphasises the necessity of eluting even the weakest components which are present on a chromatogram and which, generally, are discarded (for example: see Harborne, 1960).

The trail between the components A2-A3 yields, as expected, traces of the components A2 and A3 and, also, a distinct component A2-A3, which may (see R_f values in Bu-HCl) or may not be a component of the anthocyanins A4.

The results presented above would indicated that the BAW band A is a complex anthocyanin, which splits into several anthocyanin components, and is not a simple mixture of different anthocyanins. If A is a simple mixture, the R_f values of the HAc-HCl components upon rechromatography in the BAW solvent would have resolved around 0.15, the usual R_f region of the BAW band A. On the contrary, the R_f values of the different components vary from 0.10 to 0.33 in the BAW solvent. A similar variation was encountered when the BAW band A, after desiccation over phosphorus pentoxide was rechromatographed in the BAW solvent. Thus, the appearance of several discrete anthocyanins and also the corresponding anthocyanidin must be due to partial hydrolysis of the complex anthocyanin A. The possibility that the BAW band A may be contaminated with components of other anthocyanins will be considered next.

It was observed that the component A2 (cyanidin-3-glucoside) or other simple anthocyanins (see later) did not split. However, the components, such as A3, A4 and several others to be described later, split into two to four components. The splitting of anthocyanins may occur for two reasons. First, the studies on the potato anthocyanins (Dodds and Long, 1955) have shown that acylated anthocyanins, which usually, as a rule, are polyglycosylated, always split into two components in the Bu-HCl

solvent, the main component with a higher R_f value and the minor component with a lower R_f value (Harborne, 1959a). The deacylation occurs because of the ease of hydrolysis of the ester linkage. It is known (see also Sec. 5.3) that the R_f values of the highly glycosylated anthocyanins are exceptionally low in the Bu-HCl solvent. But owing to the greater solubility of the acyl group in the organic phase of the solvent, which is preponderant in the higher R_f regions (see Sec. 5.3), the R_f values of the acylated (polyglycosylated) anthocyanins increase in the Bu-HCl solvent. Because acylated anthocyanins split only into two components, it is clear that the glycosyl groups are not hydrolysed with the usual eluents. The usual eluents contain 1% HCl as compared to 0.03% HCl used in our eluent. The acid in the eluent was further diluted because of the addition of methanolic washings (Sec. 19.2.2). Moreover, with our techniques, the anthocyanins are eluted with only a few drops of the solvent (Sec. 16.3). Inasmuch as the HAc-HCl eluates were not dried, the mild acid concentration of our eluent, theoretically, cannot hydrolyse the acetal type linkage of the glycosides. In practice, also, no aglycones were detected on chromatograms, in any case, when the HAc-HCl eluates were not dried. Thus, the production of several components, for instance up to four components when the eluates of A4 were chromatographed, remains enigmatic. Although the data is insufficient to warrant speculation, it is clear that the anthocyanins of barley are complex and hydrolyse even in weak acid into several components.

The splitting of anthocyanins, occurring under the mild acid concentration of our eluent, leads summarily to the conclusion that similar splitting of anthocyanins must have occurred in the preparation of the original anthocyanin extracts. That this, in fact, is the case, is brought

forth by the following observations. It was observed that when the eluates of the BAW band A were rechromatographed in BAW, they usually split into 3 to 4 components. Moreover, the R_f values of all but one components were always higher than the R_f values of the original BAW band A. Consistent with these observations, it will be noted that all anthocyanins of the band A resolve, at least into four R_f value regions, namely, 0.10-0.15, 0.21, 0.28, and 0.33, in the BAW solvent. Thus, the BAW band A, as a result of splitting, during the preparation or storage of extracts, would have given rise to at least four anthocyanin components in the preparatory chromatography in the BAW solvent. The component at R_f 0.21 is approximately the R_f value region* of the BAW band B. The components at R_f 0.28, and 0.33 are about the R_f value regions* of the BAW bands D and E. It will, therefore, be necessary to scrutinize carefully the characterization data of the BAW bands B, D, and E to determine if the split contaminants of the BAW band A are present. On the same basis, it is equally plausible that the split components of the other BAW bands may have given rise to some of the components of the BAW band A. It, therefore, stands to reason that at least some of the BAW bands contain contaminants from other anthocyanins. With these considerations in the background the probable nature or the basis of the provisional identifications of the HAc-HCl components of the band A (Table 19.VIII.A) will now be scrutinized.

It is clear from the results presented already that the component A3 and A4, because they give rise to the component A2, the cyanidin-3-glucoside, must be the derivatives of cyanidin-3-glucoside. The component A3 splits into two components, a minor A2, and a major A3. Inasmuch as it

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*It is well known that the R_f values of anthocyanins, prior to and following purification vary widely (Harborne, 1959a).

splits into two components, the component A3 may be acylated on the basis of known behaviour of acylated anthocyanins (Dodds and Long, loc. cit; Harborne, loc. cit.). The probability that the component A3 may be acylated is strengthened further from two observations: (a) that the anthocyanin band A splits into only two components prior to drying and desiccation (Table 19.I). The major (of two) components gave R_f values equivalent to A3; (b) because A3 was the major component prior to desiccation and drying (Table 19.I) and because the component A4 appeared only following drying and desiccation, it seemed that A4 would have arisen as a result of deacylation of A3. This statement seems to be a consistent one because acylated anthocyanins are polyglycosylated, and because deacylated polyglycosyl components give higher Rf values than the corresponding acylated components in the aqueous solvents. However, a careful comparison of the published R_f values of acylated anthocyanins (cf., Harborne, 1959a) revealed that acylated anthocyanins, when hydrochloric acid concentration of the aqueous solvents, used in chromatography, is higher than 1%, give distinctly higher R_f values than their corresponding deacylated components. Again, the component A3 cannot be acylated because of its very low R_f value (0.05) in the Bu-HCl solvent. In Bu-HCl, only polyglycosides give such low R_f values; the acylated anthocyanins, because of the greater solubility of the usual acyl substituents, give R_f values above 0.20. The component A3 has therefore been identified provisionally as a new cyanidin-3-gluco-(?)-glycosidic derivative. Support for the newness of anthocyanin A3 is found in the R_f values which do not match any one of over 50 known anthocyanins in the five solvents.

The possibility that the component A4 is acylated is ruled out on the basis of several considerations, the principal among them

being that the known acylated cyanidin derivatives do not give such high R_f values (0.46) in the Aq-HCl, or low R_f values (0.07) in the Bu-HCl solvents. In the Aq-HCl solvent, on the basis of their published R_f values (Harborne, loc. cit.), the acylated anthocyanins give significantly lower R_f values than their corresponding deacylated polyglycosides. The situation in the HAc-HCl solvent is reversed. In HAc-HCl the acylated anthocyanins give, in general, slightly higher R_f values. Insofar as the R_f values of the component A4 do not compare with any of the known anthocyanins in the five solvents, the component A4 is also a new cyanidin-3-gluco-(?)-polyglycoside.

Because of the insufficient data of the anthocyanin A5, it is not clear if it is a component of the BAW band A. Most of the fast-moving HAc-HCl components of other anthocyanins also give similar R_f values (see later). In any event, the R_f values in the five solvents again clearly indicate that the highly labile component A5 is also a new anthocyanin. The R_f values are unusually high and compare well with the unusual glycoside, pelargonidin-3-diglucosido-7 (or 4)-glucoside, which contain sugars attached in unusual positions, either 3,7- or (3,4). Although the component A5 is not a pelargonidin derivative, the above comparability of R_f values indicates that sugars in this anthocyanin may be attached at unusual positions.

The bluish purple anthocyanin A1-A2 again is a new anthocyanin because the R_f values do not match with any of the known anthocyanins. The R_f values are identical to the anthocyanin component DE2 (Table 19.VIII.DE; also see the R_f values of the component H2, Table 19.VIII.H). It is, therefore, likely present as a contaminant. Nonetheless, it is a new anthocyanin.

The component A2-A3 (HAc-HCl, R_f 0.40) gives R_f values in all other solvents, except Bu-HCl, equivalent to cyanidin-3,5-diglucoside. However, the R_f values in Bu-HCl are significantly higher. The anthocyanin, therefore, is designated provisionally as a new anthocyanin, probably a 3,5-diglycoside. The component A2-A3 is identical to the components DE6a (Table 19.VIII.DE), 5Fi (Table 19.VIII.FG) (the BAW bands DE and 5 are identical). The component A2-A3 cannot be a contaminant from the band DE because its R_f values in BAW (0.28) preclude that possibility. On the contrary, the R_f 0.28 is the BAW region of the band DE. Therefore, it is likely that at least, a portion of the component DE6a may, in reality, be a contaminant from the band A. This possibility will be examined further along with the other components of the band DE. Because both the anthocyanin A and anthocyanins DE are complex cyanidin derivatives, the possibility, the component A2-A3 could have arisen as a product of partial hydrolysis of the anthocyanin A4 cannot yet be ruled out.

BAW Band B-400s: HAc-HCl Components

The BAW band B upon hydrolysis gave a mixture of cyanidin and peonidin: cyanidin was the major constituent of the band (Table 19.V). Although the band gave 8 HAc-HCl components (Tables 19.II and 19.VIII.8) the chromatographic data of only 4 components, namely B2, B3, B3-B4, and B4 will be examined. The component B2 yielded cyanidin; the R_f values in different solvents, and the data of partial hydrolysis (Table 19.VII) are consistent with its identification as cyanidin-3-glucoside. The component B3 is largely a peonidin derivative, though traces of cyanidin were also present (Table 19.V). It resolves as a single spot in all the solvents except BAW (Table 19.VIII.B), and has been identified as

TABLE 19.VIII.B
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND "B" OF GOPAL AND BLACK HULLESS LEAF SHEATHS

HAc-HCl Component	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
B1	F	x	2	11	14		B1	Cyanidin
B1-B2	32		6.5		36	(10)	B2	Same as B2
	42				28	(1)	B1-B2	
B2	30	24	7	25	37		B2	Cyanidin-3-glucoside
B3	34	30	9	30	43	(10)	B3	Peonidin-3-glucoside
	28	T				(1)	B2	Contaminant of B2
	38					(wk)		
	22					(t)		
B3-B4	17	6	25	51*	62	(2)	B4	Trail of B4
	23	11	17	43	62	(3)	B3-B4	Peonidin-3,5-diglucoside
	28	24	7	24	38	(1)	B2	Cyanidin-3-glucoside
B4	19	6	26	55	63	(5)	B4	Cyanidin-3-gluco-(?)-glycoside. Same as A3
	22	T		59*	69*	(t)		Trail of B5b--a peonidin derivative
	29	24	7	24	36	(1)	B2	Cyanidin-3-glucoside
B5	12-16	6	42*	69*	72*	(2)	B5	A new highly glycosylated anthocyanin
	22	9	28*	60*	67*	(3)		
	27					(1)		
	33					(wk)		
B6	10	7	48*	74*	79*	(2)		A new highly glycosylated anthocyanin
	16	7				(1)		
	21					(1)		
	27					(wk)		

peonidin-3-glucoside on the basis of the published R_f values. The component must have arisen as an artefact of partial hydrolysis (when the BAW eluates were dried and desiccated), because its R_f value in BAW, 0.34, is significantly higher than the R_f value of the band B, 0.20. in BAW.

On this basis, the component B2, BAW R_f 0.30, must also be a product of partial hydrolysis.

The weak trail between the components B3 and B4, designated as B3-B4 (Table 19.II) gave three discrete anthocyanin spots. Of the three spots, two are present as contaminants from the trail of the band B4, the third spot, designated as B3-B4 proper, has been identified provisionally as peonidin-3, 5-diglucoside on the basis of R_f values, which are identical to those published in the literature (Harborne, loc. cit.).

The component B4, upon hydrolysis, gave largely cyanidin, and only a trace of peonidin. The peonidin component (BAW R_f 0.22) is likely a trail of the sub-component B5b (Table 19.VIII.B) which appears to be a highly glycosylated derivative of peonidin. The component B4 proper gives R_f values identical to the component A3, and like A3, it yields cyanidin-3-glucoside. It is, therefore, clear that all components must have arisen as breakdown products of the components B4, which is the same as A3, a new anthocyanin identified provisionally as cyanidin-3-gluco-(?)-glycoside (see Band A, Table 19.VIII.A).

It is obvious from the results that the cyanidin derivatives of the band B arise as artefacts because of the splitting of the band A. The splitting must have occurred during the preparation and processing of the original extract. It is, therefore, necessary to develop milder methods of extraction and processing, if the in vivo state of anthocyanins is to be investigated for studying biogenesis, successive stages of glycosylation, acylation, anthocyanin ontogeny, and particularly, for studying biochemical responses to gene action.

It is likely that the component B3 (peonidin-3-glucoside) is an artefact: the R_f values in BAW suggest that it may also be found either

in the BAW bands E, or F, provided of course, the partial hydrolysis of the main peonidin derivative of the band B had occurred during the preparation and processing of the extracts. It is difficult to indicate, because of insufficient data, whether peonidin-3, 5-diglucoside (component B3-B4) is the main peonidin component of the BAW band B. The traces of peonidin obtained upon hydrolysis of the component B4 (Table 19.V) indicate that the component B5b(see Table 19.VIII.B) may be a peonidin derivative. Inasmuch as the R_f values of the BAW band B, of the component B3-B4, and of the component B5b are the same, it is not certain which of the two components, B3-B4, or B5b is the main component of the band B. The possibility that the band B, which otherwise was observed consistently in extracts obtained over several years (see (Secs. 5, 7, 8, 10, 11, 13), may entirely be an in vitro artefact will be elaborated upon later.

BAW Band DE-400s: Hac-HCl Components

The band DE is the major anthocyanin band (see preparatory BAW chromatograms, Fig. 19.1). As noted in Sec. 19.3.1, some of the HAc-HCl components, owing to poor resolution, were eluted as a mixture so that only 9 eluates were obtained totally (Table 19.II). The BAW bands D-405, E-405 and the HAc-HCl components, DE1, DE3, DE4, and DE8, upon hydrolysis gave cyanidin (Table 19.V). Although R_f values in some solvents, and also colors resemble closely petunidin, the component DE7 has been identified as cyanidin primarily on the basis of spectral characteristics. The component DE9 could not be identified conclusively: the data was meagre because the pigment was available in small quantity. It may either be petunidin or cyanidin. No suitable chromatographic solvents are yet available that differentiate petunidin and cyanidin

TABLE 19.VIII.DE

CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND DE OF GOPAL AND BLACK HULLESS LEAF SHEATHS

HAc-HCl Component	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
DE1		67	2.5	10	15	(20)	DE1	Cyanidin
			6			(1)		?
DE2		24	6	26	37	(4)	DE2a	Cyanidin-3-glucoside
		27	4	19	29	(5)	DE2b	A delphinidin derivative? same as A1-A2, K3, L3
DE3	32	26	8	27	40		DE4	Trail of DE4
DE4	33	24	9	28	40		DE4	A new complex cyanidin derivative: same as 5D
DE5	40	39		?		(?)	DE5a	See 5E i
	37	28	9	32	49	(3)	DE5b	Prob.contaminant from F4
	33			27	38	(5)	DE2a	Cyanidin-3-glucoside
	28	15*	14	38		(2)	DE6a	Trail of DE6a:same as 5Fi
DE6	?	?	19	48	59	(t)		?
	27	15	16	40	51	(3)	DE6a	A new cyanidin derivative: same as 5Fi, A2-A3
	32	26	8	27	38	(5)	DE2a	Cyanidin-3-glucoside
DE7	21	6	23	53	65	(1)	DE7a	?
	29	20	19*	45	60*	(3)	DE7b	Same as 5Gi
	33	24	65	23	41	(1)	DE2a	Cyanidin-3-glucoside
DE8cd			69*	73			DE8	Highly glycosylated anthocyanin
T*			22*					
			6	23				?
DE9T*cd			55*				DE9	Highly glycosylated anthocyanin

dependably. The components DE5 and DE6 were not available in sufficient amounts for hydrolysis.

The anthocyanin DE2a (see Table 19.VIII.DE) is most probably a cyanidin-3-glucoside, or else it may merely be a trail of the component

DE4 (see later the results of components 5B, 5C, 5D: Table 19.VIII.5). The sub-component DE2b has been identified provisionally as a delphinidin derivative on the basis of partial hydrolysis (Table 19.VII), and R_f values of its hydrolysates in a few other solvents (Table 19.V). Although it was one of the major spots of the component DE2, its concentration in relation to the total anthocyanin content of the band DE (Table 19.II), as well as the total anthocyanins of the basal leaf sheath extracts is, otherwise, negligible. The presence of delphinidin was not detected in the hydrolysates of the BAW band DE or the basal leaf sheath extracts probably because of the negligible amounts of the sub-component DE2b. It will be recalled that the component A1-A2 also gave R_f values and bluish purple colors comparable (almost identical) to the DE2b, but no delphinidin was recovered from the hydrolysis of the BAW band A. Thus, the presence of the component A1-A2 in the band A also remains confounded. Should both the components, namely, A1-A2, and DE2b, be derivatives of delphinidin, it is clear then that delphinidin does not exist as a simple 3-monoglycosidic derivative.

The R_f values of the component DE5 could not be arranged in different solvents with certainty because of poor resolution of the constituent anthocyanins (Table 19.VIII.DE). Although the results of hydrolysis are not available, it would seem on the basis of R_f values, that the constituent designated DE5b is a contaminant from the peonidin band F; its R_f values match peonidin-3-glucoside. It, therefore, is highly probable that the component DE5b is the breakdown product, B3, of the peonidin band B. The other two constituents of the component DE5 are the trails of the component DE6.

The constituent DE6a of the HAc-HCl component DE6, as indicated already, gives R_f values identical to the component A2-A3, and that it is likely an artefact originating from the band A.

The other HAc-HCl components of the band DE are basically the same as the corresponding HAc-HCl components of the BAW band 5. They will be scrutinized along with the components of the band 5 (Table 19.VIII.5).

BAW Band F-400s: HAc-HCl Components

The band F splits into 6 or more HAc-HCl components (Table 19.II). Only the major components F3 and F4 were studied in some detail. The component F3 gave a dull bluish purple color in u.v. (Table 19.VIII.F) and upon hydrolysis yielded cyanidin (Table 19.V). Consistently the component gave R_f values which differed slightly from the R_f values of cyanidin-3-glucoside on the same sheet. The component most likely is a new mono-glycoside of cyanidin (see also G3: Table 19.VIII.G) and is similar to the caryopses components 6B and 7F (Tables 19.VIII.6, and 19.VIII.7). The possibility that it may be a complex glycoside, comparable to or part of the caryopses component 5D, cannot be ruled out.

The component F4, upon hydrolysis yielded peonidin exclusively (Table 19.V) and has been identified as peonidin-3-glucoside on the basis of R_f values. The component is identical to the caryopsis component 6C and probably 6D (Table 19.VIII.6).

Although the BAW band F is a mixture of cyanidin and peonidin derivatives, it gave a bright pink color in u.v. as of course did the BAW band F-130 (Secs. 11, and 13) or BAW band 6 from caryopsis tissues (Table 19.VIII.6). The bright colors are usually given by polyglycosides. Because of the bright color and because the band split into several fast moving components in the HAc-HCl solvent (Table 19.II), it is reasonable to assume that the band F may be a mixture of polyglycosylated derivatives of peonidin and cyanidin.

TABLE 19.VIII.F and 19.VIII.G
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BANDS F AND G OF GOPAL AND BLACK HULLESS LEAF SHEATHS

HAc-HCl Component	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
F1				9				
F2				see H2				
F3	32	27	8	28	34		F3	A new cyanidin derivative See also G3, 6B and 7F
F4	37	34	9.5	33	42		F4	Peonidin-3-glucoside
G2	same as H2							F2, G2 and H2 were mixed
G3	31	28	8	28	35		G3	A new cyanidin derivative: same as F3
G4	33	29	8	28		(6)	G3	A new cyanidin derivative: same as F3
	40	41	12	37		(1)	G4a	Same as H5a
$G5^{bc}$		29	11*		36	wk		
		9	24*		60	wk		Very labile
		41	44*			(t)		

The component F2 was distinctly bluish purple and so were the components G2 and H2 (see Tables 19.VIII.G, and 19.VIII.H). Accordingly, they were mixed because the quantity of each component was too small to be processed separately. The R_f values of the mixture are given in Table 19.VIII.H. A portion of the isolate F2-400 was, however, hydrolysed and chromatographed only in two solvents (see Table 19.V). The results of hydrolysis show that the band F2 may be a mixture of two anthocyanidins

identified provisionally as petunidin (major) and peonidin or malvidin (very minor). However, the data is too meagre for conclusion. Moreover, when an aliquot of the mixture H2 was hydrolysed, no spot around the R_f of peonidin or malvidin was detected (see Table 19.V).

BAW Band G-400s: HAc-HCl Components

The BAW band G splits into several HAc-HCl components (Table 19.II). Most of the components were weak and could not be studied. The major component, G3, is a cyanidin derivative (Table 19.V). The R_f values and color reactions of the component are similar to the components F3, 6B and 7F. It will be noted that the leaf sheath bands F and G (Fig. 19.1) and so also the caryopsis bands 6 and 7 (Fig. 19.3) are distinct bands with discrete R_f values in BAW. However, their HAc-HCl components F3, G3, 6B and 7F, which are otherwise chromatographically identical (Figs. 19.2, and 19.5) give R_f values in BAW about the region of the BAW bands D and E. The conclusion is, therefore, warranted that the cyanidin derivatives of the band F and G (and also 6 and 7) are readily hydrolyzable even under the mild conditions of our elution and that they end up as artefacts in the band DE. Thus, the BAW bands A, F, G and to some extent B, all contribute components that resolve at the R_f values of the bands DE. It is primarily for this reason that, upon storage, the concentration of the band DE increases and that of the other bands decreases (Sec. 7, 23, and 24).

The bluish red HAc-HCl component G4 (HAc-HCl R_f ca. 0.39, Table 19.II) yielded two constituents, a major constituent (HAc-HCl R_f 0.28, Table 19.VIII.G), which was identical with the component G3, and a minor constituent G4a (HAc-HCl R_f 0.37). The results indicate that the component

G4 must be highly labile, because under the mild conditions of our handling, the breakdown products, G3, was the predominant constituent. The predominance of the breakdown product G3 suggests that the minor constituent G4a is the unhydrolysed portion of the original component G4. However, the R_f values of the constituent G4a approach those of pelargonidin-3-glucoside, an intriguing situation. It is intriguing (a) because the basal leaf sheath extracts of the six Greenhouse Collections of Gopal and Black Hulless did not show the orange red pelargonidin derivative in the preparatory chromatography in BAW (Sec. 19.3.1), and (b) because the leaf sheath extracts obtained from the field grown materials (Collections 130 and 138) did show the orange red pelargonidin derivative conspicuously (Sec. 10, 11, 13, and 24). It was further observed in Section 19.3.1 that when the eluates of the BAW band G were chromatographed in HAc-HCl (Fig. 19.2), the Greenhouse Collection 402 (and probably 400) did show relatively small quantities of the orange red component, the presence of which must have been masked by the predominance of cyanidin derivatives of the band G during preparatory chromatography in BAW. Although no orange red band was recovered from the other four greenhouse collections (see Sec. 19.3.1) in the HAc-HCl purification (Table 19.II), the component G4 does show the minor constituent G4a, the nature and color reactions of which could not be ascertained with certainty. It may, however, be noted that constituents K5, H5a, which give R_f values similar to the constituent G4a but which do not give pelargonidin on hydrolysis, were recovered from the bands H and K. The results of the constituents will be scrutinized in the following sections. In the meantime, it may be added that the BAW band H-130 of Gopal leaf sheaths (Table 13.II), and band 8 (Tables 19.V, 19.VIII.8-9) of Black Hulless caryopses do contain some pelargonidin, but the band H-400s (Table

19.V) does not contain pelargonidin.

BAW Band H-400s: HAc-HCl Components

The component H1 has been identified on the basis of R_f values as cyanidin. Although the component as usual, was not hydrolysed because it is an aglycone itself (cf., Table 19.II), it gave another constituent in several solvents which has been designated as a probable product of partial hydrolysis (PPPH) in Table 19.V). It has been designated PPPH, because components with identical R_f values were recovered from the hydrolysates of the caryopses anthocyanins (see later). The constituent, PPPH, shows dull absorption in ultraviolet light and in this particular case may be a contaminant of the anthocyanin components H2 and H3.

The component H2 as indicated already, is a mixture of the anthocyanin components F2, G2, and H2. All the three bluish purple (visible color) components were present in very small amounts and eluted together as a mixture. An aliquot of the mixture was hydrolysed and R_f values determined but only in two solvents. On the basis of this meagre information, the component may either be a cyanidin, or more likely petunidin derivative (Table 19.V). The component H2 splits into three constituents (Table 19. VIII.H). The weak constituent H2a may be a trail from the components H3, G3, or F3. The colors of constituents H2b and H2c are strikingly bluish purple. The constituent H2c shows dull reddish violet color in ultraviolet light. The constituents are more likely derivatives of petunidin than of cyanidin on the basis of their colors.

The component H3 is a lower part of the major component H4 (Table 19.II). The hydrolysates of H3 (see footnotes c, d, and h: Table 19.V) and H4 both showed cyanidin. The component H4 is bright magenta in

TABLE 19.VIII.H
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND H OF GOPAL AND BLACK HULLESS LEAF SHEATHS

HAc-HCl Component	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
H1							H1	Cyanidin (see Table 19.V)
H2	37		7	26	31	(1)	H2a	
	32	29	4.5*	20*	26*	(3)	H2b	Distinct bluish purple components of bands F2, G2 and H2[d]
	T*	T*	3 *	16*	22*	(10)	H2c	
H3	35	28	8	29	36		H4	Trail of H4
H4	36	28	9	30	38		H4	A new cyanidin derivative:
H5	40	40	13	37	45	(1)	H5a	Same as G4a
	36	32	9	34	43	(3)	H5b	Same as F4
	33	28	8	28	36	(7)	H4	Same as H4
H6		18	15	40*		(1)		A new anthocyanin
		28	8	28		(2)	H4	
H7		9			70*	(t)	H7a	Highly glycosylated 'nin.
		49	23*		61	(1)	H7b	A new acylated derivative
		27	20*		55	(3)	H7c	probably of cyanidin. Probably deacylated component of H7b.
			8		37	(5)	H4	Same as H4.
H8			45			wk		
			34			wk		?
			25			wk		
			8			(1)		

ultraviolet light and has been identified as a new derivative of cyanidin. Inasmuch as the BAW band H showed dull absorption, it is clear that the component H4 is a hydrolytic artefact and that the band H is labile (see also Fig. 23.1).

The components H5 to H8 were available in small amounts and therefore could not be hydrolysed, however, results of their chromatography in a few of the anthocyanin solvents are shown in Table 19.VIII.H. Although the data do not permit conclusions, it is clear from the R_f values that the constituent H7b may be an acylated anthocyanin, probably a cyanidin derivative, and the constituent H7c probably a deacylated component of H7b.

It will be noted that the constituent H5a is similar to the constituent G4a and the hydrolytic pattern of the constituent G4 and H5 are also similar

Components of the BAW Band K and L

The fast-moving BAW bands K and L were present only in the Collections 407 and 408. As already indicated (sec. 19.3.1), the desiccated BAW eluates were not purified in the HAc-HCl solvent owing to the scarcity of the materials. The results from the chromatography of the BAW isolates, which as usual were partially hydrolysed following phosphorus pentoxide desiccation are shown in Table 19.VIII.K.L. The R_f values are arranged in ascending order in each solvent, and estimated relative concentration ratio of each component in each solvent is shown in parenthesis to aid determination of the corresponding R_f values in different solvents.

The bluish red BAW band K is a mixture of two anthocyanins because it gave on hydrolysis cyanidin (major) and peonidin (minor) as shown in Table 19.V. The cromatography of anthocyanin in the five solvents (Table 19.VIII.K) shows that the resolution of the components is not uniform in the aqueous and alcoholic solvents. It is noteworthy that the anthocyanin K, which is one of the fast-moving anthocyanins in BAW, almost

TABLES 19.VIII.K and 19.VIII.L
CHROMATOGRAPHIC CHARACTERISTICS OF THE BAW BANDS K AND L FROM BLACK HULLESS LEAF SHEATHS

BAW Band	R_f x 100 (Relative Concentration)†					Component or Sub-component
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8	
K^{bef}	59(t)			55*(wk)	70*(wk)	K6
					57 (wk)	
	39(1)	42(1)	12(1)	36(1)	45 (2)	K5
	31(10)	21(10)	8(10)	28(10)	37 (10)	K4
			5* ?	19(2)	29 (2)	K3
		15(1)				K2
			2.5(1)	10(1)	15 (1)	K1
L^{bef}	50(wk)	37	21*(wk)			L6
	37(1)			30(1)		L5
	31(3)	21(10)	7(3)	25(5)	37(3)	L4
			4.5(1)	20(3)	29(1)	L3
		14(1)				L2
			2.2(2)	9(2)	14(2)	L1

† Figures in parenthesis indicate an estimate of the relative concentration of the components

disappears and the component K4 (R_f value in BAW about the region of the band DE) emerges as the major component. The trace component K6 (BAW R_f 0.59) may or may not be the original anthocyanin K. Although it is difficult to arrange the corresponding R_f values, the component designated K5 appears to be identical with the constituent G4a. The visible color of the component K5 is certainly bluish red and not orange red. The component K3 is similar to the constituent DE2b. The data, however, is too meagre to draw further conclusions.

The BAW band L is, exclusively, a derivative of cyanidin (Table 19.V). Its behaviour upon chromatography in the anthocyanin solvents

(Table 19.VIII.L) is comparable to the band K (see Table 19.VIII.K). This fast-moving anthocyanin is also highly labile and breaks down into major component L4, which gives R_f values similar to cyanidin-3-glucoside and probably similar to the component K4. The second major component L3 is similar to the component K3 and DE2b. Inasmuch as no delphinidin was recovered from the hydrolysis of the banks K, L, A and component DE, the provisional identification of the component DE2b as a delphinidin derivative is doubtful.

Although the data for the bands K and L is meagre, it would, on the basis of their original high R_f values in BAW, seem that the bands must be acylated for reasons discussed in Sections 5 and 7.

19.3.7.3 Characterization of the Caryopsis Anthocyanins

Components of the BAW Bands 1-4

The results of rechromatography of the eluates from BAW bands 1, 2, 3 (see footnote, Sec. 19.3.2, p.363) in four solvents are shown in Table 19.VIII.1.2.3. These eluates were obtained only from those chromatograms which showed good resolution. The results of rechromatography of the HAc-HCl components 1-2/A, 1-2/B, 1-2/D and 3-4/B (see Table 19.III) are summarized in Tables 19.VIII.1-2 and 19.VIII.3-4. The materials available from other components listed in Table 19.III were not available for further studies. However, almost all the HAc-HCl components of the band 4 (see Table 19.III) were chromatographed in several solvents and the results are summarized in Table 19.VIII.4.

It will be seen from Table 19.VIII.1.2.3 that the R_f values of BAW bands 1 and 2 are similar to those of the components A3 and A2 of

TABLE 19.VIII.1,2,3[gj]
CHROMATOGRAPHIC CHARACTERISTICS FROM THE BAW BAND 1, 2, AND 3 OF BLACK HULLESS CARYOPSIS ANTHOCYANINS

BAW Band	R_f x 100				Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl			
1	25	24	7	26	(1)	1A	Similar to A2
	15	12					
	13	7	25	51	(5)	1C	Similar to A3
2	15	7	23	53	(4)	1C	R_f values comparable to the BAW band 1.
	27	11 22	7	27	(1)	1A	
3		20[T*]	4	17	(5)	3A	A dephinidin-monoglycoside
			6	26	(1)	3B	

TABLE 19.VIII.1-2 and 3-4
CHROMATOGRAPHIC CHARACTERISTICS OF A FEW HAc-HCl COMPONENTS FROM THE ANTHOCYANIN MIXTURE 1-2 AND 3-4 OF BLACK HULLESS CARYOPSIS ANTHOCYANINS

HAc-HCl Component	R_f x 100					Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8		
1-2/A			4	18			Contaminant from band 3
1-2/B	31	25	7	26		1-2B	Cyanidin-3-glucoside: same as A2
1-2/D	16	7	25	56		1-2Da	New anthocyanin: R_f similar to A3
	29	23	7	26		1-2B	Same as 1-2B
3-4/B	34	29	8	28	35		Same as F3 and 6B

the BAW band A (Tables 19.I, 19.II and 19.VIII.A). Evidence was presented earlier that the cyanidin portion of the BAW band arose as an artefact, specifically, of the component A3. It would seem that the BAW band 2 originates in a similar fashion, as an artefact of the BAW band 1. This view receives support from the result of the HAc-HCl components of the mixed bands 1-2 (Table 19.VIII.1-2), and from the results summarized in Table 19.III (Sec. 19.3.2). Although the pigments of the bands 1 and 2 were available in quantities insufficient for hydrolysis, the chromatographic evidence suggests that the bands 1 and 2 are identical to the bands A and B, and, therefore, are cyanidin derivatives. It is, however, certain that the anthocyanins of the band 2 did not show any component at R_f values equivalent to those of the peonidin derivatives of the BAW band B.

The bluish red or purple BAW band 3 gave six HAc-HCl components by banding (Table 19.III and Fig. 19.4) but only two components by spotting (Table 19.VIII.1.2.3). The R_f values of the major component 3A (Table 19.VIII.1.2.3) are identical to the components 4A (Table 19.VIII.4), and 3-4/A (Table 19.III). The hydrolysates of the components 3-4A and 4A gave delphinidin (Table 19.V). The component 4A (see Table 19.VIII.4) has been identified as delphindin-3-glucoside. It is therefore clear that the major component 3A also a delphinidin-3-glucoside, and it is highly probable that a few of the other components of the BAW band 3 and also the component 3-4/D (Table 19.III) are polyglygosylated derivatives of delphinidin. The equivalent of the delphindin band was not found in the basal leaf sheaths. The HAc-HCl component 1-2/A (Table 19.VIII.1-2 and 3-4) which also gave R_f values similar to the component 3A (Table 19.VIII.1.2.3) is likely a trail of the BAW band 3.

The magenta component 3-4/B (Table 19.III) is chromatographically (Tables 19.VIII.1-2 and 3-4) identical to the anthocyanins F3 (Table 19. VIII.F), and 6B (Table 19.VIII.6) and is, therefore, a new cyanidin derivative. Inasmuch as the other components of the band on the basis of their colors (Table 19.III) appear to be delphindin derivatives, the component 3-4/B must have arisen as an artefact upon the hydrolysis of the band 6 during storage of the extracts.

It may be added that owing to degradation and several other difficulties, complete data of the components of the bands 3-4 have not been possible to present. However, one significant observation will be added. In early attempts, the anthocyanin eluates of the bands 3-4 were purified by banding in HAc-HCl and again by banding in BAW. Although the details are not available, one of the bluish red HAc-HCl components split into at least three constituents in the BAW solvents. One of the constituents at R_f ca. 0.20 was distinctly orange red, indicating the presence of a pelargonidin derivative.

The BAW band 4 appears to contain contaminants from bands 3 and 5. The band, most probably, is similar to the band D of basal leaf sheaths. In general, the bands 4 and 5 were eluted together. On the basis of hydrolysis (Table 19.V) and R_f values, the component 4A has been identified as delphinidin-3-glucoside. The component 4A may either be present as a contaminant of the band 3 (because of poor resolution) or may have arisen as a result of the usual splitting of the delphinidin band 3, a polyglycosylated derivative. The hydrolysates of the major component 4C, gave cyanidin (Table 19.V). The component has been identified as a cyanidin-3-glucoside on the basis of R_f values (Table 19.VIII.4). The component 4D (Table 19.VIII.4) is probably a trail of the anthocyanin 5D

TABLE 19.VIII.4
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND 4 OF BLACK HULLESS CARYOPSIS ANTHOCYANINS

HAc-HCl Component	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
4A	27	13	4	18			4A	Delphinidin-3-glucoside
4C	31	25	7	26			4C	Cyanidin-3-glucoside
4D	33	25				(2)		Mixture: Poor resolution: probably trail of 5D
	28		9	35		(t)		
	19	15				(2)		
4E	32	25	7	26				
	24	17	16	40			4E	Same as 4F
			13			(wk)		
4F	32	25	7	26		(5)	4C	Cyanidin-3-glucoside
	25	17	16	40		(3)	4Fi	A new cyanidin derivative similar to 5Fi
4H[f]	32	25	8	27	36	(2)	4C or 3-4/B	Cyanidin-3-glucoside or same as F3,6B
	28	T*	21	50	58	(3)	4Hi	same as 5Gi
				18		(1)	4Hii	Some labile derivative of delphinidin may also be present.
			2	10	14	(1)		Cyanidin
4J	very labile--degrades							

(Table 19.VIII.5). The component 4E is the trail of the component 4F. The component 4F gives two distinct constituents, a cyanidin-3-glucoside and a new cyanidin derivative designated as 4Fi. The R_f values of the components are identical to those of anthocyanins 5Fi, DE6a, and A2-A3. Inasmuch as the anthocyanin 4Fi yields cyanidin-3-glucoside, it is reasonable to assume that the basic structure of the new anthocyanin 4Fi is cyanidin-3-glucoside. This view is supported by the chromatographic data of the anthocyanins,

A2-A3, DE6a and 5Fi. It was shown that the anthocyanin DE6a would have arisen as an artefact of the component A2-A3 of the band A. The presence of the components 4Fi and 5Fi further support the view that the BAW band 1 is equivalent to the BAW band A of basal leaf sheaths. The possibility that the component 4Fi may also be a breakdown product of one of the complex cyanidin derivatives of the component 5D (see later) can still be accommodated by the above view. The component 4H (Table 19.VIII.4) is similar to 5G (Table 19.VIII.5) and it is likely that it also may contain a labile derivative of delphinidin as indicated by the HAc-HCl R_f 0.18 of the constituent 4Hii.

BAW Band 5: HAc-HCl Components

The relevant chromatographic data of the HAc-HCl components of the band 5 are summarized in Table 19.IV. The data of hydrolysis, partial hydrolysis, sugar components and R_f values in other solvents are summarized in Tables 19.V, 19.VII, 19.VI, and 19.VIII.5, respectively. The component 5A has been identified as cyanidin. The components 5B and 5C are largely trails of the complex anthocyanin 5D. The component 5B, however, does give though very weak, a component similar to the anthocyanin DE2b.

The anthocyanin 5D (Table 19.VIII.5) is chromatographically identical to the anthocyanin DE4 (Table 19.VIII.DE) and E3-138 (Secs. 11, 13, and 15). The identification work reported in this section was carried out early in 1962. In 1962, as will be noted from Table 19.VIII.5, the anthocyanin 5D gave only one discrete spot with no splitting whatsoever. The R_f values of the spot are comparable to cyanidin-3-glucoside except that they are consistently slightly higher in the aqueous mineral

TABLE 19.VIII.5
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND 5 OF BLACK HULLESS CARYOPSIS ANTHOCYANINS

HAc-HCl Components	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
5A		63	3	11	15	(20)	5A	Cyanidin
			6		31	(1)	5Ai	?
5B	34		8	28	39	(5)	5D	Trail of 5D
	25	26	4	19	28	(1)	5B	Similar to DE2b
5C	34	25	8	28	39		5D	Trail of 5D
5D	33	24	9	29	41		5D	New complex cyanidin derivative
5E	39	40	9	32	47	(5)	5Ei	?
	32	26				(3)		
	28	23	6.5	26	37	(10)	5Eii	Cyanidin-3-glucoside
	21	14	15	40		(1)	5Fi	See 5Fi
5F	21	14	15	38	48	(3)	5Fi	New cyanidin derivative: same as DE6a, A2-A3, 4Fi
	28	23	6.5	25	37	(5)	5Eii	Cyanidin-3-glucoside
			18*		52*	(t)		
5G	30	22	7	26	36	(1)	5Eii	Cyanidin-3-glucoside
	24	18	21	48	58	(2)	5Gi	New cyanidin derivative: deacylated product of 5Hi
	42				65*	(t)	5Hi	Prob. contaminant of 5Hi
5H			26*	T	72*	(t)		?
	41	50	17	54	67	(5)	5Hi	New acylated cyanidin derivative
	24	T*			T*	(1)	5Hii	Traces of 5Gi, 4Hi
	30	22	7	26	37	(2)		Cyanidin-3-glucoside
5K[cd]	42	60*	36*	62*	75	(1)		A new anthocyanin
			19*	?*	?*	(1)		?
	33 T	24	7	24	37	(2)		Cyanidin-3-glucoside
5L[cd]			50	74	80			

acid solvents. The anthocyanin isolate 5D, after the usual chromatography in the five solvents, was dried in a vacuum oven for storage. The isolate turned bluish on drying, an observation to be associated with the addition of methanolic washings (see Sec. 19.2.2), and the weak hydrogen ion concentration of our eluent. Thus, the amount of acid in the eluate at the time of drying was so small as to obviate significant hydrolysis. When the anthocyanin was rechromatographed in the HAc-HCl solvent in 1965, it gave rise to several hydrolytic products. For example, apart from the cyanidin component 5A, and the major component 5D, a pronounced streak extending from the component 5D to R_f around 0.90 was distinct (Fig. 15.3). In other words, several of the components of the band 5 were again recovered. The rechromatography of 1965, therefore, establishes that the anthocyanin 5D is not a simple anthocyanin such as cyanidin-3-glucoside. It is, rather, a complex derivative of cyanidin. It will be recalled that the crystalline anthocyanin E3-138 also gave similar results upon rechromatography in 1965 (see Fig. 15.3).

It will be noted from the results of partial hydrolysis included in Table19.VII, that the anthocyanin 5D shows only two spots, namely, cyanidin and cyanidin-3-glucoside even within five minutes of partial hydrolysis. The anthocyanin is highly labile and the results of partial hydrolysis support the earlier conclusions (Sec. 19.3.6) that the partial hydrolysis of barley anthocyanins should have been carried out under very mild conditions. Even the crystalline samples of barley anthocyanins undergo partial hydrolysis in storage (cf., Sec. 15).

The component 5E is similar to the component DE5 of basal leaf sheaths. The constituent 5Ei is a trail of the peonidin derivative of band 6 and the other two constituents are the trails of the band 5F. The

component 5F, as indicated already, is a new cyanidin derivative similar to the anthocyanins DE6a, A2-A3, and 4Fi.

The distinctly bluish purple components 5G and 5H (Table 19. VIII.5) will be examined in some detail. The equivalent of the component 5G is present in the basal leaf sheaths (e.g., component DE7) but an equivalent of the component 5H is not present in the basal leaf sheaths (Table 19.VIII.DE) probably due to hydrolysis (see later). The component 5G gives rise to two distinct constituents, one of which has been identified as cyanidin-3-glucoside. The other constituent designated as 5Gi is, in fact, the anthocyanin 5G. It is a new cyanidin derivative, the basic structure of which again is cyanidin-3-glucoside.

The anthocyanin constituent 5Hi gives R_f values which are typical of acylated anthocyanins. The component 5H gives another constituent 5Hii, the R_f values of which are distinct only in BAW. For instance, the BAW R_f 0.24 is identical to the BAW R_f of the constituent 5Gi. The Bu-HCl R_f 0.18 of the constituent 5Gi cannot be discerned from the overlapping R_f 0.22 of cyanidin-3-glucoside, the third constituent of the component 5H. In Aq-HCl, the constituent 5Hi shows a weak trail extending from R_f 0.17 to 0.26, which does show increased intensity around R_f 0.21, the Aq-HCl $R_f$of the constituent 5Gi. There is every likelihood that the HAc-HCl and the 3:1:8 solvents also show the presence of extra component at R_f 0.48 and 0.58, respectively, but the presence of the component is masked by strong trails of the constituent 5Hi (See Table 19.VIII.5). On this basis it appears that the constituent 5Hii is equivalent of the constituent 5Gi. The constituent 5Hi has been designated as the deacylated product of the constituent 5Hi for two main reasons: a) the R_f values of an acylated anthocyanin are always higher in butanolic solvents than the R_f values of

deacylated anthocyanins; b) after a careful comparison of the published R_f values of acylated anthocyanins and the polyglycosylated products of their immediate deacylation, it was noted that the R_f values of the acylated anthocyanins, as a rule, were lower than the deacylated components in the Aq-HCl. In the HAc-HClsolvent, the R_f values of acylated anthocyanins were in general, higher or very close to the R_f values of the deacylated components. The increase in the R_f value of acylated anthocyanins in the HAc-HCl solvent is probably due to the increased ionization of acyl components caused by the stronger acid of the HAc-HCl solvent as compared to the Aq-HCl solvent. The R_f values of the constituents 5Hi and 5Gi meet the above requirements. The absence of the constituent from the basal leaf sheaths equivalent to 5Hi may be due to deacylation.

A question arises, how could the constituent 5Hi, which has an R_f value of 0.41 in BAW arise from the anthocyanin band 5. No satisfactory explanation is offered.

The anthocyanin 5K was available in very small amounts. Its R_f values indicate that it is an acylated anthocyanin, but lack of material makes a conclusion unreasonable. It is, it should be noted, a new anthocyanin. The very labile component 5L appears to be a highly glycosylated anthocyanin.

BAW Band 6: HAc-HCl Components

The chromatographic data of the HAc-HCl components from band 6 is summarized in Table 19.IV. The data from hydrolysis, sugar determination, and partial hydrolysis with the appropriate chromatography in the several solvents is summarized in Tables 19.V, 19.VI, 19.VII and 19.VIII.6, respectively. While scrutinizing the nature and the correspondence of the

TABLE 19.VIII.6
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND 6 OF BLACK HULLESS CARYOPSIS ANTHOCYANIN

HAc-HCl Component	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
6B	30	29	8	28	34		6B	A new cyanidin derivative: same as F3, G3, 7F(?), 3-4/B
6C	29	34	9	28*	31	(1)	6B	Contaminant of 6B
	36			33?	40	(5)	6C	Peonidin-3-glucoside: same as F4, B3
6D	36	34	10	35	44		6D	Prob. Peonidin-3-glucoside (see text)
6E	40	41	13	37	44	(2)	6Ei	Pelargonidin-3-glucoside: contaminant from band 7
	36	30	8	30	40	(1)	6Eii	?
	33					(1)		?
6F[bd]			34*	62*				
	36	39	24			(2)		A new anthocyanin
	32		19	52	63	(3)		
		30						
	28		8	31	42	(2)	6Eii	Similar to 6Eii
6G[cd]				72		(wk)		
				63	70*	(wk)		A highly glycosylated anthocyanin: data insufficient
					T*			

major components of the BAW band F to the BAW band 6, it was pointed out that the component 6B is similar to the component F3. Reasons were also advanced as to why the component has been designated as a new cyanidin derivative and not cyanidin-3-glucoside. The R_f values suggest that it may resolve as a component 3-4/B upon partial hydrolysis of the original extract during storage, and thus contaminate the delphinidin band 3.

The component 6C consists predominantly of peonidin-3-glucoside though it does show a weak contaminant due to the component 6B. The component is probably equivalent to the anthocyanin F4.

The component 6D is a peonidin derivative. Though its R_f values are comparable to peonidin-3-glucoside, they are consistently slightly higher than peonidin-3-glucoside in the aqueous mineral solvents. The component is bright red to pink in u.v., an observation which increases the improbability that it is peonidin-3-glucoside which is dull in u.v. The increase of R_f values in the aqueous mineral solvents, it will be noted, is similar to the increase that was observed for the component 5D. Thus there are reasons for thinking that the component 6D may be a complex derivative of peonidin, as is 5D of cyanidin.

The component 6E splits into at least two sub-components; the constituent 6Ei, identified as pelargonidin-3-glucoside, is certainly a trail of the band 7. The nature of the sub-constituent 6Eii is not known. The component 6F is a new anthocyanin derivative and one of its constituents is 6Eii. It will be noted that equivalent of the component F2 (Table 19.VIII.F) was not recovered from the band 6.

BAW Band 7: HAc-HCl Components

The chromatographic data of the HAc-HCl components of band 7 is included in Table 19.IV. The data from hydrolysis, sugar analysis, partial hydrolysis and R_f values in other solvents is summarized in Tables 19.V, 19.VI, 19.VII and 19.VIII.7, respectively. The bands 7A and 7C have been identified as cyanidin and pelargonidin, respectively. The band 7D of the Collections 272 and 274, which originally showed yellowish red fluorescence (Table 19.IV), was available in quantity only sufficient for

TABLE 19.VIII.7
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BAND 7 OF BLACK HULLESS CARYOPSIS ANTHOCYANIN

HAc-HCl Components	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
7A		65	3				7A	Cyanidin (cf., Table 19.V).
7C			5	17			7C	Pelargonidin (cf.,Table 19.V)
7D			7			(5)	7F	Trail of 7F
			12			(1)	?	?
7E					47	(1)	7Ei	?
					36	(8)	7Eii	Trail of 7F
					76	(t)	?	
7F	33	22	8	28	36		7F	New cyanidin derivative: prob.same as 6B,F3,G3
7G	40	37	13	36	46	(5)	7Gi	Pelargonidin-3-glucoside
	37				42	(3)	7Gii	Cyanidin derivative ? see 8D
		27	9	32				
	33	T*	T*	T*	36	(1)	7Giii	Prob.same as 7F or cyanidin-3-glucoside
7H	41	38	15	38	53		7H	Prob. a complex pelargonidin derivative (see text)
P-3-g	39	36	13	35	50			R_f values of pelargonidin-3-glucoside on same sheet
7J[d]	23	12	35*	68	75	(t)	7Ji	Colors difficult to distinguish;spots appear largely orange red. ∴ mixture of polyglycosylated 'nins, mostly pelargonidin derivative(s).
	31	26	27*	58*	66*	(2)		
		21	20*	48*	58*	(3)		
	40	36	13	35	49	(6)	7Gii	Pelargonidin-3-glucoside:a breakdown product.
	31	21	8	26*	39	(1)		Prob. cyanidin-3-glucoside.

chromatography in one solvent. It will be noted (Table 19.VIII.7) that it broke down into two components neither of which showed the yellowish red fluorescence in the Aq-HCl solvent. The component 7E of the Collections 272 and 274 split into three constituents, the major constituent is the trail of the component 7F. It is, therefore, clear that the components 7D and 7E may only be trailing components rather than any new anthocyanins. The equivalents of the components 7D and 7E were absent from the other collections.

The component 7F is a new cyanidin derivative. The R_f values are identical to the components 6B, F3, G3 except that the R_f values in Bu-HCl are slightly lower than the other components.

The component 7G is a mixture of cyanidin and pelargonidin derivatives (Table 19.V). The constituent 7Gi is identified as pelargonidin-3-glucoside. The constituents 7Gii and 7Giii resolve clearly in the BAW and the 3:1:8 solvents but in the other three solvents, they resolve as a single trail. The component 7Gii may either be cyanidin-3-glucoside or it may be equivalent of the component 7F. The constituent 7Gii is a cyanidin derivative but its nature and correspondence to other components is not clear.

The component 7H is the major anthocyanin of the band 7 (Table 19.IV). It is orange red visibly and bright orange red in ultraviolet light. The R_f values of the component 7H and synthetic pelargonidin-3-glucoside (P-3-g) on the same sheet (average of several runs) are shown in Table 19.VII.7). It was observed consistently that the R_f values of the component 7H were slightly higher than the R_f values of synthetic pelargonidin-3-glucoside. The observation lends evidence to a proposal that the component 7H, like the components 6D and 5D, may be a complex

derivative of pelargonidin. Probably in barley a new series of complex anthocyanins derived from cyanidin, peonidin and pelargonidin may exist.

The component 7J (HAc-HCl R_f range 0.41 to 0.73) is a mixture of at least two to three anthocyanins. Pelargonidin was found to be the major component and cyanidin, a minor component (Table 19.V). The R_f values doubtless indicate that it is a mixture of polyglycosylated anthocyanins derived from pelargonidin and probably cyanidin. The basic structure of the polyglycosyl derivatives, moreover, is pelargonidin-3-glucoside because it was recovered as a major product of breakdown and cyanidin-3-glucoside recovered as minor product of breakdown. It is probable that the highly labile trace constituent 7Ji might have ended up as a contaminant of the BAW bands 3-4.

BAW Band 8-9: HAc-HCl Components

The chromatographic data of the HAc-HCl components of the bands 8 and 9 are included in Table 19.IV. The data of hydrolysis and R_f values in other solvents are summarized in Tables 19.V and 19.VIII.8-9, respectively. It may be stated at the outset that except for the major band 8-9C, the results in general are incomplete. The major band 8-9C is a cyanidin derivative (Table 19.V); its R_f values in other solvents are comparable to the component H4 except that in ultraviolet light, H4 is bright and 8-9C dull.

The component 8D is a cyanidin derivative. The R_f values are similar to the constituents 7Gii and 7Giii. It is likely that these constituents of the band 7 may in fact be the trails of the band 8. The component 8 and 9E is a pelargonidin derivative. The origin of the pelargonidin derivative is not clear. Owing to the scarcity of materials,

TABLE 19.VIII.8-9
CHROMATOGRAPHIC CHARACTERISTICS OF THE HAc-HCl COMPONENTS FROM THE ANTHOCYANIN BANDS 8-9 OF BLACK HULLESS CARYOPSIS ANTHOCYANIN

HAc-HCl Components	R_f x 100					Rel. Conc.	Component or sub-component	Provisional Identification
	BAW	Bu-HCl	Aq-HCl	HAc-HCl	3:1:8			
8-9C	34	27	8	27	36			A cyanidin derivative. Differs in UV from H4
8D	36				44	(1)		A cyanidin derivative: see constituents 7Hii & 7Giii
		28	8	31				
	30	T		T	36	(2)		
8F			20*			(t)		
			14	41		(1)		Same as H6
			8	27		(2)		
8G			22					
			8					
8-9H			22			(1)		
			18			(2)		
8FGH			30*		?			
	42	44	23*	53	63	(1)	8FGHi	Probably an acylated anthocyanin similar to H7b
				T*	T*			
	32	29	8	27	36	(5)		

the chromatographic data of the components 8F, 8G, 8H was obtained only in a few solvents (Table 19.VIII.8-9). However, the data obtained from the mixture of the three components (see 8FGH, Table 19.VIII.8-9) shows that the component 8FGH consists largely of cyanidin and small amounts of a pelargonidin derivative. The constituent 8FGHi appears to be an acylated anthocyanin similar to the constituent H7b of basal leaf sheaths (Table 19.VIII.H).

19.4. DISCUSSION

19.4.1 Anthocyanidins of Basal Leaf Sheaths and Caryopses

The identification of anthocyanidins of Black Hulless and Gopal basal leaf sheaths, and Black Hulless caryopses is, with few exceptions, straightforward. Cyanidin is a major, and peonidin a very minor anthocyanidin of the basal leaf sheaths grown under greenhouse conditions. Although petunidin, per se, has not been identified, there are indications (see Table 19.V) that petunidin, instead of cyanidin, may be the aglycone of a few anthocyanins. Such indications were obtained only from chromatographic characteristics: the R_f values of petunidin and cyanidin in seven solvents are very close. Whenever aglycones believed to be petunidin were available in quantity sufficient to be studied spectrophotometrically, they were identified as cyanidin. But, in cases where spectrophotometry of the suspected aglycones (particularly, a few HAc-HCl components of the band H) could not be undertaken, because of the limited availability of materials, the identifications remain inconclusive. A solvent system suitable for differentiating petunidin and cyanidin, similar to the Iso-PrOH solvent which differentiates pelargonidin and peonidin, is needed.

The distribution of cyanidin and peonidin in the Gopal basal leaf sheaths obtained in 1960 summer from the field grown materials (Collections 130, 138; Secs. 10, 11, 13) was similar to that in greenhouse grown leaf sheaths referred to above, except that peonidin was present in relatively larger quantities (compare Figs. 10.3 and 19.1). The occurrence of trace quantities of delphinidin (see HAc-HCl component DE2; Table 19.V) in the greenhouse materials is enigmatic and requires reappraisal. Delphinidin was not recovered from the corresponding Field Collections 130

and 138 either from the hydrolysates of the extracts, or the individual eluates (Table 13.II).

Cyanidin is also the major anthocyanidin of the Black Hulless caryopses which were obtained from the field conditions (see Table 19.V). Delphinidin, peonidin, and pelargonidin are minor components. The situation regarding petunidin is the same as described for the basal leaf sheaths.

It was shown in Sections 6.4.2.c that, from intact caryopses, anthocyanins are extracted from pericarp-spermoderm only. Since the anthocyanin extracts of caryopses used in this and the earlier investigation (sec. 6) are the same, they are considered as those derived from spermoderm: the contribution of anthocyanins from the pericarp is non-existent or negligible. However, at the time that caryopses were extracted for this investigation, the writer was not aware of the above fact. The caryopses, at the time, were extracted in the belief that the extracts would contain the anthocyanins both from the aleurone and spermoderm. By making a total determination and 'subtracting' the spermoderm anthocyanins, the anthocyanins of the aleurone could be established by difference. The extraction of the intact caryopses, at that time, appeared to be desirable because it eliminated the laborious peeling of aleurone absolutely free of the adhering spermoderm fragments at the furrow. However, as it turned out, the anthocyanins from aleurone were not extracted, an observation which eventually laid the basis for the selective extraction of anthocyanins from spermoderm, and aleurone without manual peeling (Sec. 22). However, a comparison of hydrolysates of the first and the third extracts from the caryopses (Collections 272, 278) showed that most of the delphinidin in the third extract of caryopses was due to

leaching of aleurone pigments through the damaged seeds (Sec. 6, Fig. 6.3). It was therefore assumed (Sec. 6) that the slight presence of delphinidin in the first extracts of caryopses (to be accurate, the extracts used for this investigation were largely a mixture of the first two extracts, the overall period of timetaken for the first two extractions are noted elsewhere: Sec. 6.3.5.c) must be due to 'contamination' from aleurone because no precautions were taken to use only the undamaged seeds. Later in 1965, it was noted during development of a method for selective extraction from spermoderm and aleurone without manual peeling (Fig. 22.2) that the hydrolysates of the peeled spermoderm did show small amounts of delphinidin. Thus, it became clear that delphinidin exists as a minor component of the spermoderm. The fact that no delphinidin was recovered from the hydrolysates of spermoderm in the 1958 (Sec. 5) and 1959 (Sec.8) appears to be due to developmental or environmental differences. The hydrolysates of the 1958 and 1959 spermoderm extracts were chromatographed by spotting. Because delphinidin was recovered as a relatively weak band in the 1965 studies (Fig. 22.2), the 1958, 1959 chromatograms were rechecked since omission in the detection of weak components on chromatograms does occur by the spotting as compared to banding technique. The rechecking did not reveal delphinidin.

The results from the studies of the caryopsis anthocyanins show that pelargonidin derivatives are the major anthocyanin(s) of the band 7, and minor anthocyanin(s) of the band 8. Thus, pelargonidin was abundant in the summer 1960 field collections. It was indicated in Sections 5 and 8 that because anthocyanidins above the cyanidin band resolved poorly (in Forestal solvent) and because other anthocyanidin solvents, such as those listed elsewhere in Table 17.I were not used, the presence of

pelargonidin derivatives in the 1958 and 1959 collections could not be confirmed. Although it has not been possible to present the results of Black Hulless spermoderm obtained from the 1961-1962 winter plantings in the greenhouse, it may be stated summarily that none of the anthocyanin bands gave pelargonidin on hydrolysis.

It was demonstrated in Section 13 that Gopal basal leaf sheath extracts of the Collections 130, 138, and specifically, for example, the bands G-130 and H-130 both gave pelargonidin in addition to cyanidin (Table 13.II). Whereas the band G-130 was orange red, the band H-130 was bluish red. In other words, the pelargonidin derivative of the band H-130 was masked by the preponderant cyanidin derivative. Inasmuch as the R_f value of the major pelargonidin derivative of the band G-130 was very similar to pelargonidin-3-glucoside, it appeared that the orange red band, G-130, could have arisen as an artefact due to splitting of pelargonidin derivatives in band H-130 over extended period of storage. Since the period of storage for the extracts 405, 407, 408 was very short as compared to those for the extracts of Field Collections 130 and 138, it seemed desirable to find out whether or not the bands H, K, or L (Collections 400s) contained masked pelargonidin derivatives. The eluates of the band K and L upon hydrolysis did not yield pelargonidin (Table 19.V). Although the BAW eluate of the band H was not hydrolysed directly, the eluates of all the major HAc-HCl components of the band H upon hydrolysis did not yield pelargonidin (Table 19.V); the trace components of the band were not available in quantity sufficient for hydrolysis. It is, however, certain that none of the HAc-HCl anthocyanin components of the band H showed the characteristic orange red color of pelargonidin derivatives. Only one very weak sub-component designated H5a (Table

19.VIII.H) gives R_f values comparable to pelargonidin-3-glucoside. Even if this component should prove to be a pelargonidin derivative, the relative amount of the component is so small that it may, at best, be a carry over from early stages of development; it was shown that small amounts of pelargonidin are present, particularly in Black Hulless leaf sheaths at an early stage of development (see the results of anthocyanin G-402: Fig. 19.1, and 19.2).

The data presented above seem to show that the absence and presence of pelargonidin from the greenhouse collections of basal leaf sheaths is due to the differences in development. However, the caryopses anthocyanins (Field Collections 217, 270, 272, 274, 278) which were obtained in the summer of 1960, and which were collected at successive stages of plant development, beginning from the stage of maximum pigment development, did show pelargonidin in the extract of all collections. Although the data included in Section 8 tend to support that stepwise development of anthocyanins may be involved in the early stage of development, it is difficult to conclude that the absence of pelargonidin, on occasion, is entirely developmental because the anthocyanins of the basal leaf sheath were also collected around and during the maximum stage of development. Thus, it is conceivable that the absence of pelargonidin from the basal leaf sheaths towards later stages of development in greenhouses may be due to variations in environments (particularly, greenhouse and field conditions) instead of variations in development. The observations listed in Section 4 tend to support the former viewpoint (see below).

The barley stocks employed in this study were planted in greenhouses in August 1961, and observations and tissue collections were

continued until April 1962. It was observed that, although the sheaths did develop color somewhat sporadically throughout the period of growth, the heads of the purple varieties, isolines and their hybrids, which emerged between November 1961, and early January 1962, did not develop anthocyanins. However, the heads that emerged after about the mid-January 1962, developed characteristic purple colors. Thus, from a single purple seed, true breeding under field conditions, tillers arose, half of which had purple heads and half of which had colorless heads (see Fig. 4.3). In view of the striking variations in expressivity of anthocyanin genes in a single genotype, it is reasonable to assume that the absence of pelargonidin in certain greenhouse materials is largely due to deviations in growing conditions.

Seikel and Bushnell (1959) have isolated a C-glycosidic flavonoid, lutonarin, from barley leaves. They report that "plant materials containing lutonarin could be obtained only under certain cultural conditions which have not yet been completely worked out. . ." It is significant Seikel and Bushnell find that only field grown crops contained lutonarin, and that indoor grown plants did not; this observation tends to support our observation that pelargonidin is absent from our greenhouse grown materials and present in our field grown materials.

It is a common practice in breeding and genetical studies to get an additional crop by growing barley in greenhouses for example, see Buckley (1930). The observations and results cited above demonstrate that the additional crop, in the case of characters which are influenced easily by environments, should be obtained only after carefully providing suitable conditions for the optimum expressivity of the character.

19.4.2. A Comparison of the Methods of Elution and Purification

It was shown in Sections 11, and 15 that when such acid concentrations as are recommended in the literature were used in the elution solvent, much degradation of anthocyanins occurred due to hydrolysis (Sec. 23, 15); irreversible modifications to 'yellow product' (Sec. 11), several spectral modifications (Sec. 12) and stationary materials (Sec. 15 and 19.3.8). The results of this investigation demonstrate the mildness of our elution and purification techniques (Sec. 16) because aglycone production did not occur. The degradation leading to the formation of stationary materials was minimal. Instability through transformation of anthocyanins to yellow products occurred specifically in those eluates which were obtained from the HAc-HCl R_f region greater than 0.65. Exploration of the causes of this instability is needed.

19.4.3. Anthocyanins of Basal Leaf Sheaths and Caryopses

Identifications of aglycones and anthocyanins by extensive chromatography in several solvents and by spectra have established that barley contains a series of new anthocyanins largely of cyanidin origin. For example, the anthocyanin A is a complex new anthocyanin which consists of at least four components, namely, A, A2-A3, A3, A4. Except for the constituent A2 (cyanidin-3-glucoside), all other constituents, as is clear from their R_f values, are new and must possess increasing numbers of glycosyl substituents. Inasmuch as the complex anthocyanin A (see Secs. 13.2.4, and 19.3.7.2) is not acylated, the splitting of the glycosylated anthocyanin A and its HAc-HCl components into several sub-constituents, even under the mild conditions of our elution is

intriguing. The reproducible patterns of splitting, and particularly, the ease of anthocyanin splitting not only of anthocyanin A, but also of the other anthocyanins of barley indicates that at least some linkages of (apparently) glycosyl substituents must be novel and highly labile. The extremely hard-to-hydrolyze glycosyl linkages of the C-glycosyl type flavonoids of barley, e.g., saponarin (Seikel and Geissman, 1957), and lutonarin (Seikel and Bushnell, 1959) and its 3-methyl ether (Seikel, Bushnell and Birzgalis, 1962) sharply contrast with the extremely labile glycosyl linkages of the anthocyanin type flavonoids of barley. The observation indicates that if glycosylation occurs following the biosynthesis of the C_6-C_3-C_6 moiety, the pathways of glycosylation of the above barley flavonoids must be different from those of barley anthocyanins. Although any further discussion of the nature of the glycosyl linkages of anthocyanins as yet is purely speculative, it is certain that not only the anthocyanin A is a new and complex cyanidin derivative but that the nature of its several constituents is also hitherto unknown. Thus, it is clear that the anthocyanin band A consists of a series of new cyanidin derivatives.

Similarly, the major constituent of the anthocyanin bands DE or 5, on the basis of 1962 chromatography, were believed to be similar to cyanidin-3-monoside. This belief was reinforced by the identification of the major anthocyanin band both from barley husks (Metche and Urion, 1961) and barley corn (Urion and Metche, 1961) of the variety violet Guimalaye, as cyanidin-3-arabinoside. Incidently, although the results of the hulls have not been presented in any detail in this thesis, they do show an equivalent of the major band DE or 5. In 1965, as already indicated, the major constituents 5D, DE4, E3-138 of the bands under

consideration were established as being highly complex. The complexity, however, is intriguing, principally because the R_f values of the major constituents, for example, 5D (Table 19.VIII.5) and its equivalents, in all the five solvents are consistent with a monoglycosidic structure. The anthocyanin 5D and its equivalents, therefore, must have a novel type of substituent, which retards the mobility of the anthocyanin, for instance, in the HAc-HCl solvent. The substituent, however, must be very labile inasmuch as the lability occurs even in storage of crystalline samples. As the substituent is removed, probably hydrolysed (?), several sub-constituents of the component (5D) with R_f values as high as 0.90 appear on the HAc-HCl chromatograms, indicating thereby that the component 5D contains several polyglycosyl derivatives of cyanidin (see Fig. 15.3). The belief in the above postulate is greatly strengthened because the components 5D, DE4 and E3-138 had been purified extensively, because they were isolated as stable entities without any apparent degradation (the BAW anthocyanin band E, even otherwise is a stable anthocyanin as compared to the other BAW anthocyanin bands (cf., Sec. 12: Fig. 12.1), and because their chromatographic behaviour prior to and following storage was identical and reproducible. Thus, the anthocyanin under scrutiny (bands DE4 or 5D) is a novel cyanidin derivative, several constituents of which, like the anthocyanin A, are also new cyanidin derivatives. From the results of several others bands, it is clear that barley is a rich source of several new cyanidin derivatives.

It will be noted that the complex anthocyanin component 5D (Table 19.VIII.5) gives very slightly higher R_f values than cyanidin-3-glucoside in the aqueous mineral solvents. Similarly the component 6D (Table 19.VIII.6), a derivative of peonidin, and the component 7H (Table

19.VIII.7) a derivative of pelargonidin are, in chromatographic behavior, identical to the component 5D: they also give slightly higher R_f values than their corresponding 3-glucosides. The reasons for the slight differences in R_f values remain to be investigated.

Tangible progress on the identification of anthocyanins would have doubtlessly resulted, had the results of sugar determinations (Table 19.VI; Sec. 19.3.5) been dependable. Subsequent to the analysis of sugars from all available anthocyanins, it was found that the chromatography paper *per se* contributed substantial amounts of sugars to the anthocyanin eluates and that the sugars contributed by the paper could be the same as those obtained from the anthocyanins (Sec. 18). However, a technique for eliminating the paper-derived glycosidic artefacts from anthocyanin eluates (which are dried *in vacuo*, presumably because crystallization is impractical (*cf.*, Harborne, 1958)), has now been worked out successfully (Sec. 18.2.3). A few of the results obtained by the technique, referred to as nylon method (Table 19.VI), indicate that some anthocyanins of barley contain arabinose and xylose as sugar substituents.

Although the results of sugar analysis are inconclusive, it has been established on the basis of R_f values that the basic structure of several barley anthocyanins is cyanidin-3-glucoside, peonidin-3-glucoside and pelargonidin-3-glucoside. Structural complexity of most barley anthocyanins is built on the 3-glucosidic unit. Inasmuch as the anthocyanins of the varieties, Gopal and Black Hulless, are highly complex, the simple anthocyanin structure of the barley variety, violet Guimalaye as revealed by the techniques used by Urion and Metche (1960) is not warranted.

19.4.4. Fast-Moving Anthocyanins

The fast-moving anthocyanins ('nins) were present in abundance on the preparatory chromatograms of basal leaf sheath extracts which were obtained from field grown barley early in November, 1958. They were absent from extracts which were obtained in December, 1958 (Sec. 7), and the summer of 1960 (Field Collections 130, 138; Secs. 10, 11, 13, and 24). It will be noted that the fast-moving 'nins are also absent from the 1961-62 winter greenhouse collections 400, 402, 404, and 405. The collections 407 and 408, however, do show relatively weak anthocyanins K and L, the R_f region of which is similar to some of the fast-moving 'nins encountered in earlier studies. It will be recalled that the 1958 and 1960 extracts were prepared with 1% conc. HCl and those of 1961-62 with 1% HCl and that the extracts were chromatographed following concentration by flash evaporation.

The fast-moving 'nins were also present in the hulls, spermoderm, and awn tips studied in 1958 (Sec. 5) and 1959 (Sec. 8); they were notably absent from the preparatory chromatograms shown in this section (Fig. 19.3). It is notable that anthocyanins in the above studies were extracted with 1% conc. HCl, and that the chromatography of the extracts shown in Figs. 5.1, 5.2, and 5.3 was carried out immediately after extraction and preparation, that shown in Figs. 8.1 and 8.2 within a few weeks of extraction and processing and that shown in Fig. 19.3 after about 2 years of extraction and processing. The fast-moving 'nins were also abundant on the 1965 preparatory chromatograms when either the intact caryopses or peeled spermoderm were extracted with a very small volume of 0.1% methanolic HCl and the extracts were chromatographed without volume reduction (Sec. 22: Fig. 22.1).

The presence or absence of the fast moving anthocyanins creates uncertainties in the delineation of the anthocyanin patterns of basal leaf sheaths or grain tissues (Sec. 5 to 8). Is the absence due to variations in development or is it artefact? This point must be resolved clearly because a serious study on the biochemical responses of gene action makes very important not only the determination of the exact structure of anthocyanin molecules but also, more importantly, their original state in vivo. The causes of differential distribution of anthocyanins on preparatory chromatograms have now become amply clear both from characterization work and studies on the instability of anthocyanins, and will be considered now.

At least two significant facts have emerged from the results of anthocyanin characterization. First, owing to the lability of barley anthocyanins, several anthocyanin bands that appear on preparatory chromatograms are artefacts. The studies on both the causes of anthocyanin splitting on paper or in storage (Sec. 15), and the influence of flash evaporation on the anthocyanin extracts which were prepared with methanol containing either 1% HCl or 1% conc. HCl (Sec. 23) provide the reasons for artefact production. It was shown that upon flash evaporation the concentration of acid in extracts increases and causes hydrolysis of anthocyanins. Secondly, the artefact production must have occurred during processing, flash evaporation and storage of concentrated extracts prior to the preparatory chromatography.

It was shown in Section 7 (see also Secs. 5 and 8) that the fast moving 'nins were highly labile because they disappeared from the extracts during brief storage. It was also adduced from the same studies that the molecules of the fast moving 'nins did not undergo

complete breakdown but were only modified, because the concentrated extracts from which they had disappeared, on rechromatography, showed several slow-moving 'nins, which were either not present prior to the disappearance, or which had undergone a striking increase in concentration following the disappearance of the fast-moving 'nins. The appearance of new anthocyanins or the changes in anthocyanin concentrations, therefore, must not be due to degradation but must be due to the modification of fast-moving 'nins to slow-moving 'nins. Experimental evidence for the contention that, upon the disappearance of the fast-moving 'nins, slow-moving 'nins appear, is furnished by the results obtained with the fast-moving anthocyanins K and L (Table 19.VIII.K.L). It will be noted from the table that upon rechromatography of the BAW eluates of the anthocyanins K and L, only traces of the original fast-moving 'nins were recovered from the eluates of K and none from those of L. Moreover, the major component resolved at the region of the slow-moving anthocyanin DE. The results, therefore, substantiate the earlier deductions that fast-moving 'nins are modified to the slow-moving 'nins and that their disappearance is not due to degradation.

Primarily on the basis of their exceedingly high R_f values in the BAW solvent, and also on the basis of several other considerations outlined in Sections 5 and 7, it was proposed that the fast-moving 'nins must be acylated with a novel type of organic residue and that the acyl linkage must be highly labile in strong acid environment of concentrated extracts (cf., Sec. 23) as indicated by the facile transformation of fast-moving 'nins to slow-moving 'nins. At the time the results of Sections 5, 7 and 8 were compiled it was not known that the slow-moving 'nins of barley are also labile; notwithstanding the mild conditions of our

handling, it is now clear that they break down into several components. It is also clear from the characterization work that most of the slow-moving 'nins are, however, less labile than the fast-moving 'nins, because the chromatographic patterns of the former, once the latter have disappeared, are reproducible. The strength of linkage of the labile components in each case must, therefore, be different. Because the slow-moving 'nins are largely polyglycosylated, the differential lability must be due to the differences in the linkages of glycosyl components (slow-moving 'nins) and the acyl components (fast-moving 'nins).

As indicated already, the appearance of the fast-moving 'nins on the preparatory chromatograms was highly variable. The fastest moving component, BAW R_f ca. 0.98, was observed only in the 1958 extracts of spermoderm, hulls, and awn tips of both Gopal and Black Hulless (Sec. 5). All those extracts were processed simultaneously, under similar conditions, using 1% conc. HCl as the extractant, and they show identical patterns of anthocyanin distribution (Figs. 5.1 and 5.2). The patterns of anthocyanins obtained in 1959 from the same tissues using the same concentration of acid in the extractant (Sec. 8, Figs. 8.1 and 8.2) differ from those obtained in 1958 (Figs. 5.1 and 5.2). The patterns obtained from the caryopses tissues of 1960 collections, which were also extracted using the same concentration of acid in the extractant (Sec. 8, Figs. 8.1 and 8.2) differ from those obtained in 1958 (Figs. 5.1 and 5.2). The patterns obtained from the caryopses tissues of 1960 collections, which were also extracted using the same concentration of acid as in the above two studies, are also identical between themselves (e.g., see the results of the five different extracts of caryopses shown in Fig. 19.3). However, none of the collections show the presence of

fast-moving 'nins and, therefore, their patterns differ markedly from those obtained from the 1958 and 1959 investigations. It is emphasized that all the above extractions were carried out on the same variety, and the same tissues, using the same range of acid concentration in the extractants, namely, 1% conc. HCl. The patterns of fast-moving anthocyanins, obtained from the spermoderm and intact caryopses, using 0.1% methanolic HCl as extractant, in 1965 (Fig. 22.1), are again identical because both the extracts were processed under identical conditions. It will be noted that the chromatograms show largely the fast-moving anthocyanins. On comparison of the 1958 (Figs. 5.1 and 5.2) 1959 (Figs. 8.1 and 8.2), 1960 (Figs. 10.3, and 19.3) and 1965 (Fig. 22.1) results, the differences in the patterns become striking. Although the results of 1961 field (extractant: 1% conc. HCl) and 1962 greenhouse (extractant: 0.1% HCl) extracts from grain tissues of parent and hybrid materials have not been presented, the patterns obtained were different not only between tissues but also between seasons (1958, 1959, 1960 and 1965).

The yearly variations in the chromatographic patterns, paradoxically, do not appear to be due to the acid concentration, because the variations occurred irrespective of the acid concentration. The fact that the patterns obtained from different tissues (Fig. 5.1), or different collections of the same tissue (Fig. 19.3), which were extracted and processed simultaneously, are basically similar, suggests that the yearly variations in patterns are likely to be due to the conditions of processing and handling of the extracts. Although the conditions of processing were uniform, the variations in the chromatographic patterns of the fast-moving 'nins must, primarily, be due to the increase in acid concentration of the extracts that occurs during flash evaporation: it

was pointed out elsewhere that it is not practicable to reduce volume of extracts to the same level of acid concentration by taking the same length of time for each extract, owing to the limitations of flash evaporators (Sec. 23). It was shown in Section 7 that from the flash evaporated extracts, the fast-moving acylated anthocyanins are the first to undergo modifications to slow-moving anthocyanins because of 'deacylation'. It is entirely possible that deacylation of fast-moving 'nins may occur completely either during the preparation of extracts for chromatography, or within a few hours or after a few days of the preparation of extracts depending upon the extent to which the increase in acid concentration occurs following flash evaporation. If the concentrated extracts are stored, deaclyation occurs and slow-moving anthocyanins are left. According to this view, then, some of the BAW bands obtained in the preparatory chromatography of caryopses anthocyanins (Fig. 19.3) and basal leaf sheath anthocyanins (Fig. 19.) are most likely the deacylated anthocyanins. Thus, the anthocyanins characterized in this section may largely be the in vitro artefacts of the fast-moving anthocyanins. Using mild extraction and handling large number of fast-moving anthocyanins were recovered in the 1965 studies (Sec. 22); the fast moving 'nins likely represent the in vivo state of anthocyanins (Sec. 24).

Fast-moving anthocyanins, it has been shown, are labile. Variations in their chromatographic patterns may be due to lability or to variations in their synthesis during plant development. The results of Section 7 were interpreted to mean that fast-moving anthocyanins are deacylated towards maturity. The conclusions will require reappraisal because the acidified extracts were flash evaporated, and because, under such conditions, the disappearance of the fast-moving anthocyanins, is possible.

Fast-moving anthocyanins were usually recovered from near neutral extracts, or extracts which were stored for brief periods, or from extracts which were not flash evaporated. Puzzling features relating to their occurrence and stability nonetheless remain. The absence of fast-moving 'nins from certain neutral extracts was surprising (Sec.24). Were they not present or was the procedure incapable of revealing their presence? Fast-moving 'nins may be tissue specific: they have not been recovered from aleurone tissue but have been recovered from spermoderm, awns, hulls and basal leaf sheaths. Curiously they were not recovered from hulls in neutral methanol but when hydrochloric acid was added to the methanolic extract two fast-moving anthocyanins were detected. Even more puzzling was the fact that the fast-moving anthocyanin bands were erratically developed (Sec. 7.3) but not so the slow-moving anthocyanin bands on the same chromatogram. It is apparent that much remains to be studied regarding fast-moving anthocyanins. Clearly in barley there is a series of anthocyanins hitherto not reported.

19.4.5. Slow-Moving Anthocyanins

On the basis of preceding results and discussion, it is now clear that some of the slow-moving anthocyanins are doubtlessly the artefacts of fast-moving anthocyanins and a few of the slow-moving anthocyanins are the artefacts of other slow-moving anthocyanins (for example, see the results of band A-400s): the artefacts must have originated during flash evaporation and storage of concentrated extracts (see discussion of the fast-moving 'nins in the preceding section). Chromatographic evidence is presented in Section 7 that several changes, particularly in the concentration of slow-moving 'nins occur within a

short period of storage following flash evaporation of the extracts. It is surmised that the changes must be due to the artefacts which originate, because of flash evaporation, from the slow-moving 'nins such as those from band A-400s. The fact that following a short period of storage, patterns of anthocyanins obtained from the same concentrated extract become almost reproducible (Secs. 7 and 8) suggests that the artefact production occurs over a short period of time when some sort of 'steady state' between the artefacts and other anthocyanins is established that the variation in patterns becomes minimal. This is clearly brought out when the concentrated extracts were chromatographed repeatedly after extended periods of storage, for example, see the results of caryopses anthocyanin collection 270 (Fig. 19.3) which was chromatographed one and two years after the preparation of the extract or those of basal leaf sheath anthocyanin Collection 130 (Fig. 10.3) which were also chromatographed after similar periods.

Although it is evident from the foregoing that anthocyanin patterns obtained in this study, in general, are far removed from the in vivo state of anthocyanins, nonetheless, on the basis of the reproducibility of the patterns obtained either immediately following flash evaporation (Sec. 5) or a few days after flash evaporation (Secs. 7 and 8), it was concluded from the early studies that anthocyanin patterns of the two varieties, Gopal and Black Hulless, were basically similar. The results of characterization of basal leaf sheaths obtained from greenhouse establish that the nature and the pattern of Black Hulless and Gopal anthocyanins are also basically identical. The presence of the additional bands K and L in the Black Hulless collections 407 and 408, as discussed already is, probably, due to slight variations in

flash evaporation and handling. It is, however, clear that the patterns of greenhouse collections, in some respects, differ basically from those of the 1960 field collections 130 and 138 (Fig. 10.3) which contained abundant pelargonidin and peonidin derivatives.

It was established in Section 5 and 8 that the anthocyanin patterns of Gopal and Black Hulless grain tissues were also similar. It was observed that the nature of anthocyanins 5 to 8 from Black Hulless caryopses (Fig. 19.3), the only bands that show clear cut resolution, is identical to Gopal basal leaf sheath anthocyanins E to H (Fig. 10.3) both of which were obtained in the summer of 1960. Thus the band 5 and E, 6 and F, 7 and G, and 8 and H are identical. It will be noted that the bands G-130, and H-130 contain pelargonidin derivatives as indeed do the bands 7 and 8. Inasmuch as the broad patterns of anthocyanin development in Gopal and Black Hulless basal leaf sheaths are identical, the patterns of anthocyanin development in Black Hulless caryopses and Gopal leaf sheaths at least for the four bands noted above are also identical: the comparisons of the other bands are not possible because of the poor resolution of caryopses anthocyanins (see Fig. 19.3).

From the comparison of the above studies, it is clear that differences in patterns do occur from year to year. On the basis of reasons presented in Section 19.4.1, it is also clear that some differences are due to development and environments. However, large differences appear to be due to the lability of barley anthocyanins, the lability was pronounced when the anthocyanins were stored following flash evaporation. In spite of the lability, however, the chromato-patterns of anthocyanins from tissues which were collected from the same plant and processed in the same way and time were in general, identical.

Therefore, under a given set of conditions, similar changes in the extracts occurred. On this basis then the striking differences that were observed in the patterns of basal leaf sheath anthocyanins of several other varieties (Sec. 7) must be real.

Although the chromato-patterns obtained under similar conditions are qualitatively similar, they do show slight quantitative variations, for example, compare the patterns of the collections 217, 270, 272, 274 and 278 (Fig. 19.3). Inasmuch as the caryopses were extracted at stages of differing maturity, the quantitative variations, may inreality, be due to variations in development. However, the conclusions cannot be regarded as certain because quantitative variations could have been caused by the differential splitting of anthocyanins following flash evaporation.

The comparative results of only a few of the tissues and stages of development presented in this section have enlarged our understanding of the complexity of barley anthocyanins. The complexity and lability of barley anthocyanins has not been appreciated by other workers (see Metche and Urion ,1961; Urion and Metche, 1961).

19.5. SUMMARY

The basal leaf sheaths of Gopal and Black Hulless, obtained from the 1961 (winter) greenhouse grown materials, gave eight anthocyanin bands, A to H, in BAW. Although the bands showed quantitative variations, they were established by extensive chromatography to be qualitatively identical in both the varieties. The sheaths from Black Hulless, however, contained two relatively fast-moving bands K and L. The ten BAW bands split into over 60 components in the HAc-HCl solvent. Of the 60 HAc-HCl

components, 49 were isolated by mild techniques (Sec. 16). The chromatographic data of the 49 anthocyanins in five solvents is given. Some of the HAc-HCl components did not split further while others did split into two to four sub-components in the five solvents. However, none of the HAc-HCl components showed the glycosidic hydrolysis. Of the 49 anthocyanin isolates, depending upon the quantity of materials available, 31 were hydrolyzed for anthocyanidins. The 31 anthocyanidins were identified by their R_f values in seven solvents, their λ_{max} values and $AlCl_3$ shifts. Of the 31 hydrolysates, only 15 could be analysed for the determination of sugars, and 10 could be hydrolyzed partially for structural elucidation.

The Black Hulless caryopses, obtained from the 1960 (summer) field grown materials, gave nine anthocyanin bands, 1 to 9, in BAW. The nine BAW bands split into over 75 HAc-HCl components. Of the 75 HAc-HCl components, 44 were isolated. The chromatographic data of the 44 anthocyanin in 5 solvents is similar to that of the basal leaf sheath anthocyanins. Of the 44 anthocyanins, 32 were hydrolyzed for anthocyanidin characterization as described above. Of the 32 hydrolysates, 24 were analysed for sugar and 15 were hydrolysed partially for structural determinations.

It is established that cyanidin is a major and peonidin a very minor anthocyanidin of greenhouse grown leaf sheaths. Of the three Black Hulless sheath collections, however, traces of pelargonidin were recovered only from one of the collections. In contrast to the 'greenhouse' sheaths, the field grown sheaths (Sec. 13) showed distinctly pelargonidin, relatively larger amounts of peonidin and also cyanidin. The absence of pelargonidin is due to the differences either in environment or development or both. It is necessary that further studies are carried

out under controlled environments.

Cyanidin is a major and delphinidin, peonidin and pelargonidin are minor anthocyanidins of Black Hulless caryopses. Delphinidin originates most likely from aleurone. The spermoderm extracts from 1958 (Sec. 5) and 1959 (Sec. 8) did not show delphinidin. But the 1965 spermoderm extracts (Sec. 22) did show traces of delphinidin. The discrepancies are likely due to developmental or environmental differences.

Although the identification of anthocyanidins is mostly clear cut, that of anthocyanins remains inconclusive because of the difficulties of glycosidic analysis mentioned already in Section 18. Nevertheless, on the basis of anthocyanidin determinations, chromatographic properties in five solvent systems and data from partial hydrolysis, the provisional nature of most of the complex anthocyanins has been advanced, and the simple anthocyanins, such as cyanidin-3-glucoside, cyanidin-3, 5-diglucoside, peonidin-3-glucoside, peonidin-3, 5-diglucoside and pelargonidin-3-glucoside, have been identified. It is also established that the cyanidin and peonidin derivatives of Gopal and Black Hulless leaf sheaths are identical to those of Black Hulless caryopses. The delphinidin derivatives of the caryopses, which originate most likely from the aleurone, were not recovered from the sheaths. The pelargonidin derivatives of the caryopses were also not recovered from the sheaths obtained from the greenhouse grown sheaths. It is clear from the results that barley is a rich source of several new cyanidin derivatives, and also some new peonidin and pelargonidin derivatives.

The major anthocyanin band of the caryopses (band No. 5) and that of the leaf sheaths (band No. DE) are identical and are derived from cyanidin. The hulls and other tissues also show a similar major

band. Urion and Metche (1961) have identified the major band from barley corn, of the variety violet Guimalaya, as a simple cyanidin-3-arabinoside. The caryopses band No. 5 is likely the same as the major band of the variety Guimalaya. The band No. 5, following desiccation over P_2O_5, split into over 13 HAc-HCl components, which are chromatographically distinct. The major of the split components, designated 5D, is the same as the anthocyanin band 5. In 1962, immediately after isolation, the component was regarded as a 3-monoside because it gave a single spot in the five solvents with R_f values very similar to those of cyanidin-3-glucoside. The R_f values were, however, found to be consistently, though very slightly, higher than cyanidin-3-glucoside. In 1965, when the same isolate was rechromatographed, it split into a large number of HAc-HCl components. The band is therefore very complex and not a simple monoglycoside.

The anthocyanins are so labile that within five minutes of partial hydrolysis only one or two simple components are left. An understanding of lability and complexity was aided greatly either because of the drying over P_2O_5 or the period of storage prior to the chromatography resulted in mild partial hydrolysis of the anthocyanin isolates. It is very likely that anthocyanins possess a novel type of 'glucosidic' linkage because the splitting occurs, notwithstanding the mild conditions of handling. The ease of splitting indicates that the splitting must have occurred even during the preparation of original extracts for the preparatory chromatography. This view is substantiated by the results described in this section. For instance, it is established that the cyanidin derivatives of the band B (a mixture of cyanidin and peonidin derivatives) arise as artefacts of the band A. Similarly, several

cyanidin components of the bands A, B, F and G give R_f values in BAW at the R_f value region of the band DE. It is for this reason that the concentration of the band DE increases and that of the other bands decreases following storage of the extracts, and the bands DE or 5 emerge as the major bands. It is therefore clear that the bands obtained in the preparatory chromatography do not represent the in vivo state of anthocyanins.

The extremely labile glycosyl linkages of barley anthocyanins contrast sharply with the extremely hard-to-hydrolyse linkages of C-glycosyl flavonoids of barley, namely, saponarin, lutonarin and its 3-methyl ether. Thus, if glycosylation occurs following the biosynthesis of C_6-C_3-C_6 moiety, the pathways of glycosylation, in the two cases, must be different.

The fast-moving anthocyanins (see Sec. 5, 7 and 22), except the two weak bands K and L, were not recovered. The facile transformation of the bands K and L to slow-moving anthocyanins indicates that the absence of the fast-moving anthocyanins both from the leaf sheath and caryopses extracts, may be due to the inadequacies of the techniques rather than doe to differences in development or environment. According to this view then, the anthocyanins characterized in this section are likely the in vitro artefacts of the fast-moving anthocyanins. However, several disconcerting features of the fast-moving anthocyanins are discussed and they remain to be resolved.

Total study indicates that some of the slow-moving anthocyanins are the artefacts of the fast-moving anthocyanins, and a few of the slow-moving anthocyanins are the artefacts of the other slow-moving anthocyanins e.g., the origin of the cyanidin derivatives of the band B:

the artefact production must have occurred during flash evaporation and storage of the concentrated extracts. The observations emphasize the need for appraisal of factors affecting anthocyanin stability (see Secs. 23 and 24).

PROCEDURES DEVELOPED FOR THE STUDY OF PEDIGREE STOCKS

(SECTIONS 20 to 22)

Following the characterization of anthocyanins, described in Section 19, the characterization of parental and hybrid anthocyanins was undertaken. The results of this work will be compiled at a later date. In this section, however, three new methods to aid the study of pedigree stocks are presented.

PROCEDURES DEVELOPED FOR THE STUDY OF PEDIGREE STOCKS

20. MICROTECHNIQUES FOR HYDROLYZING AND CHARACTERIZING ANTHOCYANINS ON PAPERGRAMS FOR THE STUDY OF BARLEY HYBRIDS

In order to facilitate comparisons of anthocyanins obtained from hybrids and their corresponding parents, a technique of partial and complete hydrolysis of anthocyanins on the paper and a clamping technique* was developed to aid in characterizing the hydrolyzed products. The techniques effected immense saving of time and helped to avoid the instability problems associated with elution. The earlier techniques associated with identification of anthocyanins and anthocyanidins directly on paper (without elution), (a) by sewing (Sec. 13) and (b) by spectral analysis (Sec. 14) were greatly extended.

Direct hydrolysis on the paper has been carried out with

*Clamping Technique has also been used by Schlogl and Siegel (1953): see Lederer and Lederer, p. 129.

compounds that hydrolyze easily with enzymes (Chargaff and Kream, 1952; Williams and Bevenue, 1951). However, direct hydrolysis on the paper with acidic reagents presents two major difficulties, the first being the need to simulate a liquid state on the paper and the second being to avoid the pastiness and frangibility of the paper associated with acid hydrolysis. The first problem was resolved by the addition of glycerol to the hydrolytic reagent and by carrying out the hydrolysis in a specially designed hydrolysis chamber. Glycerol, because of its high boiling point, effects the hydrolysis quickly. The second difficulty was overcome by hardening the chromatopaper prior to rechromatography by a clamping technique.

The value of the technique in a large scale characterization will be illustrated briefly using the spermoderm extracts of a hybrid, 33-B1B1-13 ♀ x Black Hulless ♂ (Greenhouse Collection 441) and the colored parent, Black Hulless (Greenhouse Collection 456) and several other flavonoid glycosides available commercially.

20.1. AN APPARATUS AND TECHNIQUE FOR HYDROLYSIS

The hydrolysis chamber (Fig. 20.1) utilized a pyrex tray (18" x 12" x 2½") the rim of which was etched with carborundum powder and glycerol against a lid of mirror glass (18½" x 12½" x ¼"), with a to-and-fro movement so that the lid was also etched uniformly at least an inch wide around its perimeter. A frame, 14½" x 8" x 1½", was constructed of pyrex glass rods. Thirteen removable glass rods 10½" long

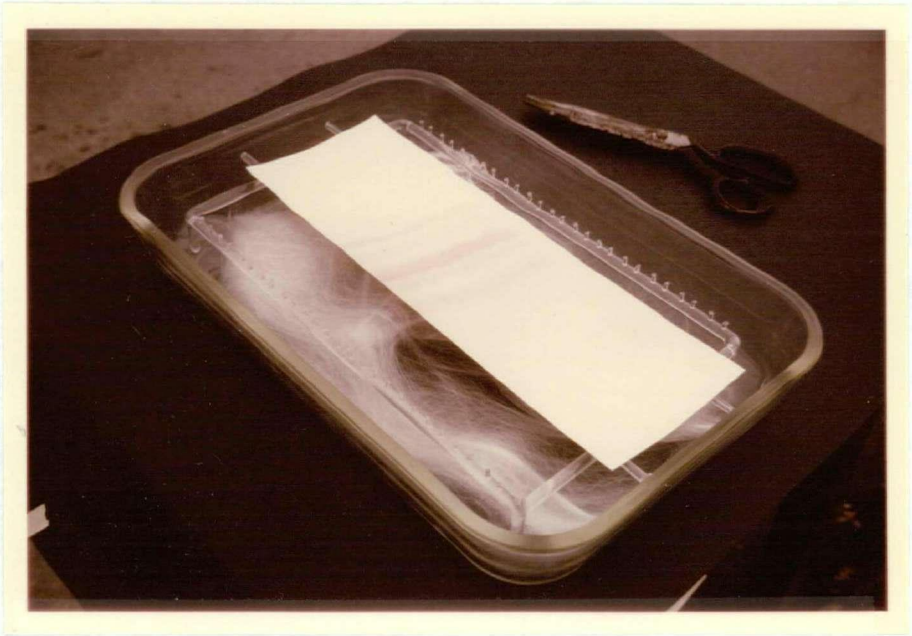


Fig. 20.1.
Apparatus for hydrolyzing anthocyanins and related glycosides on chromatopaper.

were placed 1" apart across* the frame to serve as a bed for supporting chromatopaper. The rods were kept in place by small glass stops fused to the longitudinal bars of the bed. A layer or two of pyrex glass wool was laid on the floor of the tray and the bed placed on top. The glass wool and the glass rods are thinly sprayed with N HCl, primarily, to prevent sticking of the paper to the bed after the hydrolytic operation.

Chromatograms developed on Whatman paper No. 3 showing several anthocyanin bands (Fig. 20.1; see also Fig. 20.3) are sprayed liberally with a hydrolytic reagent consisting of 3N HCl and glycerol (3:1,v/v), and placed on the glass bed. The tray rim as well as the etched surface

*On the basis of subsequent experiences, the cross rods were dispensed with because they cause interference in the detection of colorless compounds. Instead, several glass rods 16" long were placed at equal distances across the bed but along the length of the bed as shown in Fig. 20.1.

of the glass lid are smeared with Dow Corning high vacuum silicone grease. The lid is sealed tightly and the hydrolysis chamber is placed in an oven at 110°C for 30-45 minutes for complete hydrolysis or for a few minutes for partial hydrolysis.

After hydrolysis, the bed along with the chromatogram is removed from the chamber carefully and placed on a hot plate at low temperature (ca. 80°). The width of hot plate should be smaller than the width of the bed so that the frame (not the legs) are in direct contact with surface of the hot plate. The transference of the bed to the hot plate is performed carefully because the paper at this stage is pasty and frangible. In the course of the low temperature drying, the paper is hardened but must be handled with care. Glycerol, because of its high boiling point and hygroscopic nature keeps the paper moist even after hardening, and thus reduces the frangibility of the paper.

As soon as the paper is hardened, and this generally takes 15 to 30 minutes, several band segments of equal sizes (see later) are excised using metal templates. The excised segments are chromatographed by a clamping technique described next.

20.2. CLAMPING TECHNIQUE

The paper segments carrying the hydrolyzed anthocyanins, despite the hardening operation at low temperature, require skillful handling for rechromatography in different solvents by the sewing technique described earlier (Sec. 13). If the band segment is stitched tightly to secure intimate adposition, the paper crumbles. The difficulty was resolved by clamping, instead of sewing, the band segments to new chromatopaper. It

may be added in passing that clamping as a technique has been used by other workers (see Lederer and Lederer, 1957, p. 129), but the clamping as described below is simple, and meets the needs of this work.

The equipment consists of 1" long teflon clamps*, and an assortment of 0.1" thick glass slides (microscopic slides are unsuitable), with buffed edges, in the following sizes:

7/16" x 3"	10/16" x 3"
7/16" x 4"	10/16" x 4"
7/16" x 5"	10/16" x 5"

Different methods of clamping anthocyanidin and anthocyanin band segments are shown in Fig. 20.2: A, B, and C. If the pigment in the band segment is strong, only one band is clamped. However, when the pigment is weak, up to 4 band segments of equal size, half below and half above the new chromatosheet (Whatman paper No. 3) are clamped so that they are uniformly opposite one another. The uniformity is facilitated by performing the operation against a light source after moistening the glass slides slightly with appropriate solvent. Uniform and intimate contact of the band segments with the paper is essential for maximum resolution.

The intimate contact of unhydrolysed anthocyanin band segments, with fresh chromatopaper is better achieved in another way. Usually a 3/8" x 1" band is cut out with a template, folded in half along its length, with small incisions, approximately 1/8" long, made at either end of the fold as shown in Fig. 20.2. A slit, approximately 14/16", is cut across the starting line of the chromatography paper so that it is equidistant

*The teflon clamps distributed by California Laboratory Equipment Co., 1717, 5th Street, Berkley, Calif., U.S.A., Catalogue No. 103, possess appropriate tension and are most suitable for the purpose.

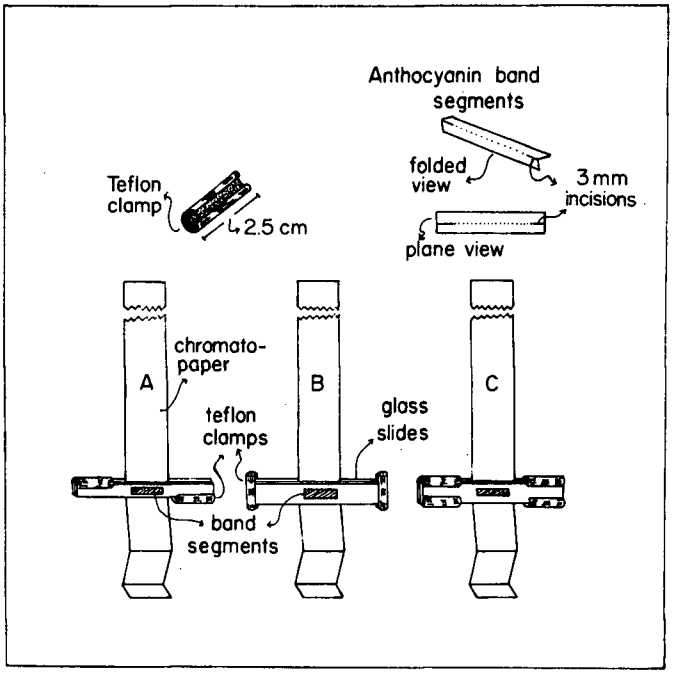


Fig. 20.2
Methods of clamping anthocyanin and anthocyanidin bands to fresh chromatopaper.

from either side of the chromatopaper. The folded anthocyanin band is pushed through the slit so that the folded surfaces are on opposite sides of the chromatography paper. The incisions on the fold are dovetailed into the ends of the slit to secure the band on the starting line. A pair of slides are then clamped on to secure uniform contact of both the folds to the chromatography paper.

It is essential that anthocyanin band segments obtained from Whatman paper No. 3 be clamped to Whatman paper No. 3, and band segments from Whatman paper No. 1 to Whatman paper No. 1 for rechromatography.

During chromatography, the band segments and the chromatopaper swell, when the irrigation solvent passes beyond the starting line. The teflon clamps, being flexible, act like springs and expand to accommodate

the swelling paper. The pressure generated in the swelling paper against the glass slides is sufficient to crack the slides, in the middle, during chromatography, if elastic bands or other means of clamping are used but not when the flexible teflon clamps are used. Through the flexibility of the teflon clamps, an even pressure is achieved throughout the surfaces of contact between the band and the paper. Of the three possible ways of clamping shown in Fig. 20.2:A, B, and C, the cross-clamping using only one pair of clamps, as shown in B, gives optimum flexibility and uniform contact and hence best results.

20.3. THE TECHNIQUE IN USE

The technique as used on comparing the hybrid anthocyanin bands (Greenhouse Collection 441) with those of the corresponding colored parent (Greenhouse Collection 456) is illustrated in Fig. 20.3: chromatograms A to I. The chromatogram A (Collection 456) shows at least 10 anthocyanins and the chromatogram F (Collection 441) about 8 anthocyanins. In the interest of brevity, the comparative chromatography of only the Band No. 1 in a few solvents will be presented. In order to determine the correspondence of the parental and hybrid anthocyanins, a portion of each anthocyanin band was excised and chromatographed by clamping in different anthocyanin solvents (Table 10.II). The results from band No. 1 of Collections 456 and 441 in the HAc-HCl solvent are shown in Chromatogram B and G respectively. In both cases, band No. 1 yields a major anthocyanin R_f0.32 and three weak anthocyanin trails. The spot No. 1, in both the cases are, therefore, identical. The chromatogram A was hydrolyzed for about 15 minutes and the chromatogram F, for about 40 minutes in the

Fig. 20.3
Partial and complete hydrolysis technique as used in a comparison of spermoderm pigments in Black Hulless (Collection 456) and its hybrid with 33-B1B1-13, (Collection 441). N1N, anthocyanin. D1N, anthocyanidin.

hydrolysis chamber. The results of only the band No. 1 after chromatography by clamping in the HAc-HCl, Forestal (Table 17.I) and Formic acid -4NHCl (1:1) solvents are shown in Fig. 20.3. Whereas the band No. 1/441 (chromatograms H and I) shows complete hydrolysis to the parent aglycone, cyanidin, the band No. 1/456 (chromatograms C, D and E) shows only partial hydrolysis. It may be added that the R_f values given by clamping technique following hydrolysis are within acceptable limits of accuracy. Using these techniques, a great many comparisons of hybrid and parental anthocyanins have been made.

The hydrolysis and clamping techniques, in addition, were used

successfully with several flavonoid glycosides such as apigenin-7-glucoside, quercitrin, and rutin.

20.4. DISCUSSION

The ease with which anthocyanins and other flavonoid glycosides can be hydrolysed and characterized by the technique of hydrolysis on paper greatly extends the usefulness of paper chromatography. In studying the flavonoid glycosides of barley, which are many, the ease and rapidity of the techniques cannot be stressed too highly. Thus, if 15 glycosides are present per chromatogram, and three chromatograms are hydrolysed at a time, the hydrolysis of 45 glycosides may be effected within an hour. The hydrolysed glycosidic bands, after excision, may be chromatographed right away in several solvents. The technique circumvents many of the problems posed by the general instability of flavonoids because elution, flash evaporation, hydrolysis in solution, as steps in processing, are eliminated.

The amenability of the technique to partial hydrolysis of anthocyanins on paper extends its value in detailed identification work. However, stringently reproducible conditions for controlled partial hydrolysis will have to be established. For instance, the time required for the hardening operation, at low temperature, is not uniform: chromatograms, which are sprayed liberally with the hydrolytic reagent require a longer period of time for hardening. Longer the period of time required for hardening, the greater will be the hydrolysis.

The hydrolytic reagent remains stable for a long time. The strength of acid in the reagent may be adjusted to suit the requirements.

The determination of R_f values by sewing (Sec. 13), or clamp-

ing (Sec. 20.2), the measurements of spectral characteristics of anthocyanins and anthocyanidins directly on the paper (Sec. 14) and further characterization of anthocyanins by partial and complete hydrolysis on the paper, as shown in this section, greatly enhance the ease with which comparative studies can be carried out. The techniques were extensively used in determining quickly the correspondence of one pigment band to another pigment band, because, at several occasions, the resolution of anthocyanins in the preparatory chromatography (Sec. 10) of crude plant extracts is variable.

20.5. SUMMARY

A micro-method for carrying out acidic hydrolysis of anthocyanin and related flavonoid glycosides was developed to aid hybrid and parental pigment comparisons in conjunction with the techniques described in Sections 13 and 14. The hydrolysis on paper poses two major problems, namely, accomplishing a sustained liquid state on the paper for quicker hydrolysis and controlling the frangibility of the paper following the hydrolysis. The liquid state is accomplished by the addition of glycerol to the hydrolytic reagent, and the frangibility of the paper is controlled both by a hardening operation prior to chromatography, and by carrying out chromatography by a clamping technique. The hydrolysis is carried out in a specially designed chamber.

A large number of flavonoid glycosides appearing on a preparatory chromatogram, and in fact several such chromatograms, are hydrolysed in the chamber within an hour. Hence, elution, and hydrolysis in solution, of each glycoside separately are eliminated: an immense saving of time, indeed.

PROCEDURES DEVELOPED FOR THE STUDY OF PEDIGREE STOCKS

21. A METHOD FOR COLOR CLASSIFICATION OF BARLEY ALEURONE LAYERS

Although the genetics of color in barley kernels has been extensively investigated, the results lack uniformity (see reviews: Smith, 1951; Nilan, 1964). Smith (ibid.) felt that the variability in results was ascribable to the difficulties in accurate distinction of pericarp color owing to the superimposed glume color, and recommended the use of naked varieties for color analysis. Buckley (1930), in order to make color classification "more nearly correct," used naked strains of barley.

Dependable genetic classification of aleurone color may not be achieved even by the use of naked varieties for three principal reasons: (a) Harlan (1914) observed that "the color of aleurone is influenced to a very high degree by climatic conditions. It is very difficult to

separate blue aleurones from white in kernels produced under humid conditions especially if the blue color is of low intensity. Under arid conditions, separations of blue and white colors are easily made, and here it is even possible to distinguish even three or four shades of blue," (also see Wiebe and Reid, 1961). (b) Observations made by the writer on many colorants of barley (Sec. 4) by visual examination of aleurones after laboriously peeling outer tissues (Sec. 3) reveal that aleurone possesses several shades of colors until late hard dough stage (Sec. 4). Cream colored aleurone tissues may become red, owing to the presence of anthocyanin pseudo bases, in several cases on exposure to acid (Table 4.IV). Thus, those aleurones, which are potentially anthocyanogenetic, may be classified erroneously as anthocyanin-free. Deep, or dark olive buff, vetiver green and serpentine green aleurones (Sec. 4.3.2.8, Appendix: Tables 27.I to 27.XX), which simulate blue anthocyanin colors of aleurone, may develop much to no anthocyanins, when tested with acid. Thus, there is a possibility that aleurones, which in fact may not contain anthocyanins, but, which seem to be anthocyanogenetic by surface examination, may be classified erroneously as anthocyanin-containing. (c) The transient appearance of anthocyanins in spermoderm and aleurone tissues (Secs. 4.3.2.7, and 4.3.2.8) creates further problems in genetic classification. Anthocyanins do appear in these tissues, yet they may not be detected because of the superimposed pigmented or non-pigmented tissues.

Thus, in order to (a) substantiate Harland's (loc. cit.) observations of the influence of environments on the expressivity of color in the aleurone, (b) provide a dependable basis of genetic classification of aleurone, (c) study the probable appearance and disappearance of aleurone pigments during development (Sec. 4.3.2.8) and (d) gain an

understanding of the physiological functions, and economic role of the aleurone layer, it is essential that the aleurone layer must be accessible to direct visual and biochemical examination.

The direct accessibility of the different tissues of barley to physical and chemical examination was first made possible by the peeling technique (Sec. 3). However, the technique is of limited usefulness because of the heavy labor input and because it cannot be used after hard dough stage. To follow pigment physiology in aleurome tissues after hard dough stage (Sec. 6), and to classify mature and dough stage aleurone pigments dependably, another method was developed latterly, and is described below.

21.1. BACKGROUND OF THE METHOD

The purple variety, Black Hulless, is rich in anthocyanins in spermoderm and aleurone layers: the anthocyanins in spermoderm are red (flavylium salt state), and in aleurone blue (anhydro base state). The studies described in Section 6 indicated that when the intact developing seed of Black Hulless are placed in acidified methanol, the anthocyanins are extracted selectively from the outer coverings of the seed, and that blue aleurone anthocyanins remain *in situ* even after six years of standing in acidified methanol (Sec. 6.4.2.c).

The selective extraction is attributable to the outer coverings (pericarp, spermoderm) of barley caryopses, which are semi-permeable to common mineral acids (Brown, 1907; Collins, 1918). Brown (*ibid.*) believed that the semipermeability was due to the nucellar epidermis (see Figs. 3.1, 3.3 and 3.7), whereas Collins (1918) maintained that it was

associated primarily, with the tegmen. The seat of semi-permeability does not appear to have been precisely determined (see Sec. 22). Collins (ibid.), however, did establish that there was little passage of water through the outer coverings and that absorption of water occurred mainly through the sheaf of chalazal cells. Brown (1907) made another relevant observation. He noted that when undamaged grains of blue barley are steeped in dilute acidic solutions, although the contents of the grain become soft and swollen, the aleurone cells retain their original blue color for at least a week. However, the seeds on extended steeping in a N sulfuric acid or N hydrochloric acid become red within a period ranging from 7-24 days (Brown, 1909). Thus, the impermeability, either of the coverings, or of the absorption region (chalaza), or both, is impaired after about a week when the acid, along with water, seeps through.

Subba Row, Bains, Bhatia and Subrahmanyan (1953) used 3N hydrochloric acid for chemical peeling of wheat grains. Pollock, Essery and Kirsop (1955) used 50% sulfuric acid steeping for removing barley husks as an aid in the germinative capacity test of barley. Morgan, Barta and Kilpatrick (1964) used 20% sodium hydroxide for loosening wheat bran.

Thus, owing to the differential extractability of anthocyanins as a consequence of the impermeability, the aleurone pigments could be exposed to direct physical examination, without any modification, by peeling the outer coverings chemically. The 50% sulfuric acid reagent of Pollock et al., (ibid.) was selected finally for chemical peeling because it does not cause any apparent physiological impairment of the seed, and because dilute sulfuric acid is widely used to break the

dormancy of hard seed coats e.g., sweet clovers and several other leguminous seeds. Hydrochloric acid was not used because it requires a longer period of time for decortication and also because it seriously affects germinability.

21.2. MATERIALS

Flint state kernels of the hull-less stocks, namely, 71-Pr Pr-10 (purple), 71-pr pr-10 (white), Black Hulless (purple) and Gopal (purple) and stocks with hulls, 33-B1B1-13 (blue), 36-B1B1-21 (blue), Montcalm (blue), Trebi (peculiar blue), 33-b1b1-13 (white), 36-b1b1-21 (white), Golden Pheasant (white), Lion (black) and Gatami (black) were used. In one case, developing kernels of Black Hulless around mid hard dough stage were also used.

21.3. CHEMICAL PEELING

In all cases, 50% sulfuric acid was used for steeping barley kernels. The hull-less flint state kernels of the stocks used as well as the young developing seeds of Black Hulless (intact with lemma and palea) were steeped for 2 hours, the hulled varieties other than the blacks, for 5 hours and the black varieties for about 15 hours. At the end of the steeping, the kernels were washed repeatedly with water; the hulls and the outer coverings were removed partially by shaking vigorously during washing in a flask. Finally, the outer coverings were removed and aleurones and embryos exposed completely by gentle rubbing with hands in rubber gloves. The seeds were dried by spreading over paper towels for a few hours.

A few of the kernels with exposed aleurones and with intact embryos were allowed to germinate in petri dishes.

The aleurone colors were classified by direct examination and again after dipping the kernels for about an hour in 1% methanolic HCl.

21.4. RESULTS

21.4.1. Chemical Peeling

Although the periods of 5-hour, and 2-hour steeping for the varieties with and without hulls gave tolerably good decortication of the outer coverings, the black varieties required 15-hour steeping. The developing caryopses of Black Hulless (with hulls intact) required much less time, usually 1 to 2 hours, than the mature kernels. The results of chemical peeling for only two varieties, Black Hulless (purple) and Gatami (black) are shown in Fig. 21.1. The aleurones of both the varieties remained blue after steeping for a period of 2, and 15 hours, respectively, in the sulfuric acid.

It was observed that immediately after steeping, the outer coverings of the purple varieties become red. The acid gradually corrodes the outer coverings but its scarifying and solublizing action does not reach the aleurone or embryo, both of which remain unaffected. The decorticated seeds retain their viability, and germinate in petri dishes readily even after a 15-hour steep. The red anthocyanins in the purple varieties are localized almost exclusively in spermoderm. It was pointed out in Section 3, that spermoderm can be peeled apart, around soft dough stage, into two layers consisting probably of tegmen (facing

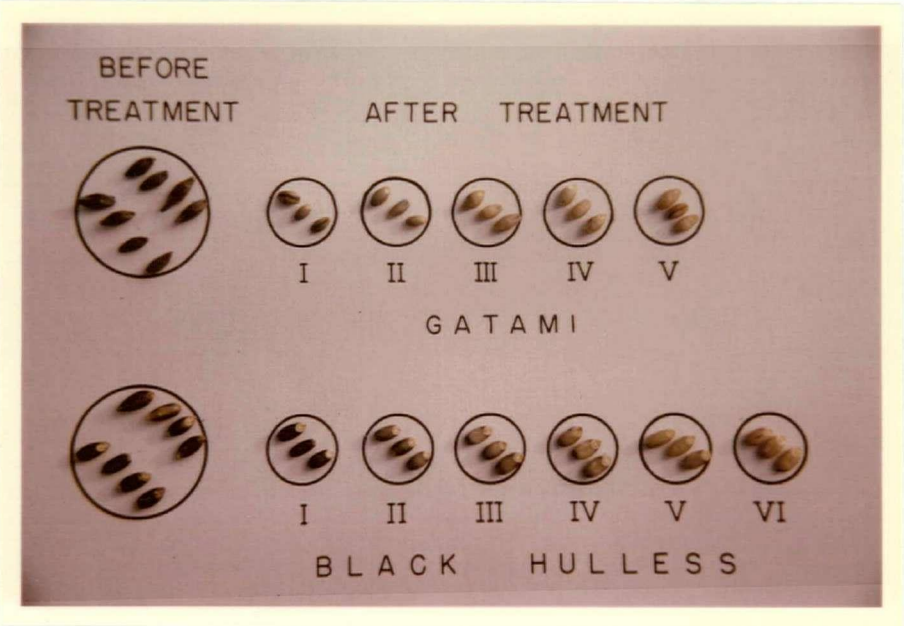


Fig. 21.1
Chemical removal of the outer tissues of barley caryopses to reveal aleurone tissue colors. Grains are of the black variety Gatami and the purple variety Black Hulless before and after treatment.

the pericarp) and nucellar epidermis (facing the aleurone envelope). It is certain that the layer facing the pericarp is pigmented heavily, and sulfuric acid acts upon this layer readily. Thus it appears that the tegmen is not impervious to the action of sulfuric acid. These observations therefore support the observations of Brown (loc. cit.) that it may be the nucellar epidermis alone or the nucellar epidermis and the aleurone envelope jointly, that may block the penetration of sulfuric acid. It will be interesting to determine biochemically, what makes the cell walls impervious to the acid. It was noted that if the seeds are steeped for a shorter period of time, small shreds, probably of the nucellar epidermis, remain attached, in situ, over the aleurone envelope. If the shreds are not completely removed from the blue aleurones during the decortication process, the areas of the aleurone covered by the

shreds, upon treating with 1% methanolic HCl, do not become red. The presence of the shreds, therefore, must be checked for dependable classification of the aleurone colors. The shreds can be removed easily with a tweezer when wet. Work on the chemo-histology of the tissues might throw light on mechanisms of tissue permeability.

The chemical peeling worked satisfactorily on developing as well as mature kernels. The developing kernels, with hulls intact, of Black Hulless around hard dough stage required about 2 to 3 hours for decortication. It was noted that aleurones became harder after the steep. If the steeping is continued for 15 hours, the young aleurones become flinty obviously because of dehydration. However, after the 15-hour steep, the blue aleurones become slightly purplish red indicating that the impermeability of the young kernels is destroyed at a faster rate than that of the mature kernels.

21.4.2. Color Classification of Flinty Aleurone Tissues

The availability of aleurones on large scale afforded an opportunity to resolve (see Secs. 4, 6, and 9) whether the delayed development of reddish color in colorless aleurones upon exposure to 1% methanolic HCl could, in part, have been due to leucoanthocyanins. The white aleurones (flint state maturity) of the isoline 36-b1b1-21 were placed in the acidified methanol for 48 hours. No red color appeared during this period. The solvent, however, did extract yellowish materials from the aleurone. Over a period of 48 hours, the wheat colored aleurones are almost bleached white. It was established from the observations noted in Section 22.2, that true leucoanthocyanins of barley aleurone do not change color in dilute acid, and the red color that develops in the acid

treated aleurone of many 'leuco or colorless' varieties must be due to anthocyanins in the pseudo base or anhydro base states (Secs. 4, 6 and Appendix).

The aleurones of the white isolines 36-b1b1-21, 33-b1b1-13, 71-prpr-10 were found to be uniformly white and showed no segregation at flint state maturity, both before and following the acid treatment. Although the aleurones of the black variety Lion were generally white, aleurones from a few kernels did show dark shades, possibly to be designated as a weak blue. Such kernels were put in the acidified methanol for 48 hours. Anthocyanins did not develop, and all aleurones were bleached white. Thus, the black variety Lion also possesses aleurones which are anthocyanin-free like those of the white isolines. About 700-1000 seeds of each stock were peeled chemically and used for the above observations.

The aleurones of the blue isolines 36-B1B1-21, and 33-B1B1-13 showed a great deal of variation in blue color. The aleurone colors of 36-B1B1-21 were arbitrarily grouped into five color classes. Of the 604 aleurones, which were obtained by chemical peeling, 194 showed good blue (Class I), 252 medium blue (Class II), 108 slightly less blue (Class III), 36 near white (Class IV), and 14 white (Class V). The range of blue color variation in this isoline (and also in other stocks) is so gradual that discrete classes are set with difficulty. The aleurones which may possess very weak blue colors cannot be readily distinguished from white aleurones because the shade of weak blue blends in imperceptibly with the background of wheat-colored aleurones. Thus, it is not only the classification of blue colors <u>per se</u>, but also the differentiation of anthocyanin-free aleurones that adds to uncertainties

in the classification.

It was observed that the presence or absence of anthocyanins is better ascertained by treatment with the acidified methanol because the reagent dissolves away the yellowish materials from the aleurone, but not anthocyanins from the mature flinty aleurones (see Sec. 6). The often yellowish background colors of aleurone tissues become relatively light and provide a better background for the red anthocyanins. The acid treatment is necessary for at least two other principal reasons: (a) to confirm that the bluish shades are due to anthocyanins, because aleurones of certain varieties, as was demonstrated in Section 4.3.2.8, do develop non anthocyanin off-blue colors, and (b) it was also shown (Table 4.IV) that aleurone colors, such as, deep and/or dark olive buff (Ridgway, 1912) or vetiver green (Ridgway, ibid.) may show significant variation in anthocyanins upon exposure to acid. Thus, the classification of aleurone anthocyanins without the acid treatment may be potentially misleading in the case of certain stocks.

For reasons stated above, half of the aleurones of each of the five color classes of the isoline 36-B1B1-21 were placed in 1% methanolic HCl for 12 hours. Following the acid treatment, the color definition of the aleurones which were difficult to classify, particularly, Classes IV and V became clear cut. Five color classes, based on the intensity of red color in aleurones were again arbitrarily decided: the Class I beginning with the maximum red color to Class IV with barely detectable red color and Class V without red color, i.e., white. The following class counts were obtained with and without the acid treatment:

	Color Class					
	I	II	III	IV	V	Total
Without acid treatment	97	126	54	18	7	302
With acid treatment	96	149	50	6	1	302

Of the 7 aleurones which were classified very carefully as pure white (Class V), only one aleurone remained pure white upon acid treatment; all others developed red colors belonging to Classes III and IV. Similarly, several adjustments had to be made with the 18 kernels of Class IV after the acid treatment. Accordingly, all aleurones after the acid treatment were mixed and reclassified. The classifications and reclassifications mentioned above were arrived at after several attempts. The observation stresses the difficulties of delineating distinct color classes of aleurones.

The other blue isoline 33-B1B1-13 did not show white aleurone segregates in 383 kernels (aleurones) that were obtained by chemical peeling. However, the blue color did show a range of variation similar to that found in 36-B1B1-21. Of the 383 aleurones, 132 showed good blue color (the color was slightly less than the Class I of 36-B1B1-21), 121 medium blue, 109 slightly less blue and 21 near-white or barely dectectable blue.

The "peculiar blue" variety, Trebi, was divided into 3 colored (blue) and one colorless (white) classes. Of the 589 aleurones, 262 showed good blue color (the color was as strong as that of the good blues of 33-B1B1-13), 225 medium blue, 90 light blue, and 12 white. When the 12 white aleurones were exposed to acid, all turned reddish within 2 hours. The observation again emphasizes the necessity of the

acid treatment for the detection of anthocyanins. Trebi has been classified as a peculiar blue variety by the breeders. The aleurones of the variety, however, do not exhibit any "peculiar" blueness on treatment with acid.

The kernels of the blue variety Montcalm possess a relatively large proportion of light blue and white aleurones, though a few aleurones of the variety do show as strong blue color as that of the 33-B1B1-13 aleurones. A portion of the white aleurones (27) was treated with acid. Of these 24 turned red, and 3 remained colorless. Thus, the variety shows slight segregation for aleurone colors.

Although the aleurone of black variety Lion is true white, the aleurone of the other black variety Gatami is blue (Fig. 21.1). In general, the blue color is more intense or deeper than that of 33-B1B1-13 or 36-B1B1-21. Of 767 aleurones, approximately 33 showed good blue, 344 medium good blue, 340 medium light blue, 40 very light blue and 10 white. The whites on treatment with acid turned red. Thus, the variety does not show any anthocyanin-free white aleurone segregates.

The white variety Golden Pheasant (in this stock only) does not appear to be true white. Of the 465 aleurones examined, 42 showed good blue colors equivalent to the good blues of 36-B1B1-21, 90 were good medium blue colors equivalent to the good blues of 33-B1B1-13, 131 medium blue and 202 near white. Of the 202 near white aleurones, upon the acid treatment, 190 became red. Several of these kernels showed a remarkably good development of red color. The good color development must be due to anthocyanin pseudo bases (cf., Secs. 4.3.2.8 and 4.4). The observation again emphasizes the necessity of the acid

treatment not only in the differentiation of anthocyanins from anthocyanin pseudo bases, but also in the determination of the color intensity for the purposes of genetic classification. Twelve aleurones of Golden Pheasant were 'white' on superficial examination. It was noted that several of these aleurones were covered with the 'shreds', probably of the nucellar epidermis (see Sec. 21.4.1). The shreds were removed with a pair of tweezers and the aleurones were again placed in the acidified methanol; 4 aleurones turned red, but the remainder were white. Thus, out of the 465 aleurones examined, only 8 showed white color, the other 457 were blue. The variety, therefore, should be classified as a 'blue' instead of a 'white'.

The kernels of the varieties described thus far develop anthocyanins only in aleurone, but not in spermoderm and pericarp. Special precautions are needed with the purple varieties which do develop anthocyanins in spermoderm and pericarp as well. Usually it was observed that the pericarp-spermoderm tissues remained adhering at the furrow, hence, a little longer steeping time should have been used (see later). The decorticated kernels of the purple varieties were first placed in 0.01% methanolic HCl for 2 hours. The treatment removes bulk of the spermoderm anthocyanins and also the yellowish materials from the aleurones. The solvent, however, does not extract the anthocyanins of the aleurone which retains its bluish color. The treatment with weak acid is repeated as often as necessary to selectively remove the anthocyanins of the spermoderm. The aleurones are then placed in 1% methanolic HCl as usual.

The purple variety, Gopal, was classified initially as a variety with largely white aleurone but with occasional segregates

possessing very light blue colors (Sec. 4 and Appendix). Because of the relative ease of observing a large number of aleurones by chemical peeling, the classification must now be changed. The variety possesses largely blue aleurones but also some pure white aleurones. Although the counts on a large sample will be compiled later, a small sample of 170 aleurones, showed 124 blue and 46 white aleurones. The blue color of this variety in general is very weak. In fact, it is the weakest of the blue aleurone varieties investigated thus far. The observations on aleurone colors of Gopal clearly bring out the value of acid treatment as an accompaniment to visual assessment. The peeling technique used previously is limited only to hard dough stage when the color development of aleurone is still progressing. The discrepancies in results could be due to variations in the physiological age of the kernels and not solely to the small size of the sample. Extensive observations on the aleurone of this variety were made during manual peeling but, in general, the aleurones were found to be white.

The aleurone of the purple variety Black Hulless possesses a deep prussian blue (Ridgway, 1912) color (Sec. 4 and Appendix). The blue color is indeed very intense in most kernels, more intense by far than the blues of the good blue aleurone variety, Gatami (Fig. 21.1). The aleurones of this variety were divided into six color classes based on intensity of color (Fig. 21.1). Of 986 aleurones, 285 showed maximum blue color all over the aleurones, 319 about the same or slightly less blue color but with patches of light blue and white shades, 168 medium blue which was equivalent to the maximum blue of 36-B1B1-21, 88 still lighter blue and 14 white. The white aleurones looked to be "good" white, yet upon treatment with the acid, all turned red. Although,

the variety shows a wide range of variation in blue color, there are no true white segregates.

21.5. DISCUSSION

The results demonstrate that chemical peeling as a technique for classification of aleurone pigments, both during development and at maturity, is very simple and highly dependable. Manual peeling of the outer coverings of flinty kernels, for exposing the aleurones to direct observations, is impractical. Chemical peeling reduces the labour demand literally from months to hours.

In the earlier work (see Sec. 4.3.2.8), observations on the aleurone colors of a large number of varieties were made using 3 to 5 aleurones obtained by manual peeling of kernels slightly past hard dough stage. Although variation of aleurone colors within each stock was noted occasionally, the variations at that time were ascribed either to variations in the physiological stage of color development or environments. The possibility that the variation could be due to genetic segregation was not explored primarily because of the limited obtainability of the peeled aleurones and not because it was believed that the stocks were genetically uniform. The easy observation of large numbers of aleurones, at dough and flinty stages of development, by chemical peeling should ensure greater dependability of color investigations in barley and aid in the study of the causes of color variations in aleurone, one of the prime reasons for undertaking this study on barley anthocyanins. It will now be possible to discern whether the segregation of blue color into several classes, even in the blue isolines, is due to genetic or

environmental differences.

It was observed in the course of color classification of hybrid aleurones obtained from the greenhouse plantings (the results are not included in the thesis) by manual peeling, that the kernels at the top of the head were generally colorless. The observation suggests that the variations may be microclimatic or may be developmental in origin. This point should be kept in view in future investigations: each head could be divided vertically into several regions and seeds obtained from each region classified separately. The results obtained from Black Hulless, and also from several other blue aleurone varieties showed visually white aleurones which upon the acid treatment turned red: the red colors, upon the acid treatment, however, did not develop in the pure white isolines. The observation suggests that anthocyanins despite their low intensity or quantity do develop in the pure blue aleurone varieties. The differences in quantitative expression may be environmental or developmental. Genetic variation essentially within pure lines cannot be entirely ruled out; certainly 'blue color' may be associated with several 'intensity factors'. For instance, over a period of several years, it was observed that the aleurones of Black Hulless always developed intense prussian blue color; the intensity of blue color in all other varieties was always low. Inasmuch as anthocyanin color in barley leaf sheaths is not a homogeneous genetic character (Sec. 7), it would be instructive to determine if biochemically, the anthocyanins are similar in different color classes of the same stock and between the stocks. In any event, the method proposed for aleurone classification will greatly extend further studies not only on the genetic and environmental analysis of aleurone pigments but also the analysis of other entities of biochemical and physiological importance.

That the physiological activity of the seed is not destroyed is one of the best features of the method.

21.6 SUMMARY

The observations on (a) the differential extractability of anthocyanins from the first and the third extracts of the caryopses and (b) the inability of the acidic solvent to penetrate through the caryopses investments to the aleurone even over a six-year period (Sec. 6) were exploited in the development of a dependable method determining aleurone colors at flint state maturity by direct visual examination. The outer investments of the caryopsis are peeled chemically by 50% H_2SO_4 to reveal the aleurone. The labor demand by chemical peeling, in contrast to manual peeling, is reduced literally from months to hours.

It is demonstrated that the blue color of the aleurone shows almost a continuous variation, and it is difficult to group the color variation into discreet classes. The observations reveal that the current classification of some varieties requires revisions. That the germinability of the chemically-peeled seeds is not impaired holds promising possibilities for biochemical, genetical and physiological studies on pigment and other substances of the aleurone layer.

The observations are also applicable to similar studies on the aleurone of wheat grains by chemical peeling.

PROCEDURES DEVELOPED FOR THE STUDY OF PEDIGREE STOCKS

22. A METHOD OF EXTRACTION FROM THE SEPARATE TISSUES OF BARLEY GRAIN WITHOUT MANUAL PEELING

The nuclei in the pericarp and spermoderm (Sec. 3) of the barley caryopsis are of maternal origin and those of aleurone and endosperm of maternal and paternal origin. Investigations into the biochemistry and genetics of pigments in these tissues demand cognizance of this fact. Originally, manual peeling was used to separate the tissues with different genomic backgrounds (Secs. 5 and 8), but this required a great deal of effort and skill. Moreover, the quantities of tissues obtained were insufficient for detailed characterization of the anthocyanins. In order to extend studies on the nature of the anthocyanin pigments, a method is presented in this section that extracts selectively and separately the pigments from the maternal and maternal-paternal tissues and eliminates manual peeling as a step in the collection of tissues.

The method is based on the frequent observation that upon preliminary extraction of intact developing seeds of purple varieties, e.g., Black Hulless, with methanol containing 1% conc. HCl, the pigments are extracted from the outer coverings, but that the aleurones of undamaged seeds remain blue (Sec. 6.4.2.c). On the basis of chromatographic observations on the first and the third extracts obtained from the same batch of seeds of Black Hulless, it was inferred that what came in the first extract was largely the anthocyanins of pericarp-spermoderm and that what came in the third extract was partly the anthocyanins from the aleurone of the damaged seeds (Sec. 6.5.4). These observations were fully substantiated in the course of investigations on the development of a method for the classification of aleurone pigments (Sec. 21). In order to take advantage of these observations, it is necessary to present evidence that anthocyanins obtained from spermoderm by peeling are those obtained from the intact seeds.

22.1. MATERIALS AND METHODS

The purple variety, Black Hulless, which contains liberal quantities of anthocyanins both in the spermoderm and aleurone, was used in this investigation. Spermoderm was peeled manually from 8 young seeds at hard dough stage grown under Field conditions in the summer of 1965. The spermoderm peels and 10 intact seeds of Black Hulless obtained from the same head were extracted with 0.1% methanolic HCl separately under identical conditions. An aliquot of each extract was chromatographed in the BAW solvent by banding. In order to keep the pigment changes during extraction and handling to the minimum, the extraction and chromatography were comp-

leted within about 3 to 4 hours.

The pigment extraction from the aleurone had to be undertaken separately because the rate of extraction, even from the young aleurone, is very slow. About 50 seeds were extracted with 0.1% methanolic HCl. The extract was discarded. The extracted seeds were peeled chemically by the method described in Section 21 to obtain aleurones. The aleurones were extracted in enough 1% methanolic HCl (see Sec. 6) so that the solvent was barely above the surface of the peeled grains. An aliquot of the extract was chromatographed directly in the BAW solvent by ascent as described elsewhere (Table 10:II).

The remaining extracts of the spermoderm and aleurone were hydrolysed in 4 N HCl for 10 minutes, and processed further as described in Section 19.2.3. The hydrolysates were chromatographed by banding in the Forestal solvent. Appropriate anthocyanidin bands obtained in Forestal were excised and rechromatographed probably in the Iso-PrOH solvent (Table 17.I).

22.2. RESULTS

22.2.1. Comparison of the Extracts from Spermoderm and Outer Investments of the Seeds

The chromatographic results from the spermoderm peels, the intact seeds, and the aleurones obtained by chemical peeling are shown in Fig. 22.1: Chromatograms No. I, II and III respectively. Each of the Chromatograms No. I and II show at least four major bands which in R_f values, colors and relative concentration ratios are identical to one another. This is good evidence that (a) what is extracted from the intact seeds are the antho-

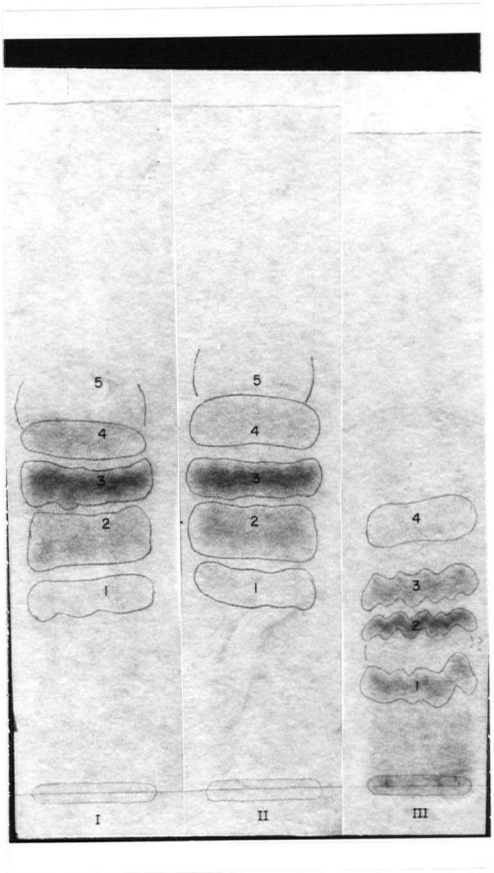


Fig. 22.1
Anthocyanins from Black Hulless caryopses; I, spermoderm peeled manually and extracted, II, spermoderm from intact caryopses extracted, and, III, aleurone tissue from which spermoderm has been removed chemically by 50% H_2SO_4 and then extracted. Spermoderm manually peeled and extracted and that extracted intact, yield identical chromatoband patterns, mainly composed of cyanidin derivatives. Aleurone yields mainly delphinidin derivatives.

cyanins of the spermoderm, and that (b) peeling as a step in the extraction of pigments from the spermoderm is unnecessary.

In the course of the peeling process, it was also noted that the pericarp as usual was colorless but for some small patches of pigments which appeared to be due to lacerated spermoderm. If the pigments develop in pericarp, their quantity is a minute fraction of the pigments present in the spermoderm. Thus, the contribution of anthocyanins from the pericarp is negligible. This view is further supported by the results of the peeled spermoderm and the intact seeds shown in Fig.22.1: no additional

pigment contribution is noted from the pericarp tissue of the intact seeds. Furthermore, it was shown in Sections 5 and 8 that if traces of pigments do develop in pericarp, then they are chromatographically identical to those of the spermoderm. Thus, a single extraction from the two surficial tissues is valid for determining the pigments of the maternal tissues of the caryopsis.

It will, however, be noted that the patterns and number of spermoderm anthocyanins obtained in Fig. 22.1 are quite different from those obtained elsewhere (Figs. 5.1, 8.1, and 20.3). The differences most likely are due, in part, to variations in environment between plants grown under field and greenhouse conditions. It is equally likely that the differences are also due to differences in physiological state. It is unlikely that the differences are due to partial hydrolytic breakdown, though the possibility cannot be ruled out (see Sec. 23). These points yet remain to be resolved unequivocally.

22.2.2. Extraction from the Aleurone Layer

Progress on the study of aleurone pigments, as pointed out in Section 6, was hindered greatly because of the difficulties of obtaining aleurones in substantial quantities for dependable investigations. A prime difficulty arises in obtaining aleurones from flinty kernels. The chemical peeling technique (Sec. 21.3) has greatly facilitated further studies on aleurone. A few observations will be described briefly.

The chromatographic patterns of anthocyanins obtained from the young aleurones (hard dough stage) of Black Hulless by chemical peeling are shown, along with those of the spermoderm, in Fig. 22.1. The R_f values and colors of the aleurone anthocyanins are quite different from

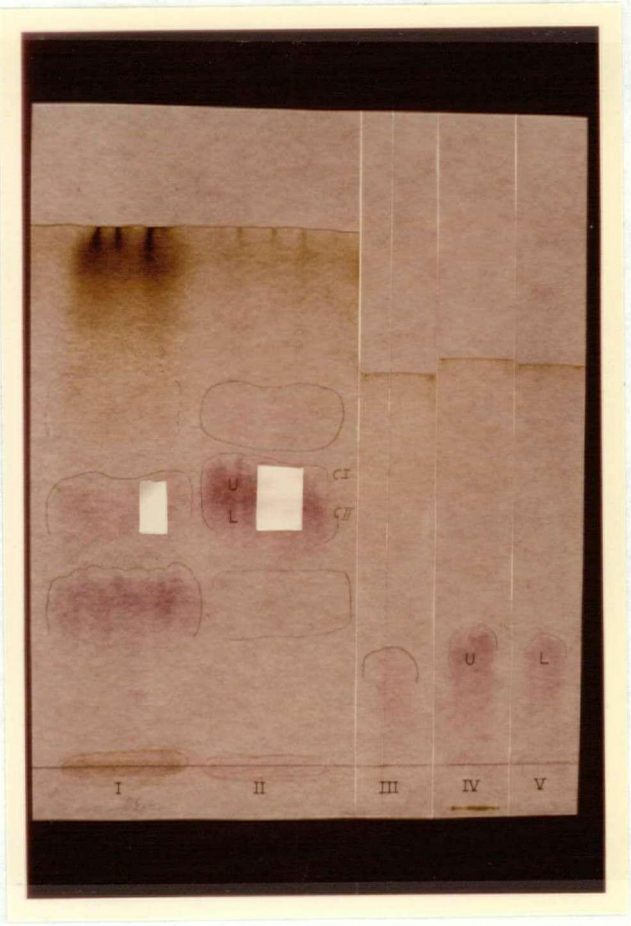


Fig. 22.2
Anthocyanidins from manually peeled spermoderm and from aleurone from which spermoderm has been removed chemically; I, Aleurone yields delphinidin and petunidin; II, Spermoderm yields cyanidin; III, substantiates the identification of petunidin excised from I; IV and V, excised from the upper (U) and lower (L) parts of the broad cyanidin band of II substantiates the pure cyanidin identification.

those of the spermoderm anthocyanins. The hydrolysates of the aleurone and the spermoderm anthocyanin extracts (aliquots of which are chromatographed in Fig. 22.1) upon chromatography in Forestal (Fig. 22.2) show basic differences. The aleurone shows at least two distinct anthocyanidins at R_f 0.35 (purple in visible and ultraviolet light), and at R_f 0.52 (mauve in visible and ultraviolet light). The purple component on the basis of earlier studies (Sec. 6.4.2.c and Sec. 19.3.3) is identified as delphinidin. The mauve component gave R_f 0.08 in the HAc-HCl solvent by clamping chromatography and, on the basis of color reactions and R_f values (Table 17.II), is provisionally identified as petunidin. The major anthocyanidin of the spermoderm (Fig. 22.2) as expected is cyanidin.

The color reactions of the cyanidin component are distinctly different from those of the petunidin component (see Fig. 22.2). In order to confirm the differences, the uppermost (U) and the lowermost (L) regions (discarding the central region) of the cyanidin band were chromatographed by clamping in the Iso-PrOH solvent (Fig. 22.2, chromatograms IV and V), which gives usually greater spread of R_f values between cyanidin and petunidin (see Table 17.II). Both the uppermost and the lowermost regions of the band resolved exactly at the same R_f values indicating that the cyanidin band of the spermoderm was not a mixture. The R_f values of the petunidin spot from the aleurone hydrolysates in the Iso-PrOH solvent (Fig. 22.2, chromatogram III), however, as expected was lower than that of the cyanidin.*

The spermoderm hydrolysates, in addition to cyanidin, also show another minor anthocyanidin R_f 0.69, which most likely is peonodin (Sec. 5.2.4), and a very weak, but nonetheless distinct band due to delphinidin. Again, pelargonidin was not recovered from the spermoderm. Although cyanidin is absent from the aleurone, there is, in all likelihood, a component at R_f 0.69, probably a peonidin, present in aleurone. Thus, it is clear from the results that cyanidin is a major pigment of the spermoderm tissue and is absent from aleurone. Delphinidin is a major pigment of the aleurone and is present only in traces in the spermoderm tissues. Petunidin, appears to be localized only in aleurone. Peonidin is present in spermoderm and may also be present in aleurone. The above

*The R_f values in the Iso-PrOH solvent from different determinations are highly variable (see Table 17.II); however, different anthocyanidins do show proportionate differences in R_f values, when they are chromatographed under identical conditions.

results hold for the hard dough stage kernels of Black Hulless which were grown in the summer of 1965 on the University Farm.

It may be added that the presence of delphinidin, even though it is weak, in the hand peeled spermoderm is not due to contamination from the aleurone. The delphinidin and petunidin in the aleurone arise from the aleurone anthocyanins and not leucoanthocyanin. This was determined by placing the chemically peeled white aleurones of the isoline 33-b1b1-13 in 1% methanolic HCl. The aleurone and endosperm of the isoline contain rich deposits of leucoanthocyanins. It was noted that the white aleurones did not turn red even after standing for a week in the solvent. The extracts upon hydrolysis did not yield anthocyanidin.

22.3. DISCUSSION

The results demonstrate that through chemical peeling the pigments from the maternal and maternal-paternal tissues of barley grain can be extracted separately. The basis of the separation lies in the 'impermeability' of the nucellar epidermis (Sec. 21.1). The elimination of highly laborious manual peeling as a step for differentiating the anthocyanin phenotypes of the maternal and maternal-paternal tissues of barley grain, doubtlessly, will extend further studies on the biochemical genetics of barley pigments. The method appears to be valid, not only for the studies on anthocyanins, but also for studies on other compounds of biochemical, physiological and commercial importance.

The pigments from wheat seed coats have been extracted by manual peeling (Miyamoto and Everson, 1958). It has been determined that our method can be extended to wheat kernels with equal ease. The

impermeable layer of the wheat grains may, likewise, be the nucellar epidermis.

It is necessary that healthy, undamaged, and preferably hand-picked seeds be used in large scale extraction for reasons discussed elsewhere (Sec. 6 and Sec. 21). Precautions are taken when the caryopses are divested of hulls: the hulls should be peeled from the apical end. If the hulls are peeled from the embryo end, occasionally the outer coverings of the seed are damaged and thus contaminants from the aleurone may also be extracted.

22.4. SUMMARY

It is demonstrated that the anthocyanins extracted from the intact and undamaged caryopsis and from the hand-peeled spermoderm are chromatographically identical. The chromatographic patterns of anthocyanins obtained from the chemically-peeled aleurones differ markedly from those of the spermoderm. Whereas the major anthocyanidin of the spermoderm is cyanidin, that of the aleurone is delphinidin. Surprisingly, traces of delphinidin were also recovered from the spermoderm hydrolysates. The second major anthocyanidin of the aleurone is identified tentatively as petunidin. It is demonstrated that petunidin does not occur in the spermoderm. Thus, it is clear that the pigments from the intact caryopses are extracted selectively from the spermoderm and not from the aleurone. The selective extraction is due either to the impermeability of the nucellar epidermis (Sec. 21) or the nucellar epidermis and aleurone envelope (Sec. 3) acting together. The selective extraction cannot be due to the impermeability of the tegmen (cf., Collins, 1918) because the anthocyanins from the

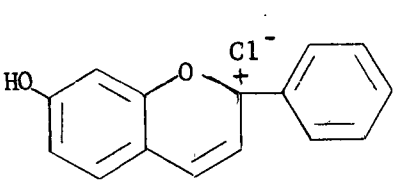
tegmen, which is the outer layer of spermoderm, are extracted completely.

With the development of the method for selective extraction, from the tissues of differing genetic background, the time-consuming manual peeling is dispensed with as a step in obtaining extracts from the separate tissues of the caryopsis.

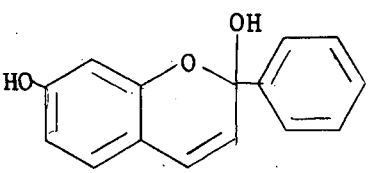
APPRAISAL OF ANTHOCYANIN STABILITY

(SECTIONS 23 AND 24)

Anthocyanin instability has been a problem, from the beginning, in elution and purification. Although the techniques developed in this laboratory to insure pigment stability made possible the work reported thus far, they were devised by trial and error. Appraisal of the anthocyanin instability had to be undertaken because the existing beliefs on the stability of flavylium (I), pseudo base (II), and anhydro base (III) states of anthocyanins contradicted either the



I



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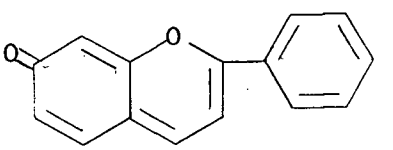


III

empirical basis of our techniques or served to confuse the instability problem. For instance, it is a well established belief that anthocyanins are best "preserved" (Robinson and Robinson, 1930) in acidic solvents below pH 1 (Ca. 1% HCl) and that pseudo bases of anthocyanins are relatively unstable. The studies described in Sections 10 to 15, however, indicated that acid concentrations, such as, 1% HCl were rather high and mainly responsible for degradation. It was for this reason that the anthocyanins in this study were eluted eventually with 0.03% methanolic HCl (Secs. 16 and 19). The acid concentration was reduced further because

of the washings with unacidified methanol (Sec. 19.2.2). At that low acid concentration, it was observed frequently that the anthocyanin eluates turned bluish red. The changes in color indicated that a portion of the anthocyanins in eluates existed as flavylium salts, a portion as anhydro bases and a portion, probably, as pseudo bases. Since the anthocyanins, empirically, remained more stable under these mild conditions and not when 1% HCl was used, a detailed study of factors affecting the stability of flavylium, anhydro base and pseudo base states of anthocyanins was undertaken. Although it has not been possible to include all these studies in the thesis, it will, nonetheless, be demonstrated that the current methods of processing anthocyanins, which are based on the belief in the stability of flavylium state, require modifications.

APPRAISAL OF ANTHOCYANIN STABILITY

23. INFLUENCE OF ACID ON THE STABILITY OF ANTHOCYANIN EXTRACTS AND ELUATES DURING FLASH EVAPORATION

Since Robinson and Robinson (1933), it is accepted that 1% HCl extracts and preserves anthocyanins. The same range of acid concentration is used generally for anthocyanin extraction, processing and elution. The extracts or eluates are finally concentrated or dried by flash evaporation by several workers, for instance, Asen, Siegelman, Stuart (1957), Harborne (1958, 1959a, 1960), and others. In this section, it will be demonstrated that this range of acid concentration in the extractants and eluents drastically affects the stability of anthocyanins primarily because of the concentration and drying by flash evaporation.

23.1. MATERIALS AND METHODS

23.1.1. Effect of Flash Evaporation of Anthocyanin Extracts to Dryness

As shown elsewhere, in order to bring the extraction procedure in line with those of the acknowledged authorities, for instance, Harborne (1958, 1959a, 1960), the basal leaf sheaths of several stocks grown in the University Greenhouses in 1961 were extracted with 1% methanolic HCl (Sec. 19.2.1). For the purposes of this investigation, aliquots of the concentrated anthocyanin extract of the basal leaf sheaths of Gopal and Black Hulless (Greenhouse Collections 400 and 402; Sec. 19.2.1) were flash evaporated to dryness at 30°C. The dried extracts were chromatographed from 1% methanolic HCl.

23.1.2. Comparison of Extractions with Methanol Containing 1% and 0.1% Hydrochloric Acid

23.1.2.1. The pigment rich dusts, obtained by pearling (Sec.6.3.1.c) the mature kernels of the hull-less purple varieties, Gopal and Black Hulless, were used for this investigation. The pearled dust of Gopal was extracted and processed within one day, as described in Section 5.1.2. To an aliquot of the concentrated extract was added, after four days, 1% methanolic HCl (5 x v). The diluted extract was reconcentrated (but not dried) in vacuo at 30°C for chromatography.

23.1.2.2. The pearled dust of Black Hulless was extracted separately, at the same time, with two solvents, viz. 1% and 0.1% methanolic HCl, at the total rate of 1 gram dust per 20 ml solvent. Initial extraction of the dust was carried out by shaking for a period of 48 hours using half the amount of solvent viz., 10 ml per gram dust. The extract was

obtained by centrifugation at 15,000 rpm. The anthocyanin-rich centrifuged pellets (see Sec. 6.3.1c) were re-extracted four times using a quarter of the remaining solvent each time. All the five extracts of the corresponding extractions (viz., 1% and 0.1% HCl) were combined separately. The extract containing 0.1% HCl was dried in vacuo at 30°C, but the extract containing 1% HCl was only concentrated to about 1 ml per gram dust and not dried at any stage. The dried extract of the 0.1% HCl extraction was made up to the volume of the 1% HCl extract with 0.1% methanolic HCl prior to chromatography.

All extracts were chromatographed by banding on Whatman paper No. 3 in the BAW solvent by ascent as described in Section 10.2.2.

23.2. RESULTS

23.2.1. Effect of Flash Evaporation of Anthocyanin Extracts to Dryness

Chromatographic evidence of the 'adverse' effects of flash evaporating 1% methanolic HCl anthocyanin extracts to dryness is shown in Fig. 23.1, Chromatograms 400 and 401. The results shown in Chromatogram 400 (see also Fig. 19.1) were obtained by concentrating the 1% methanolic HCl extracts as described in Section 19.2.1, and those in Chromatogram 401 by flash evaporating an aliquot of the concentrated extract 400 to dryness. The Chromatogram 400 contains 7 anthocyanin bands, viz., A, B, D, E, F, G, H and I: the details of identification of these bands are described in Section 19.3.7. For the purposes of this presentation, it may be added that the band I/400 is a mixture: the major component of the mixture, however, is cyanidin (Sec. 19.3.1).

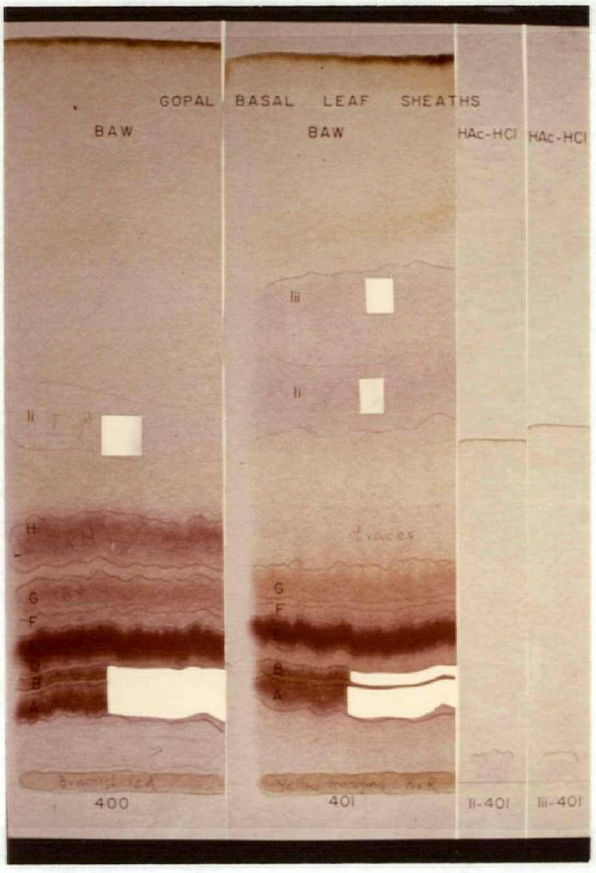


Fig. 23.1
Alteration of chromatoband numbers and sequences by flash evaporation. Anthocyanin extract 400, concentrated but not dried before BAW chromatography; 401 same extract, flash evaporated before BAW chromatography. Note especially the loss of band H and note the appearance of anthocyanidins Ii, and Iii in chromatogram 401. That Ii and Iii are anthocyanidins and not 'fast-moving' anthocyanins is shown in chromatograms Ii/401 and Iii/401 from HAc-HCl.

Conspicuous changes occur when the extract is flash evaporated to dryness as seen in Chromatogram 401. One of the major bands, H/400, and also the band D/400 disappear. Moreover, the intensity of the cyanidin band Ii/401 increases several folds over that of I/400, and also a new band Iii/401, which, more or less, is confounded with the band Ii/400 appears. The bands Ii/401 and Iii/401 by clamping chromatography give R_f values below 0.1 in the HAc-HCl solvent and are mixture of aglycones. Identical patterns were obtained upon flash evaporating the 1% methanolic HCl extracts of the Greenhouse Collection

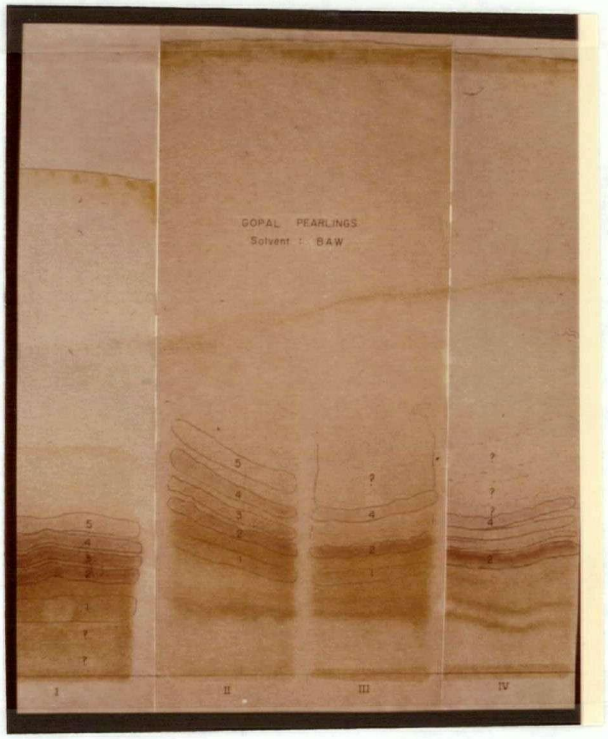
402 (basal leaf sheaths of Black Hulless: see Sec. 19.3.7) to dryness.

The results indicated that it was due to the increase in acid upon flash evaporation (Sec. 15.2.1) that the hydrolytic degradation occurred. In order to confirm that the concentration of acid was responsible for the modifications, the anthocyanins were extracted with 1% and 0.1% methanolic HCl. The results of this investigation are presented next.

23.2.2. Comparison of Extraction with Methanol Containing 1% and 0.1% Hydrochloric Acid

The pearled dust of Gopal was extracted with 0.1% methanolic HCl. The extraction, processing, and chromatography were completed in one day. The distribution of anthocyanins is shown in Fig. 23.2: Chromatogram No. I. After 4 days, to an aliquot of the concentrated 0.1% methanolic HCl extract was added 5-fold 1% methanolic HCl. The volume of the diluted extract was reduced *in vacuo* to about the original volume. The results of chromatography of the 4-day old 0.1% methanolic HCl extract, and after it was brought into 1% methanolic HCl are shown in Fig. 23.2, Chromatograms No. II and III, respectively. The maximum number of anthocyanins present in the 1% methanolic HCl extract is shown in chromatogram No. IV, which was obtained by applying about twice the amount of concentrated extract to that applied in chromatogram No. III. On comparison of chromatogram No. I and II, it becomes clear that no changes occurred in the 0.1% methanolic HCl extract in storage over a period of 4 days. Comparison of the chromatograms I, II on the one hand, and III, IV on the other, shows that when 1% methanolic HCl is added to the extract, the anthocyanin bands No. 3 and 5 almost

Fig. 23.2
Anthocyanins from Gopal pearlings extracted in 0.01% HCl-MeOH and chromatographed in one day (I); same extract after 4 days, then chromatographed (II); same extract after 4 days, diluted with 5x 1% HCl-MeOH and reduced *in vacuo* to original volume (III); same as (III) but applied in double concentration (IV). Note disappearance of bands 3 and 5 in (III) and (IV) and the appearance of two or more 'fast-moving' bands in (IV).



completely disappear. It is to be noted that in the preceding investigation (23.2.1), the degradation occurred when the 1% methanolic HCl extracts were dried *in vacuo*. The results of this investigation show that changes occur due to excess acid, because none of the extracts was dried at any stage during processing, and, therefore support the earlier conclusion that acid increases in the final stages of flash evaporation.

In the results presented thus far, the acid concentration of the extracts was increased either by flash evaporation or by the addition of extra acid but anthocyanins were not extracted directly using different amounts of acid in the extraction solvent. Thus, in another investigation, the pearled dust of Black Hulless was extracted separately with methanol containing 1% and 0.1% methanolic HCl under identical

conditions at the same time (see Sec. 23.1.2.2). The extracts after volume reduction were chromatographed. Although, the chromatograms of the 0.1% methanolic HCl are not traceable, the results of the two different extractions did show variations in anthocyanins comparable to those shown in Fig. 23.2. In order to determine the influence of flash evaporation to dryness, the remaining concentrated extract, obtained with 0.1% methanolic HCl, was evaporated to dryness. The results of this 0.1% methanolic extract after drying, and those of the 1% methanolic extract without drying are shown in Fig. 23.3, chromatograms I and II, respectively. It will be noted that whereas the chromatogram I shows two bluish red components, (2, and 3), the chromatogram II shows only one bluish red component, (b) but in very strong concentration. The bluish red anthocyanins No. 1 (chromatogram I) and anthocyanins No. a (chromatogram II) were identified as delphinidin derivatives by the techniques described elsewhere (Sec. 20). The delphinidin derivatives would have originated from the aleurone layer (Secs. 6.4.2.c, and 22.2.2). The color reactions of the anthocyanin No. 2 (chromatogram I) also appear to be those of delphinidin. Thus, in the presence of the commonly used concentrations of acid, namely, 1% methanolic HCl some hydrolytic modifications take place that instead of the two delphinidin derivatives obtained when 0.1% methanolic HCl is used, only one delphinidin derivative, but in relatively strong concentration, is obtained. It is notable that in the work reported previously (Mullick, Faris, Brink and Acheson, 1958), only one bluish red anthocyanin spot was noted to be present in the variety Black Hulless. The reasons for the discrepancy are self-evident.

The concentrations of the other bands in the two chromatograms

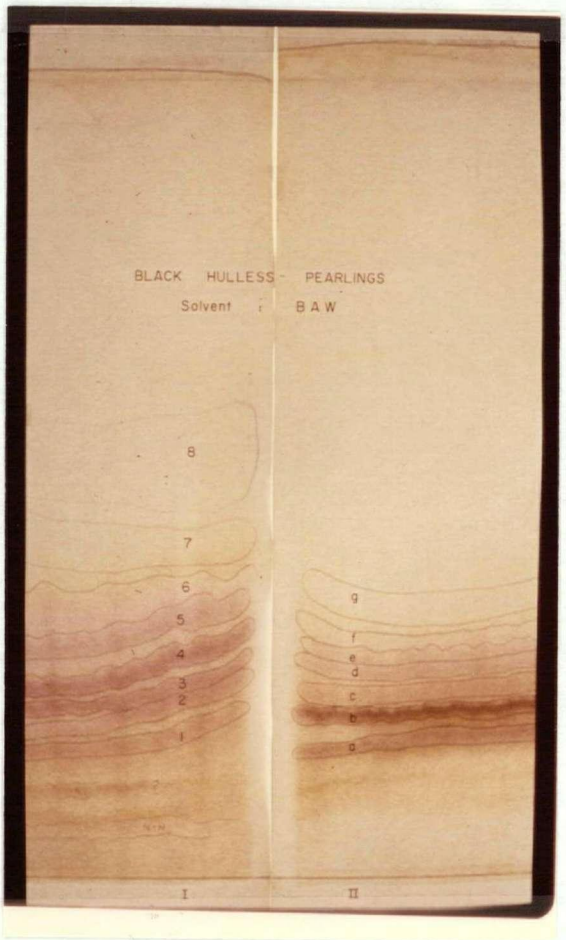


Fig. 23.3
Pearlings from Black Hulless extracted in 0.1% HCl-MeOH and dried in vacuo (I) and in 1% HCl-MeOH not evaporated to dryness (II) and chromatographed from BAW.

(Fig. 23.3) are so different that it is difficult to relate the identity of the corresponding bands. The band No. b (chromatogram II) emerges as the major spot. No such major spot is present in Chromatogram I. The Chromatogram I shows additionally, the presence of the bands No. 7 and 8 which were neither detected in Chromatogram II nor in the earlier work reported elsewhere (Mullick et al., ibid.).

23.3. DISCUSSION

The results demonstrate that extensive modifications of anthocyanins occur when the extracts are flash evaporated to dryness. The disappearance of several anthocyanins from the 1% methanolic HCl extract 400 (Fig. 23.1), and the appearance of the aglycone, cyanidin band Ii/401 in excessively large quantities upon flash evaporation is good evidence that (a) glycosidic hydrolysis occurs during flash evaporation, and (b), the hydrolysis is pronounced upon drying but minimal when the 1% methanolic HCl extracts are merely 'concentrated' in vacuo (note band I/400 (Fig. 23.1)). The conclusion is consistent with the key observation stated in Section 15.2.1 that upon flash evaporation to dryness of acidified methanolic extracts the odour of concentrated hydrochloric acid is pronounced. Thus, the concentration of hydrochloric acid increases towards the end of flash evaporation and it is due to increase in the acid concentration that partial hydrolysis occurs. The results of extractions with methanol containing 1% and 0.1% HCl further confirm that several modifications of anthocyanins occur when extractions are carried out with 1% methanolic HCl: some modifications, theoretically, should occur even when the extract is only concentrated and not dried.

The observations indicate that if acidic solvents are to be used, anthocyanins must be extracted with as little solvent as possible so that upon concentration in vacuo, minimal increase in acid concentration occurs. The detrimental influence of the solvent on anthocyanin stability was appreciated only towards the conclusion of this research program. The anthocyanins in early studies, for instance, from pearled dust of barley kernels had to be extracted repeatedly with the fresh solvent by

sonication (see Sec. 6, and also Mullick, et al., 1958), because the aleurone anthocyanins are extremely hard to extract. Thus, upon the in vacuo concentration the anthocyanins were stored literally in 'concentrated' HCl (see Sec. 15.2.1). It is primarily for this reason that the results obtained in the 1958 studies, or later in the studies reported in this section show variations. The variations from the biological materials, otherwise, could have been ascribed to several other causes.

The studies presented earlier in the thesis, were largely of a qualitative nature. Emphasis was placed primarily on extraction of anthocyanins from living tissues with as little change as possible. It was understood that slight variations in the quantity of solvent to the weight of tissues would have no bearing on the stability of anthocyanins. Thus, in order to minimize autolysis of anthocyanins, the quantification of extraction had to be compromised. The autolysis of anthocyanins in the peeled grain tissues (Secs. 5, 8) would have ensued had the tissues not been inactivated and extracted immediately after peeling. Again, the quantification was not undertaken because it should have been based on the exact amount of anthocyanins present in tissues at each stage of development so as to standardize the extraction ideally on the basis of amount of acidified solvent per unit weight of anthocyanins. Although the extractions could not be fully standardized for reasons stated above, the extracts, following the in vacuo concentration, however, were quantified to a standard volume based on the dry weight of tissues following preliminary extraction. In the course of large scale comparative work, even the tissue collection is highly time consuming. For example, the collection of about 300 different tissues and stages of development of several stocks in 1960 (see Sec. 10) took a period of over two months.

The processing and concentration of the extracts *in vacuo* took a similar period of time. The concentrated extracts were refrigerated (-15°C) for several months before they were standardized for chromatographic comparisons. Several hydrolytic changes must have occurred. It is thus clear that the concentration of the acidified anthocyanin extracts as well as the storage of the concentrated extracts is a potential source of variability due to degradation or artefact production.

If acidic solvents are to be used in extraction, the standardization of extraction on the basis of amount of acidified solvent per unit weight of anthocyanins becomes a necessity for studies on the stepwise development of anthocyanins (*cf.*, Sec. 8). Relatively, large quantities of tissue, and therefore larger quantities of *acidic* solvent would be required to extract tissues where anthocyanins are just beginning to form (stage I) as compared to tissues which show maximum development of pigment (stage III) and, therefore, require relatively a small amount of both tissue and solvent to extract comparable amounts of anthocyanins (Sec. 8). Upon chromatography of the extracts at different developmental stages, it was observed consistently in three separate investigations (see Sec. 5 and Sec. 8; the results of the third investigation are not presented in the interest of brevity) over a period of three years that stage I contained fewer anthocyanins and anthocyanidins as compared to stage III. Although, the anthocyanins at stage I were fewer in all the three investigations, they showed significant variations in R_f values, indicating, on the basis of the above observations that partial hydrolysis of anthocyanins could have occurred during preparation of the extracts. Thus, the variability of anthocyanins caused by the differential flash evaporation (see also Sec. 19) or by storage of concentrated extracts, if

solvents containing 1% methanolic HCl are to be used in extraction, may confound the developmental variability.

Apart from the problems involved in the developmental work, several practical difficulties are encountered with the use of acidic solvents in strictly comparative work. For instance, if the tissues had been extracted on large scale, the concentration of the extracts may have to be undertaken in several batches. In practice one batch is liable to be concentrated more than the other and thus could be a potential source of variability. Even when batch concentration is not necessary and anthocyanins are extracted from equal amounts of tissues using equal amounts of the solvent, differential breakdown or modification of anthocyanins still occurs in the extract (cf., Sec. 24). Thus, when everything else is strictly comparable, the comparability breaks down for lack of control in the currently available flash evaporators for concentrating the acidified extracts exactly to a desired volume and over a fixed period of time. The rate of evaporation of acidified methanolic extracts is relatively fast in the early stages of flash evaporation but it is considerably slowed down in the final stages. Thus, anthocyanins remain, in the final stages of flash evaporation, in an environment of high acid concentration and high temperature, for instance 50°C (cf., Harborne, 1958) for a long period of time. It is precisely around this stage of flash evaporation that differential degree of hydrolysis could occur in otherwise strictly comparable samples.

The longer time taken in the final stages of flash evaporation to dryness could be one of the reasons for the extensive breakdown of anthocyanin eluates encountered in the early work (Sec. 11). The breakdown of anthocyanins eluted from the paper may be due to other causes as well.

For example, it was shown in Section 18 that anthocyanins eluted from the paper may contain large amounts of paper derived sugars. The anthocyanin breakdown is accelerated in the presence of sugars (Tinsley and Bockian, 1960). Oxygen has been shown to cause rapid breakdown of anthocyanins in acidic solutions (Huang, 1956; Lukton, Chichester and Mackinney, 1956; Robertson, 1959, and others). In a flash evaporator, although the pressure of oxygen is low, nonetheless, in the presence of paper derived substances, the presence of residual oxygen and agitation due to continuous rotation of the flash evaporator flask, some breakdown of anthocyanins could have occurred. However, the potential source of breakdown still appears to be due to the acidic hydrolysis. The eluates of several anthocyanins did show the presence of aglycones when the eluates were obtained with the elution solvents used normally (Table 11.I). The eluates of the anthocyanin E3-138, even after 3 purifications (Sec. 15), still showed the presence of the aglycones. Depending upon the substituents, some anthocyanins elute quickly while others very slowly. Thus, there is, importantly, no control on the amount of eluent used in the elution. Again, in an effort to elute weak anthocyanins as completely as possible, relatively larger amounts of eluents are used. Thus, those anthocyanins which move faster in the eluent used, are eluted in small quantity of the eluent and therefore, a small quantity of acid, and accordingly suffer little degradation during flash evaporation. The anthocyanins eluted with a larger quantity of the eluent, on this basis, would undergo some hydrolysis due to increase in the acid concentration. Supporting evidence for this point of view comes from the anthocyanin eluates which were processed and finally eluted with 0.03% methanolic HCl by the techniques described elsewhere (Sec. 16). The eluates, in general, did not show the presence of

aglycones because not only the strength of acid in the eluent was relatively very weak, but also the amount of the eluent used for elution was minimal.

A few of the salient difficulties that arise in the developmental and comparative work, when acidified extracts or eluates of anthocyanins are concentrated or dried by flash evaporation, have been presented above. Why did other workers not encounter such difficulties when the usual extraction, elution and isolation procedures for anthocyanins employ solvents ranging from 1% to 5% hydrochloric acid (reviews: Sheshadri, 1962; Hayashi, 1962)? Such concentrations of acid may be tolerable because the anthocyanins are isolated in a dry state either by ether precipitation, derivative formation, displacement into other solvents or eventual crystallization but, in general, without resort to concentration by flash evaporation. The above range of acid concentration for processing anthocyanins was established prior to the advent of chromatography, and in the belief that it was the flavylium salt state and not the pseudo base state of anthocyanins (see Sec. 24) that was stable. With the advent of chromatography, the concentration of extracts by flash evaporation has become almost a necessity and a part of the chromatographic techniques. In biological work, the samples occasionally are so small that sometimes they require experienced hands even for crude crystallization by ether. Flash evaporation under the circumstances is ideal, provided a solvent of low acid concentration is used. Indeed, in our elution and concentration technique (Sec. 16), the flash evaporation of anthocyanins eluted with 0.03% methanolic HCl did not cause detectable hydrolysis. In view of the studies described in the following Sections, it appears that, contrary to the existing beliefs, the anthocyanins may be more stable in the pseudo base state than the flavylium state. Thus, the requirement of acid may be

none to minimal. The acid may be required only, for practical considerations, for promoting the solubility of anthocyanins (Sec. 24.3).

23.4. SUMMARY

Since Robinson and Robinson (1933), 1% aqueous HCl has been used for anthocyanin extraction. This range of acid concentration was originally found satisfactory because anthocyanins were concentrated by displacement into small volumes of acidic solvents or were isolated in a dry state by derivative formation, crystallization or ether precipitation. With the advent of chromatography, the concentration of extracts and eluates has become a necessity, and usually flash evaporation, because of its convenience, is employed routinely for concentration of anthocyanin extracts and drying of anthocyanin eluates in many laboratories. In order to hasten evaporation, 1% methanolic HCl instead of 1% aqueous HCl is used generally for anthocyanin extraction and elution. It is demonstrated chromatographically that following flash evaporation of the acidified methanolic extracts to dryness, significant hydrolytic breakdown of anthocyanins occurs owing to the drastic increase in the acid concentration in the final stages of flash evaporation.

The influence of flash evaporation in reference to the total study presented in the thesis is scrutinized briefly.

APPRAISAL OF ANTHOCYANIN STABILITY

24. EFFECT OF NEUTRAL AND ACIDIC SOLVENTS ON ANTHOCYANIN STABILITY IN STORAGE

It was demonstrated that glycosidic hydrolysis is one of the principal causes of anthocyanin modifications in acidic solutions (Sec. 23), in adsorbed state on the paper (Sec. 15) and even in crystalline state (Sec. 15). Although, the breakdown of anthocyanins in 0.1% methanolic HCl extracts, as compared to that of the 10% methanolic HCl, is minimal (Sec. 23), it is no indication that the in vitro breakdown in the former extracts would not have occurred. Accordingly, in this section the stability of anthocyanin extracts in 'neutral' and acidified methanol, ethanol and water after a two-year storage will be explored. It will be demonstrated that, contrary to the existing beliefs, the anthocyanins are stable as pseudo bases in 'neutral'solvents and unstable as flavylium salts in acidic solvents.

24.1 MATERIALS AND METHODS

The basal leaf sheaths of Gopal were obtained from the 1960 growing season as described in Section 10.1. Freshly harvested sheaths, showing maximum color development*, were chopped to about 1/8" pieces, divided, approximately into six equal parts, and added immediately to 6 amber bottles containing 100 ml of six different solvents, namely methanol, acidified methanol, ethanol, acidified ethanol, water, acidified water. Each of the acidified solvents contained 1% conc. HCl. The solvents were kept cool (ca. 2°C) prior to addition of the chopped sheaths. The amber bottles were placed in the dark at -15°C immediately. The extracts were processed along with the other 300 extracts of the 1960 collection, concentrated in vacuo, and stored at -15°C as described elsewhere (Sec.10. 2.1). The concentrated extracts, prior to chromatography, were standardized to 12.5 ml per gram dry weight of the tissue at room temperature following the preliminary extraction (see Sec. 10.2.1 and also Table 10.I). The dry weight of the sheaths at room temperature following the preliminary extraction is shown in Table 24.I.

TABLE 24.I
DRY WEIGHT OF GOPAL BASAL LEAF SHEATHS FOLLOWING PRELIMINARY EXTRACTION

1960 Field Collection No.	Extraction Solvent	Weight Grams
156	EtOH	0.52
157	EtOH-HCl	0.40
158	H_2O	0.34
159	H_2O-HCl	0.38
160	MeOH	0.48
161	MeOH-HCl	0.40

The standardized extracts were chromatographed after two years,

*The basal leaf sheaths were collected from the same planting which was used for the Collection No. 130 (see Sec. 10.1), except that the sheaths for the Collection No. 130 were harvested on July 11, 1960, those used for this study, on July 15, 1960.

in the summer of 1962, in the BAW solvent by ascent as described in Section 10.2.2.

24.2. RESULTS

The chromatographic results of the basal leaf sheath anthocyanin extracts in the neutral and acidified aqueous, ethanolic and methanolic solvents after two-year storage, in the dark and at low temperature, are shown in Fig. 24.1. The results obtained from the acidified solvents are shown in chromatograms 161 (MeOH-HCl), 157 (EtOH-HCl), and 159 (H_2O-HCl), and from the neutral solvents in chromatograms 160 (MeOH), 156 (EtOH), and 158 (H_2O). The R_f values and colors in visible and ultraviolet light of the anthocyanins extracted with different solvents are shown in Table 24.II.

The chromatogram 161 (acidified methanol) shows all the bands shown by the extracts of the Field Collection 130 (Sec. 10; Fig. 10.3), which were obtained in the same growing season and which were extracted and processed also under similar conditions. The results obtained with neutral methanol (Chromatogram No. 160), however, show two* extra anthocyanin bands (R_f 0.09 and 0.11) which were not recovered from the acidic extracts of the Collections 130, 161, 138 nor from any other collection of the basal leaf sheaths. In this connection, however, the splitting of the band A-130 into two components (see Fig. 10.3) is noteworthy. The anthocyanin band DE (a complex cyanidin derivative) is the major band both in the acidified and unacidified extracts. Although it is relatively

*The two anthocyanins are indicated by question mark in chromatogram No. 160 for lack of suitable designations.

Fig. 24.1
Chromatograms of anthocyanins from basal leaf sheaths of Gopal stored for two years in acidic and two years in neutral solvents, 156-EtOH, 157-EtOH-HCl, 158-H_2O, 159-H_2O-HCl, 160-MeOH, 161-MeOH-HCl. The anthocyanins stored as flavylium salts solutions degrade to one or a few bands; the anthocyanins stored as colorless pseudobases degrade very little, or not at all, and show a full spectrum of chromatobands.

weak in the unacidified extracts, the question arises why then are the anthocyanin bands A and B in the unacidified extract stronger in intensity than those of the acidified extract (see later). The anthocyanin band F (a peonidin derivative), is present in both the chromatograms. The orange red anthocyanin band G (a pelargonidin derivative) is present clearly in Chromatogram 161; its presence in Chromatogram 160, however, is made doubtful because of the superimposition of the mauve colored anthocyanin, probably, the anthocyanin H. It is noteworthy that the anthocyanin H can scarcely be detected in the acidified methanolic extract (Chromatogram 161).

TABLE 24.II

R_F VALUES, AND VISIBLE AND ULTRAVIOLET COLORS* OF THE GOPAL BASAL LEAF SHEATH ANTHOCYANINS, EXTRACTED WITH SEVERAL NEUTRAL AND ACIDIFIED SOLVENTS, AFTER CHROMATOGRAPHY IN BAW.

COLLECTION NO. 161: MeOH-HCl
COLLECTION NO. 159: H_2O-HCl

Spot #	R_F x100	Colors V	UV
A	14	Vio-M	DVio
B	18	R	R
E	23	M	M
F	26	Pk	bPk
G	29	OR	wk
H		?	?
I?	44	?	?

COLLECTION NO. 160: MeOH

Spot #	R_F x100	Colors V	UV
?	9	Mv	wk
?	11	Mv	wk
A	16	Mv	lY**
B	18	R	R
E	23	M	M
F	26	Pk	bPk
?	28	Mv	?
H?	34	Mv	wk

COLLECTION NO. 157: EtOH-HCl

Spot #	R_F x100	Colors V	UV
E	24	M	M
?	26	wk	wk

COLLECTION NO. 156: EtOH

Spot #	R_F x100	Colors V	UV
A	16	Mv	wk
B	18	R	wk
?	23	PR	Mv
?	25	PR	P
?	27	R-Mv	?
?	29	wkR	?
?	32	R-Mv	?
H?	?	R-Mv	DMv

COLLECTION NO. 158: H_2O

Spot #	R_F x100	Remarks
?	14	
?	20	Very poor resolution
?	24	Nonetheless large
?	31	quantity of antho-
?	36	cyanins are present in neutral solvent

*Abbreviations are listed in Table 4.II.

**Probably due to superimposition of a copigment.

The chromatographic results with acidified (Chromatogram 157), and unacified ethanol (Chromatogram 156) provide for an interesting comparison. The acidified ethanolic extract shows only the anthocyanin band DE and probably the anthocyanin band F, which, however, is very weak. On the other hand the unacidified ethanolic extract shows at least 8 anthocyanins. It will be noted from Table 24.II that although the bands A-156 and B-156 give colors in visible and ultraviolet light identical to those of the bands A and B of the methanolic extracts already discussed, the color reactions of the other bands are different from those shown in Chromatogram 160. For instance, the bands at R_f 0.23 and 0.25 are present in equal quantity as compared to the corresponding bands in unacidified methanol. In particular, the band F-160 (R_f 0.26), which is a peonidin derivative, is a bright pink band which can be very easily recognized. Instead of the bright pink band, the corresponding band on Chromatogram 157 R_f 0.25) is purple in ultraviolet light. In addition to the above discrepancies, the chromatogram 156 shows at least two extra bands, the equivalents of which do not seem to be present in the chromatogram 160. It is also clear from Fig. 24.1 that the two bands R_f 0.9 and 0.11 present on chromatogram 160 are absent from chromatogram 156. The basis for these discrepancies in extraction with unacidified ethanol and unacidified methanol remains to be explored.

The chromatographic results with acidified water (chromatogram 159) are identical to those with the acidified methanol (see Table 24.II). The anthocyanins did not resolve well with unacidified water (chromatogram 158). Nonetheless, the anthocyanins remain 'stable' in unacidified water as carbinol bases during storage over a period of two years.

24.3 DISCUSSION

It will be observed on comparison of the Chromatograms 161 and 160 that the band DE is the major band both in the acidified and unacidified methanolic extracts. However, the band DE is stronger in the acidified than the unacidified extracts. In relation to the intensity of the band E, the intensity of the anthocyanin bands A (cyanidin derivative) and B (mixture of cyanidin and peonidin? derivatives) may now be compared. Although the intensity of the band DE-161 (acidified methanol) is strong, the intensities of the bands A-161 and B-161 are relatively very weak. On the contrary, even though the intensity of the band DE-160 (unacidified methanol) is weak, the bands A-160 and B-160 are almost twice as strong as those in the acidified methanol. It was shown in Section 13 by stitching chromatography and in Section 19 in the course of characterization studies, that each of the anthocyanin bands A and B yields, either owing to partial hydrolysis on paper, or in solution, major components A2 (Table 19.VIII.A) and B2 (Table 19.VIII.B). The components A2 and B2 in the BAW solvent yield the same R_f values as that of the major component DE4 (Table 19.VIII.DE) of the anthocyanin band DE. Thus, on comparison of the results shown in chromatograms 161 and 160, it is clear that the relative increase in the intensity of the band DE-161, and decrease in the intensities of the bands A-161 and B-161 is most likely due to a partial hydrolysis that would have occurred in the storage of the concentrated acidified extracts. That partial hydrolysis would have occurred is supported by the demonstration that upon concentration of acidified methanol, the acid concentration increases and causes hydrolytic breakdown of anthocyanins (Sec. 23). Support for the view that hydrolytic breakdown of anthocyanins occurs in

acidified solvents is provided by the barely detectable presence or complete absence of the band H in the acidified extracts (chromatograms 161, 157 and 159) and presence in the unacidified extracts. In this connection, it is noteworthy that the band H-400 disappeared completely as a consequence of increase in acid concentration upon flash evaporation of the extract to dryness (Fig. 23.1).

Similarly, the disappearance of all anthocyanins, except DE-157, from the acidified ethanolic extract (chromatogram 157) and the presence of several anthocyanins in the unacidified ethanolic extract (chromatogram 156) testifies to the degradative influence of the acid on anthocyanin stability. The differential degradation in the acidified ethanolic-extracts as compared to the acidified methanolic-or aqueous-extracts may be due to the differential concentration of the acidified extracts in vacuo: the volumes of the concentrated extracts were standardized only sometime prior to chromatography in 1962. Thus, anthocyanins of the three acidified extracts would have remained in an environment of different acid concentration during storage (see Sec. 23).

The presence of the band I-159, particularly in the acidified aqueous extract appears to be due to an anthocyanidin. Equivalents of the band I were not recovered from any of the unacidified extracts. The possibility that anthocyanidins were present originally in the extracts but had suffered irreversible degradation (Robinson, 1937) because of pseudo base formation in neutral extracts though remote cannot be ruled out.

In contrast to the hydrolytic breakdown of anthocyanins in the acidified extracts, the hydrolysis of anthocyanins in the unacidified extracts could not have occurred upon concentration in vacuo. Indeed, all the three unacidified extracts (chromatograms 160, 156 and 158) remain

'stable'. However, the reasons for the discrepancies in the number and color reactions of anthocyanins obtained by the three solvents still remain to be investigated. Nonetheless, on the basis of the assumption that no hydrolysis occurs in the unacidified extracts, and on the indications that apart from hydrolysis, several structural transformations of anthocyanins also occur in acidic solutions (Sec. 12, Mullick and Brink, in press), the belief is advanced that the anthocyanins extracted with unacidified solvents may represent the nearest state of anthocyanins in vivo.

It was established early by the Robinson school that pseudo bases of anthocyanidins are highly unstable in neutral and alkaline solvents and that their colors are best preserved as flavylium salts in acidic solvents. It is likely that, partly because of the stability of the aglycones in the flavylium salt state, and partly because of the natural occurrence of anthocyanins in the colored state (usually, flavylium salt state) that both anthocyanins and anthocyanidins have been as a rule extracted or processed in acidic solutions. The results presented in this section demonstrate that contrary to the prevalent belief, the flavylium salt state of anthocyanins is relatively unstable. The instability in acidic solutions is principally due to glycosidic hydrolysis during flash evaporation or several other structural modifications (see Secs. 12, and 23) during storage. The results presented in this section demonstrate that in contrast to the pseudo bases of anthocyanidins, the pseudo bases of anthocyanins are almost indefinitely 'stable' when processed and stored in darkness.

It is, therefore, obvious from the results that anthocyanins should not have been extracted in acidified solvents particularly those containing 1% HCl. The extractions should have been carried out in neutral

or near neutral solvents from young plant materials. The pH of the cell sap of most plant materials is slightly acidic (Hayashi, 1962). The slight acidity promotes the solubility and hence extraction of anthocyanins from the tissues. The extraction from alkaline tissues, such as aleurone (review: Wiebe and Reid, 1961), particularly mature aleurones, still poses problems. The anthocyanins in aleurone layer exist as anhydro bases. The anhydro bases are found to be almost insoluble in neutral solvents. Therefore, solvents will have to be acidified enough to promote the solubility and extraction of anthocyanins from aleurone layer. Although, the anthocyanins from the aleurone are currently extracted with 1% methanolic HCl (Sec. 22), the acid concentration will have to be modified or acid from the extract removed by selective adsorption of anthocyanins on resins such as polyclar A.T. (Antara Chemicals). The removal of acid is necessary because repeated extractions are required for extracting even a small fraction of the soluble anthocyanins of aleurone.

Inasmuch as the findings that the anthocyanins are relatively unstable in the flavylium salt state as compared to the pseudo base state are in direct contradiction to the current beliefs, further studies had to be undertaken to substantiate the findings. The substantiation led the writer to a detailed study of the factors affecting the stability of anthocyanins, pseudo bases, and anhydro bases. This work fully supports the above conclusions and will be presented at a later date.

24.4 SUMMARY

It is well established that the pseudo base state of anthocyanidins and anthocyanins is unstable and that the degradative modifications of

anthocyanins (for instance: see Lukton *et al.*, 1956) occur through the pseudo base state. Accordingly, both anthocyanins and anthocyanidins are extracted, processed and stored in their flavylium salt state.

In this study, anthocyanins were extracted with 'neutral' methanol, ethanol and water, and also with acidified (containing 1% conc. HCl) methanol, ethanol and water under identical conditions. The extracts were concentrated *in vacuo*, and stored in a refrigerator (-15°C) for two years. It is established chromatographically that, contrary to the existing beliefs, anthocyanins remain more stable, in neutral solvents, as pseudo bases than in acidic solvents as flavylium salts over the two-year storage period. Several other spectral studies (not included here) support this view.

It is suggested that in order to obtain anthocyanins as near their *in vivo* state as possible, the anthocyanins should be processed in near 'neutral' systems. Traces of acid will have to be added to extract anthocyanins from the alkaline aleurone.

25. CONCLUSIONS

1. A technique for separating pericarp, spermoderm and aleurone tissues from a developing barley grain was developed to study localization and chemical nature of pigments in these tissues.

2. The anthocyanin and chlorophyll competent tissue of the developing grain is spermoderm and is not, as currently believed, pericarp.

3. Pigment cycles are altered markedly by changing environment. The genetically competent purple variants, and their hybrids, produce deep anthocyanic pigmentation under summer field conditions. Under winter greenhouse conditions, single plants produce anthocyanin-free heads until January, and anthocyanic heads after February. Environmental manipulation as an aid in studying anthocyanin biogenesis is valuable.

4. Leucoanthocyanins do not occur in the pericarp, spermoderm and other maternal tissues; the finding confirms that they occur exclusively in the aleurone and endosperm. Leucoanthocyanins are not related biogenetically to anthocyanins in the maternal tissues.

5. Dynamic patterns for polyphenolic compounds in the awns, hulls, pericarp and spermoderm of several color variants are demonstrated.

6. When aleurone, intact after peeling off pericarp and other tissues, is placed in 0.1% methanolic HCl, the anthocyanins change progressively from blue to red to colorless. It is concluded that the anthocyanins exist in the alkaline aleurone as anhydro bases and finally in 0.1% methanolic HCl as colorless pseudo bases.

7. In living tissues, anthocyanins occur as colorless pseudo bases in the developing aleurone of several color variants, and the hood veins of the black variety, Gatami. Visual examination of the tissues, thus, is insufficient for the detection of anthocyanins.

8. Preliminary studies establish that the blue anthocyanins of the aleurone are of two types: those that are soluble and those that are tissue bound. During dough stage, a major part of the anthocyanins is soluble and a minor part tissue bound. The situation is reversed later at flint stage, when a major part exists in the bound state.

9. The inextractability of the aleurone anthocyanins from the intact seeds even over a six year exposure to acidified methanol is due to the impermeability to solvent of the nucellar epidermis and possibly an aleurone envelope.

10. It is concluded that the first extract from the intact seeds, which contain anthocyanins both in the spermoderm and aleurone, contains anthocyanins from the spermoderm, the third extract largely from the aleurones of chipped and unhealthy seeds.

11. After the conclusions of 9 and 10, a method for the selective extraction of anthocyanins from the spermoderm and aleurone without manual peeling was developed.

12. A method for genetic classification of aleurone by chemical peeling is presented. The method does not affect the germinability of the seeds. The blue color of the aleurone varies continuously. The aleurone color classification of some varieties requires revision.

13. The aleurone anthocyanins differ markedly from those of the spermoderm anthocyanins. The aleurone anthocyanins, for example, of Black Hulless, are derived largely from delphinidin and, tentatively, petunidin. The spermoderm anthocyanins are derived largely from cyanidin and peonidin.

14. The leaf sheaths, awns, hulls and spermoderm of Black Hulless and Gopal show identical anthocyanin patterns with standard techniques. The identity of patterns supports the previously reported genetics of lemma and pericarp (now spermoderm) tissues. Three of the anthocyanins from the above tissues showed novel chromatographic characteristics (R_f 0.5 to 0.99 in BAW). Such exceedingly 'fast-moving' anthocyanins were absent from the flinty caryopsis tissues.

15. The chromatographic patterns of the above tissues vary from season to season with standard techniques.

16. In maternal tissues noted in 14 above, the cyanidin derivatives are formed first and then peonidin and/or pelargonidin derivatives. It is concluded that firm decisions on stepwise biogenesis are contingent upon controlling the environmental variation as well as the factors affecting anthocyanin stability during extraction and processing.

17. The anthocyanic pigmentation in barley is biochemically and genetically complex. It is concluded from investigations on the basal leaf sheaths of several color variants that, although the visual phenotypes of the pigments are undifferentiable, the biochemical phenotypes

are differentiable.

18. Basal leaf sheaths from several color variants obtained early in November showed varying anthocyanin patterns, but all showed the two to three fast-moving anthocyanins. By early December, equivalent tissues did not show the fast-moving anthocyanins. It is demonstrated that the fast-moving anthocyanins, because of their lability and instability are transformed to slow-moving (R_f 0.5 in BAW) anthocyanins. A firm conclusion, as to whether or not the absence of fast-moving anthocyanins is associated with the development of the plant, is contingent upon controlling their instability.

19. Different solvents give a varying number of anthocyanin bands on preparatory chromatograms. It is concluded that a choice of a suitable solvent is necessary and that the number of anthocyanin bands appearing on chromatograms may give no indication that the same number of anthocyanins exist in vivo.

20. Barley anthocyanins are very labile. They split into several components, and give rise to several degradation products following elution and flash evaporation using standard techniques.

21. Several barley anthocyanins eluted with methanol containing 0.01% conc. HCl give rise to degradative peaks around 360 mu which resemble those of chalcones.

22. Anthocyanin losses occur also through the formation of reddish and brownish materials, which remain at the starting line during chromatography.

23. Anthocyanin eluates are transformed to brownish and jet black products during drying. These brownish products are similar to the stationary materials of 22.

24. A series of three methods, namely, a sewing or a clamping technique for chromatographic characterization of anthocyanins, an improved method for studying anthocyanin spectra of even weak spots directly on paper, and a method for hydrolysing anthocyanins on the paper were developed to circumvent the instability inducing steps, elution and flash evaporation. Additionally, the methods effect great saving of time and plant materials, particularly, in the comparative characterization of anthocyanins from hybrids and their parents.

25. It is concluded (a) that the "irreversible adsorption" of anthocyanins during paper chromatography is due to the glycosidic hydrolysis that ensues, gradually, on the paper, (b) that the component adsorbed irreversibly is corresponding aglycone, and (c) that glycosidic hydrolysis on paper is the principal cause of the anthocyanin splitting (splitting occurs even when the sewing technique eliminates the elution step).

26. The anthocyanin E3-138 undergoes glycosidic hydrolysis when stored at room temperature in the crystalline state.

27. A mild technique for purifying anthocyanins in several solvents without elution, and a technique for eluting anthocyanins in only a few drops of 0.03% methanolic HCl were developed for isolating anthocyanins. The techniques were used extensively and do not cause glycosidic hydrolysis of anthocyanins during isolation.

28. Two new solvents were developed for anthocyanidins. It was found that two well-known anthocyanin solvents could be used advantageously to characterize anthocyanidins.

29. It was established that glucose, galactose, arabinose and xylose are eluted from chromatopaper as artefacts. The glycosidic analysis of anthocyanins, therefore, is confounded, because the same

sugar components were recovered from the anthocyanins. A method for eliminating the glycosidic artefacts from anthocyanin eluates is presented.

30. Also eluted from the chromatopaper within a few hours are unknown yellow compounds, which show increasing spectral absorptions in the wavelength range 300-400 mu, and affect dependability of the ratio E_{440}/E_{max} used in differentiating 3-monoglycosides from 3, 5-diglycosides.

31. The anthocyanins of barley are complex: the ten BAW anthocyanin bands from the basal leaf sheaths of Gopal and Black Hulless split into over 60 HAc-HCl components, and the nine BAW bands from the Black Hulless caryopsis split into over 75 HAc-HCl components due to partial hydrolyses. Chromatographic data of 93 anthocyanin components in five solvents is given. Anthocyanidins from 63 components are identified by R_f values in seven solvents and by spectral means. 39 components were hydrolysed for sugar analyses, and 25 for structural elucidation by partial hydrolyses. The simplest of the complex anthocyanins are identified as 3-monoglucosides of cyanidin, peonidin and pelargonidin. Barley is a rich source of a number of new cyanidin derivatives. Putative evidence for the existence of new derivatives of pelargonidin and peonidin is presented.

32. Pelargonidin derivatives, present plentifully in Gopal leaf sheaths - under summer-1960 field conditions, were absent in the same sheaths - under fall-1961 greenhouse conditions. On the basis of this and several other observations (see Conclusions 3 and 16), it is concluded that chemicogenetical and developmental studies on anthocyanins should be carried out under controlled environments.

33. The major anthocyanin band from the seeds of the variety Violet Guimalaya has been identified by Urion and Metche (1961) as a simple cyanidin-3-arabinoside. The major band from similarly pigmented varieties,

Gopal and Black Hulless, which initially we believed to be a cyanidin-3-glucoside (Mullick et al., 1958) split into more than 13 HAc-HCl components.

34. Barley anthocyanins, extracted and processed using the currently accepted methods little resemble, in the main, their in vivo state, because they undergo artefact production due to transformation to simpler anthocyanin components.

35. Drying of 1% methanolic HCl extracts by flash evaporation is a principal cause of artefact production and hydrolytic breakdown of anthocyanins.

36. It is demonstrated chromatographically that anthocyanins extracted with unacidified methanol (in pseudo base state) remained more stable over a two-year storage than those extracted with acidified methanol (in flavylium salt state).

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27. APPENDIX

Detailed observations of pigment changes in the grain and spike tissues, namely, awn tip and awn remainder, glume tip and glume remainder, lemma veins and lemma interveins, pericarp, spermoderm, and aleurone at five stages of kernel maturation (see Table 4.I) for twenty barley varieties and isolines (mentioned below) are recorded(Tables 27.I to 27.XX):

27.I	Black Hulless	(purple)
27.II	Gopal	(purple)
27.III	71-PrPr-10	(purple)
27.IV	C.I.5628	(purple)
27.V	36-b1b1-21	(white)
27.VI	C-54-55	(white)
27.VII	Golden Pheasant	(white)
27.VIII	Deficiens	(white)
27.IX	Vantage	(white)
27.X	33-b1b1-13	(white)
27.XI	5090-10-4	(near white)
27.XII	5090-2-3	(gray or near white)
27.XIII	36-B1B1-21	(blue)
27.XIV	Montcalm	(blue)
27.XV	Trebi	(peculiar blue)

27.XVI	C-54-22	(pale blue)
27.XVII	33-B1B1-13	(blue)
27.XVIII	Gatami	(black)
27.XIX	Lion	(black)
27.XX	Kitchen	(black)

As indicated in Section 4.2.3, the simplest of color designations were used to specify pigment changes occurring in all tissues except the aleurone tissue. Abbreviations used for simple color designations are included in Table 4.II. However, the aleurone colors, which are first records, were designated by comparison with Ridgway's Color Charts (1912). The Arabic numerals used for designating Ridgway's colors are included in Table 4.III. Other abbreviations and symbols used in the Tables 27.I to 27.XX are given below.

v = Color in situ, without treatment
s = Color in situ, when treated with 1% ethanolic HCl.
# = Color attributable to anthocyanins
* = Anthocyanin pigments absent
** = Anthocyanins not visible until tissues are placed in acidified ethanol

TABLE 27.1

BLACK HULLESS
Purple
6 rowed

Tissues	STAGES OF DEVELOPMENT									
	I		II		III		IV		V	
	Colour		Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s	v	s
Awn tip	dP	#	dP	#	fP	#	Y	*		
Awn remainder	G	*	GP-YP	#	P	#	fP	#		
Glume	P	#	P	#	P	#	Y	*		
Lemma veins	G	*	G	*	P	#	P	#		
Lemma interveins	G	*	P	#	P	#	PY	#		
Pericarp	C	*	C-LY	*	See discussion					
Spermoderm	G	*	G&P	#	P	#	dk P	#		
Aleurone	W-Cr	*	Partly Bl	#	Bl	#	Tips(3)	(5)#	Tips(22) Centre(23)	# #
Anthocyanin[i] Concentration in aleurone							+++++			+++++ +++++

[i] Estimated values only (see Sec.4.2.3)

TABLE 27.II

GOPAL
Purple
2 rowed

Tissues	STAGES OF DEVELOPMENT									
	I		II		III		IV		V	
	Colour		Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s	v	s
Awn tip	P	#	dP	#	fP	#	DY	*		
Awn remainder	G	*	G	*	P&GY	#	DY	*		
Glume tip	P	#	P	#	fP	#	Y	*		
Glume remainder	G	*	Not Recorded							
Lemma veins	G	*	G	*	P	#	P	#		
Lemma interveins	G	*	P	#	dP	#	YV	#		
Pericarp	C	*	C	*	C	*	See Discussion			
Spermoderm	G	*	G&P	#	bP	#	bP	#		
Aleurone	W	*	Cr	*	CrY	*	(8) Centre (4) tips only	* #	16	*
Anthocyanin[i] Concentration in aleurone								+ + +		

[i] Estimated values only (see Sec. 4.2.3)

TABLE 27.III

71-Pr-Pr-10
Purple
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		II		III		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	P & Bk	# *	M & Y	**	M	*		
Awn remainder	G	*	GY	*	P	#		
Glume tips	P & Y	#	P	#	dP	#		
Glume remainder	G	*	YG	*	CrG	*		
Lemma veins	G	*	bR	#	P	#		
Lemma interveins	R & GY	#	R	#	P	#		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	Partly R	#	PR	#		
Aleurone	Cr	*	Cr	*	(7)	*		

Not Available

TABLE 27.IV

C.I. 5628
Purple
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		II		III		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	VR	#	Vg	#	DY	*	DY	*
Awn remainder	Not Available				Y	*	Y	*
Glume tip	V	#	Vg	#	MY	**	Y	**
Glume remainder	G	*	GY	*	Y	**	Y	**
Lemma veins	G	*	P	#	P	#	Y	**
Lemma interveins	G	*	PG	#	P	#	Y	*
Pericarp	C	*	C	*	C	*		Inseparable
Spermoderm	Y&G	*	P	bR #	P	#		
Aleurone	YW	*	B1	#	B1	#		

TABLE 27.V

36-b1b1-21
White
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	II		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	P	#	Partly P	#	Partly M	*		
Awn remainder	G	*	G at base	*	Y	*		
Glume tips	P & M	# *	MBr	*	Y	*		
Glume remainder	G	*	BlG	*	WY	*		
Lemma veins	G	*	P&Y	#	R	#		
Lemma interveins	GY	*	Y	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	Slit P	#	Slit MBr	*		
Aleurone	Cr	*	?	*	(8)	**	(15)	**
Anthocyanin[i] Concentration in aleurone					$<0.1^+$		$<0.1^+$	

[i] Estimated value only (see Sec. 4.2.3)

TABLE 27.VI

C-54-55
White
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	P	#	MY	**	mostly Y	**		
Awn remainder	G	*	GY	*	Y	*		
Glume tip	P	#	P	#	Y	**		
Glume remainder	G	*	GW	*	Y	*		
Lemma veins	G	*	P	#	Br	**		
Lemma interveins	G	*	Y	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	Slit P	#	Slit Br	*		
	G	*	GY	*	pY	*		
Aleurone	Cr	*	Y	*	(10)	*	(17)	#
Anthocyanin[i] Concentration in aleurone								

$< 0.2^{+}$

[i]Estimated value only (see Sec. 4.2.3)

TABLE 27.VII

GOLDEN PHEASANT C. I. 2488
White
2 rowed

Tissues	STAGES OF DEVELOPMENT			
	IV		V	
	Colour		Colour	
	v	s	v	s
Awn tip	M	slightly #		
Awn remainder	Y	*		
Glume tip	P	#		
Glume remainder	Y	*		
Lemma veins	R	#		
Lemma interveins	Y	*		
Pericarp	C	*		
Spermoderm	LM	*		
Aleurone	(8)	*	(14)	*

TABLE 27.VIII

DEFICIENS
White
2 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		II		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	M	*	M in patches	*	M in patches	*		
Awn remainder	G	*	Y	*	Y	*		
Glume tip	M	*	M	*	Y	*		
Glume remainder	M	*	Br	*	Y	*		
Lemma veins	G	*	GY	*	BrY	*		
Lemma interveins	G	*	GY	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	GY	*	Y	*		
Aleurone	W	*	CrW	*	(2)	*	(9)	*

TABLE 27.IX

VANTAGE
White
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	G	*	Y	*	MBr (slightly)	*		
Awn remainder	G	*	GY	*	Y	*		
Glume tip	G	*	Y	*	Y	*		
Glume remainder	G	*	VBk	**	Y	*		
Lemma veins	G	*	slightly R	#	R	#		
Lemma interveins	G	*	YG	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	C	*	C	*		
Aleurone	W	*	GY	*	(7) or (8)	** **	between (16) & (17)	#
Anthocyanin[i] Concentration in aleurone						+		(1.5)[+]

[i]Estimated value only (see Sec. 4.2.3)

TABLE 27.X

33-blbl-13
White
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		II		III		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	bY	*	BrY	*	Y	*		
Awn remainder	green	*	GY	*	Y	*		
Glume tip	Y	*	Y	*	Y	*		
Glume remainder	G	*	Y	*	Y	*		
Lemma veins	G	*	GY	*	Y	*		
Lemma interveins	G	*	GY	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	GY	*	Cr	*		
Aleurone	W	*	Cr	*	(8)	**	between (14 & 15)	*[ii]
Anthocyanin[i] Concentration in aleurone						0.1[+]		*

[i]Estimated value only (see Sec. 4.2.3)

[ii]Aleurone stage V; No anthocyanins even in 3% HCl-MeOH.

TABLE 27.XI

5090-10-4
White and Dirty White
6 rowed

Tissues	STAGES OF DEVELOPMENT									
	I		II		III		IV		V	
	Colour		Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s	v	s
Awn tip	dk Br	?	PV	#	M	**	Y	*		
Awn remainder	G	*	GY	*	YG	*	Y	*		
Glume	G	*	G	*	Br	**	Y	*		
Lemma veins	G	*	R	#	R	#	Y	*		
Lemma interveins	G	*	G	*	GY	**	Y	*		
Pericarp	C	*	C	*	C	*	C	*		
Spermoderm	G	*	G	*	?	*	?	*		
Aleurone	W	*	Cr	*	?	*	(7)	**	(14)	#
Anthocyanin[i] Concentration in aleurone								0.5+		0.3+

[i] Estimated value only (see Sec. 4.2.3)

TABLE 27.XII

5090-2-3
Gray or dirty White

Tissues	STAGES OF DEVELOPMENT							
	II		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	Y	*	Y	*	Y	*		
Awn remainder	G	*	Y	*	Y	*		
Glume tip	?	?	Y	*	Y	*		
Glume remainder	G	*	Y	*	Y	*		
Lemma veins	G	*	R	#	Y	*		
Lemma interveins	G	*	YG	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	YW	*	dY	*		
Aleurone	pW	*	pg	**	(6)	#	(17)	#
Anthocyanin[i] Concentration in aleurone				very little		+ + +		(1.5)+

[i] Estimated value only (see Section 4.2.3)

TABLE 27.XIII

36-B1B1-21
Blue
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	II		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	Y	*	MBr	*	Y	*		
Awn remainder	G	*	YG	*	Y	*		
Glume tip	Y	*	Y	*	Y	*		
Glume remainder	G	*	Y	*	Y	*		
Lemma veins	G	*	R	#	R	#		
Lemma interveins	GY	*	YG	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	R	#	R	#	?	*		
Spermoderm slit	pG	*	p	*	pY	*		
Aleurone	LB1	#	B1G	#	(19)	#	(6)	#
Anthocyanin[i] Concentration in aleurone						(1.5)+		(0.3)+

[i] Estimated value only (see Section 4 .2.3)

TABLE 27.XIV

MONTCALM
Blue
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	dP	#	fP	#	Y	*		
Awn remainder	G	*	Y	*	Y	*		
Glume tip	dP	#	fP	#	Y	*		
Glume remainder	G	*	Y	*	Y	*		
Lemma veins	G	*	P	#	Y	*		
Lemma interveins	G	*	YG	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	pale	*	pale	*		
Spermoderm slit	G	*	P	#	?	*		
Aleurone	W	*	slightly Bl	#	(19) & (25)	#	(20)	#
Anthocyanin[i] Concentration in aleurone						+		(0.2)+

[i]Estimated value only (see Section 4.2.3)

TABLE 27.XV

TREBI
Peculiar Blue
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	P	#	M&Y	*	Y	*		
Awn remainder	G	*	Y	*	Y	*		
Glume tip	P	#	Y	*	Y	*		
Glume remainder	G	*	Y	*	Y	*		
Lemma veins	G	*	R	#	BrR	#		
Lemma interveins	G	*	YG[ii]	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	P around slit tips Bl	# #	C	*		
Aleurone	pW	*	Centre Y	#	(6) & (21)	#	(24)	#
Anthocyanin[i] Concentration in aleurone						+ +		(0.2)+

[i] Estimated value only (see Section 4.2.3)

[ii] Occasionally lemma and palea appear as reddish violet due to the color of the underlying spermoderm. Actually the colour of the lemma and palea is yellow.

TABLE 27.XVI

C-54-22
Pale Blue
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	P	#	YBk	*	M patches	*		
Awn remainder	G	*	Y	*	Y	*		
Glume tip	P	#	P	#	Br	occasionally		
Glume remainder	G	*	GY	*	Y	*		
Lemma veins	G	*	YG	*	Y	*		
Lemma interveins	G	*	GY	*	Y	*		
Pericarp	C	*	C	*	C	*		
Spermoderm	G	*	P patches	#	C	*		
Aleurone	W	*	?	?	(19) & (20)	#	(20)	#
Anthocyanin[i] Concentration in aleurone					+		(1.5)+	

[i]Estimated value only (see Section 4.2.3)

TABLE 27.XVII

Only stage IV is available

33-B1B1-13
Blue
6 rowed

Tissues	STAGES OF DEVELOPMENT			
	IV		V	
	Colour		Colour	
	v	s	v	s
Awn tip	Y	*		
Awn remainder	Y	*		
Glume tip	Y	*		
Glume remainder	Y	*		
Lemma veins	R	#		
Lemma interveins	Y	*		
Pericarp	C	*		
Spermoderm		*		
Aleurone	17 & 20	#	20 & 13	#
Anthocyanin[i] Concentration in aleurone		+ + + + +		(0.2)+

[i]Estimated value only (see Section 4.2.3)

TABLE 27.XVIII

GATAMI
Black

Tissues	STAGES OF DEVELOPMENT									
	I		II		III		IV		V	
	Colour		Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s	v	s
Awn tip	P	#	gV	*	Y	*	Bk	*		
Awn remainder	G	*	Y	*	gY	*	Bk	*		
Glume	?	#	V		g	*	Bk	*		
Lemma veins	G	*	YBr	*	Br	* not definite	Bk	*		
Lemma interveins	G	*	GBk	*	Bk	*	Bk	*		
Pericarp	C	*	C	*	C	*	C[ii]	*		
Spermoderm	G	*	YG & small dark patches	*	?	*	?	*		
Aleurone	W	*	LBl	#	Bl	#	(24)& (21)	(1)#	(6)	#
Anthocyanin[i] Concentration in Aleurone								+ + + + +		(0.5)+

[i] Estimated value only (see Sec. 4.2.3)

[ii] Pericarp probably colorless; considerably dry.

TABLE 27.XIX

LION
Black
6 rowed

Tissues	STAGES OF DEVELOPMENT							
	I		III		IV		V	
	Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s
Awn tip	P	#	P	#	MBk	*		
Awn remainder	G	*	GY	*	MBk	*		
Glume tip	P	#	?	*	MBk	*		
Glume remainder	G	*	W	*	MBk	*		
Lemma	G	*	P & VBk	# **	Bk	*		
Lemma interveins	G	*	MBr	*	Bk	*		
Pericarp	C	*	slightly g	*	dg	*		
Spermoderm	G	*	Y slit R	* #	gBk	*		
Aleurone	W	*	W	*	7	**	(6)	*
Anthocyanin[i] Concentration in aleurone					< 0.05 to Nil	+		

[i] Estimated value only (see Section 4.2.3)

TABLE 27.XX

KITCHEN
Black
6 rowed: Hooded

Tissues	STAGES OF DEVELOPMENT									
	I		II		III		IV		V	
	Colour		Colour		Colour		Colour		Colour	
	v	s	v	s	v	s	v	s	v	s
Hood veins	G	**	MBk	*	MY	*	Y	*		
Hood remainder	G	*	GY	*	M	*	M	*		
Glume tip	P	#	P	#	MP	*	M	*		
Glume remainder	?	?	M	*	M	*	BlBk	*		
Lemma veins	G	*	YG	*	M	*	MBk	*		
Lemma interveins	G	*	GY	*	M	*	MBk	*		
Pericarp	C	*	C	*	MBr	*	Bkg	*		
Spermoderm	G	*	Y	*	W	*	Br-Bk	*		
Aleurone	W	*	YW	*	LBl	#	(8) and (11)	*	(11)	*