Flavonoids and Related Phenolic Compounds Produced in the First Internode of Sorghum vulgare Pers. in Darkness and in Light [1]

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Knowledge of C_{15} intermediates in the biosynthesis of anthocyanins is scanty or controversial. Recently, chalcones (7) and leucoanthocyanins (2) have been postulated as intermediates from evidence based on kinetic studies and isotope experiments. Downs and Siegelman (3) have studied the photocontrol of anthocyanin synthesis in seedlings of *Sorghum vulgare*. The anthocyanin has been identified as an acylated cyanidin-3-glucoside, and possible C_9 precursors of this pigment have been studied with isotope techniques (1).

Since the first internode (mesocotyl) of *Sorghum* can be easily grown in the dark and forms large amounts of the red anthocyanin only in the light, this material was examined in hopes that intermediates in anthocyanin biosynthesis might be accumulated under appropriate conditions. It was necessary first to know what phenolic compounds were present in completely dark-grown internodes and what changes took place upon illumination.

Materials and Methods

Seeds of *Sorghum vulgare* Pers. var. Wheatland milo were obtained through the courtesy of Dr. H. W. Siegelman. They were surface sterilized for 30 to 60 minutes with a saturated solution of calcium hypochlorite followed by a solution of Orthothrix (California Spray Company) at a concentration of 1 g/liter. The rinsed seeds were placed on moist filter paper in covered trays at 20° to 25° for 4 days in the dark. The first internodes (each about 2–3 cm in length and weighing about 2.5 mg dry wt) were cut above the scutellar node and below the coleoptilar node. They were analyzed directly (called zero time controls) or were surface sterilized 2 minutes with the above Orthothrix solution, and were placed on moist filter paper for further incubation in the dark or light. In some cases, only the roots or the root and coleoptile were removed, and the resulting seed-shoots or seed-internodes were incubated, the internodes being completely excised just before analysis. Fresh weight determinations of the internodes were made just before analysis, and were converted to dry weight values using an average conversion factor of 13 % fresh weight as dry weight.

For white light treatment of low intensity (100–300 ft-c or 1000–3000 lux), the internodes were kept in the laboratory at about 20° to 25° under 40 w cool white fluorescent lamps. For high light intensity (1000–1200 ft-c or 10,000–12,000 lux), they were kept at 25° in a growth chamber equipped with high intensity fluorescent lamps (Sylvania F72T12 VHO/CW).

The general extraction procedure is summarized in figure 1. The fresh internodes (20–400 mg dry wt) were held in hot methanol for 1 to 2 minutes, cooled, and then were ground in a mortar with methanol containing 0.05 % or 1 % HCl (w/v). The residue was successively extracted with a similar solution of HCl-methanol, but containing about 20 % water

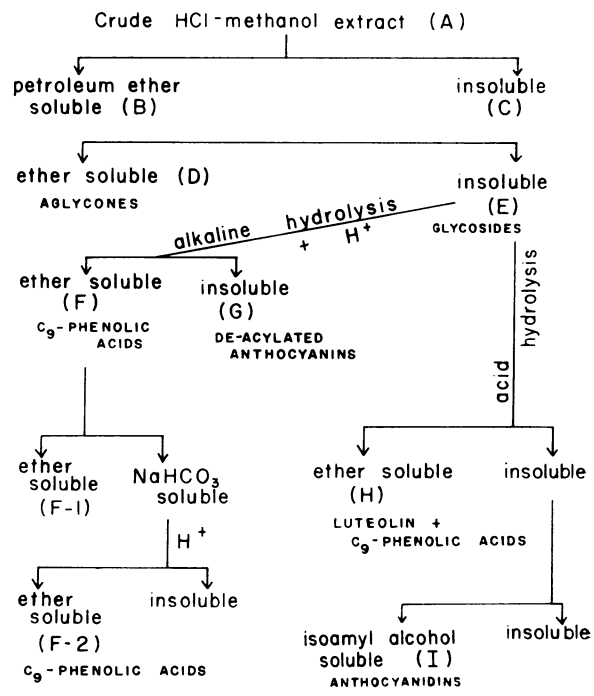


FIG. 1. Extraction procedure.

[1] Received July 20, 1964.

[2] National Science Foundation Senior Postdoctoral Fellow, 1963–64.

[3] The author gratefully acknowledges the aid of Dr. T. A. Geissman in this research, and the financial support of the Public Health Services (GM-03667 of T. A. Geissman) during this work in the Chemistry Department at the University of California at Los Angeles.

(v/v). After centrifugation, the supernatant extracts were combined and concentrated if necessary under reduced pressure at 30° or less to remove most of the methanol. After extraction with petroleum ether (or skelley B), the remaining aqueous solution (C) was chromatographed directly or was extracted with ethyl ether. The ether fraction D containing aglycones was evaporated and the residue dissolved in ethanol. The aqueous fraction E was chromatographed or hydrolyzed with alkali or acid. Alkaline hydrolysis at 20 to 25° in 1 N NaOH in the dark under N_2 for 1 hour was sufficient to convert the acylated red anthocyanin to cyanidin-3-glucoside. After acidification, the aqueous phase was extracted with ether to obtain the free phenolic acids (F), and the acidified aqueous fraction (G) contained the deacylated anthocyanins. When only the ether soluble fraction was studied, hydrolysis was sometimes done in 2 N NaOH for 1 to 5 hours at room temperature under N_2. The ether fraction F was treated with a solution of 5 % $NaHCO_3$. The ether phase (F-1) contained neutral and basic compounds. The $NaHCO_3$ phase contained phenolic acids which were reextracted into ether after acidification (F-2). Ether extracts were evaporated to dryness with air and the residues were dissolved in ethanol for chromatography.

Acid hydrolysis was made in 1 to 2 N HCl in the dark in a hot water bath for 30 to 60 minutes. The hydrolysis mixture was extracted with ethyl ether or ethyl acetate to obtain flavonoids such as luteolin and phenolic acids (H). A subsequent extraction of the hydrolysis mixture with isoamyl alcohol removed the anthocyanidins (I).

For 2-dimensional paper chromatography, aliquots of extracts equivalent to 1 to 50 mg dry weight of original tissue were placed on sheets of Whatman No. 1 paper about 20 × 20 cm in size. Chromatograms were developed in darkened containers in an ascending direction for 1 to 3 hours depending on the solvent. Large scale separations were chromatographed and eluted sequentially as bands on Whatman No. 3 MM paper. Concentrated HCl was added to solvents with extracts containing anthocyanins or anthocyanidins. The following solvents were used individually or in pairs (v/v): 1-butanol-acetic acid-water-concentrated HCl (60: 10: 20: 1) = BAW-HCl; acetic acid-water-concentrated HCl (87: 10: 3) = 10 % A-HCl; formic acid-water-concentrated HCl (25: 15: 10) = F-W-HCl; acetic acid-concentrated HCl-water (30: 3: 10) = Forestal; benzene-acetic acid-water (40: 10: 1, organic phase) = BeAW4: 1; or another mixture (42: 24: 1) = BeAW2: 1; 1-butanol-ammonium hydroxide-water (40: 5: 5); sodium formate-formic acid-water (10 g: 1 ml: 200 ml).

When thin layer chromatography (TLC) was used, silica gel G was the adsorbent and the solvent generally used was ethyl acetate-formic acid-water-concentrated HCl (85: 6: 8: 1) = EA-F-W-HCl.

Both white and short and long wavelengths of UV light, with and without fuming with ammonia, were used to detect spots. Useful sprays were $AlCl_3$ (5 % w/v in ethanol), Fast Red (5 mg/10 ml water) followed by 25 % w/v Na_2CO_3 and a fresh mixture of equal volumes of 1 % $FeCl_3$ plus 1 % $K_3(FeCN)_6$ (w/v).

The author is grateful to the following people for samples of compounds; Dr. J. B. Harborne for apigeninidin-5-glucoside, apigeninidin and luteolinidin, L. Jurd for apigeninidin, E. Conn for dhurrin, and and T. A. Geissman for all other standards cited.

Spectra were determined with an Hitachi Perkin Elmer spectrophotometer. To obtain samples of flavonoids that chromatographed as 1 visible compound, crude extracts (C or E) were chromatographed as bands on Whatman No. 3 MM paper in 10 % A-HCl. Bands representing the major flavonoids were eluted in 70 or 100 % methanol containing 0.01 to 0.05 % HCl and were rechromatographed as bands 2 to 3 times in BAW-HCl, F-W-HCl or 10 % A-HCl. Paper blanks for spectrophotometric analysis were made only with the last solvent used. To obtain shifts with $AlCl_3$, it was necessary to elute with methanol alone to reduce the acid concentration. Additions were made as follows to a 1.5 ml volume in a cuvette: 0.05 ml 5 % $AlCl_3$ in ethanol, 10 μl 10 N NaOH and sodium acetate as a solid.

Identification of Flavonoids in the First Internode. Four flavonoids, formed under different conditions discussed below, have been identified. Three have the anthocyanidin nucleus, the other a flavone (fig 2).

One was the red anthocyanin formed in blue and red light studied by Downs and Siegelman (3), and postulated as an acylated cyanidin derivative by Arnold and Brown (1). R_F values are shown in table I for the acylated form (C-1), for the product of alkaline hydrolysis (C-2) which cochromatographed with cyanidin-3-glucoside (chrysanthemin), and of acid hydrolysis (C-3) which cochromatographed with cyanidin. Spectral characteristics of this anthocyanin before and after alkaline hydrolysis were similar (fig 3), indicating the lack of a typical aromatic acyl group (1). After acid hydrolysis, the long wavelength peak shifted from about 525 to 535 mμ, and after the addition of $AlCl_3$, to about 545 mμ (5, 8). All data support the conclusion that the major red anthocyanin formed in light of high intensity is an acylated cyanidin-3-glucoside. The acyl group has not been identified. Cyanidin-3-glucoside or the aglycone cyanidin was found in the extracts only after hydrolysis occurring during preparation. If the temperature was kept at 30°, only 1 acylated form was detected on chromatograms.

A second flavonoid was an orange or red-orange pigment which appears to have the basic structure of one of the rarer anthocyanins without a hydroxyl group in the 3 position; i.e., luteolinidin (5, 8, 9, and fig 2). The pigment existed in unhydrolyzed extracts in 3 forms, indicated as L-1, 2 and 3 in table I. The major spot L-1 was the aglycone which cochromatographed with a known luteolinidin standard in several solvents. Two minor spots were L-2, presumably the 5-glycoside, and L-3, an unidentified form. Hy-

drolysis with alkali indicated the absence of an alkali-sensitive group, but there was always a loss of pigments even in the presence of N_2. The unidentified form, L-3, was found along with the aglycone in acid hydrolyzates, and both were extracted into isoamyl alcohol, but not into ether or ethyl acetate. Spectra of the major form L-1 and its glycoside L-2 are shown in figs 4 and 5, and may be compared with those of a known sample of luteolinidin and its 5-glycoside. The spectrum of the major component L-1 exhibited a peak at long wavelengths of about 495 mμ. The addition of NaOH or solid sodium acetate shifted this peak to a broad one at 568 mμ and $AlCl_3$ to about 545 mμ. All are similar to those reported by Harborne (5, 8). The long wavelength peak of the glycoside and L-3 are similar, both being shifted slightly toward the shorter wavelengths. With UV, all luteolinidin compounds could be detected by their orange fluorescence which intensified to a pink-orange when fumed with ammonia. With visible light, the orange to red-orange spots turned violet to purple.

The third flavonoid was a yellow anthocyanin whose aglycone was similar in all respects to apigeninidin, another anthocyanin without a hydroxyl group in the 3 position (5, 8 and fig 2). R_F characteristics of the 3 forms found are listed in table I. A-1 was the major spot in extracts before and after hydrolysis and it cochromatographed with known samples of apigeninidin in several solvents. A-2 was found only in unhydrolyzed extracts and it cochromatographed with the 5-glucoside, gesnerin. The third form, A-3, found both in unhydrolyzed extracts and in the amyl alcohol fraction of hydrolyzed extracts, was not identified, but appeared to be an acid stable form comparable to L-3. Spectra of the aglycone, the monoglycoside and standards are shown in figures 4 and 5. The addition of NaOH shifted the peak in the spectrum of the aglycone from 480 to 535 mμ as reported by Harborne (5, 8). The glycoside was unstable in alkali under aerobic conditions. Apigeninidin could be detected by an orange yellow color which turned pink or red upon fuming with NH_3, and under UV, there was a similar change in the color of the fluorescence. The glycoside changed from a yellow to pink in white light, and fluoresced a very bright yellow changing to a bright red with NH_3. A-3 was similar

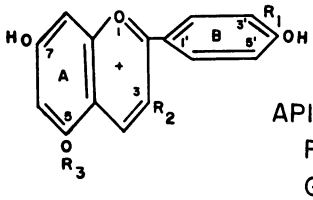
Table I. *Chromatographic Characteristics of Flavonoids Found in Sorghum and of Known Compounds*
R_F values are multiplied by 100.

Compounds	BAW-* HCl	10 % A-* HCl	F-W-HCl*	Forestal*	TLC** EA-F-W-HCl
Red anthocyanin					
C-1, acylated cyanidin-3-glucoside	37	20	62	...	20
C-2, cyanidin-3-glucoside***	23	15	55	...	5
C-3, cyanidin***	50	5	34	56	50
Orange anthocyanin					
L-1, luteolinidin***	42	10	53	70	38
L-2, 5-glycoside	21	35	70	...	...
L-3, ?	42	15	65	85	29
Yellow anthocyanin					
A-1, apigeninidin***	60	20	68	85	54
A-2, 5-glycoside***	25	45	90	...	5
A-3, ?	60	28	79	94	44
Flavone					
Compound A, luteolin*** or 5-glycoside	70	5	35	...	94
Standards:					
Chrysanthemin	23	15	55	...	5
Cyanin	10	28	90	...	0
Cyanidin	50	5	34	56	50
Pelargonidin	81	15	51	70	70
Delphinidin	28	3	25	38	...
Apigeninidin	60	20	68	85	54
" 5-glucoside	25	45	90	...	5
Luteolinidin	42	10	53	70	38
Luteolin	70	5	35	...	94
Dhurrin	60	85	...	...	...
Chlorogenic acid	50	66	...	...	...
Tyrosine	30	90	...	...	...
Phenylalanine	50	95	...	...	...

* Paper chromatography. Solvents described in materials and methods.
** Thin layer chromatography. Solvent described in materials and methods.
*** Cochromatographed with known standards in several solvents in both 1 and 2-dimensional chromatography.

to A-1 in white light, but exhibited a bright yellow white fluorescence on paper when acid which changed to a pink fluorescence with NH_3 fumes.

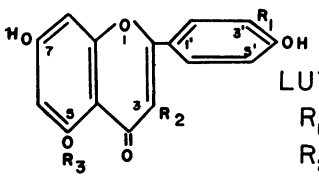
The fourth flavonoid (compound A) found in the smallest amount was probably the flavone, luteolin (fig 2). R_F characteristics are shown in table I.



APIGENINIDIN
R_1, R_2, R_3 = H
GESNERIN
R_3 = GLUCOSE

LUTEOLINIDIN
R_1 = OH
R_2, R_3 = H
5-GLUCOSIDE
R_3 = GLUCOSE

CYANIDIN
R_1, R_2 = OH
R_3 = H
CHRYSANTHEMIN
R_2 = O-GLUCOSE



LUTEOLIN
R_1 = OH
R_2, R_3 = H

FIG. 2. Flavonoid nuclei and substitutions (R) of the types identified in the first internodes of *Sorghum*.

Compound A cochromatographed with known luteolin in several different solvents, and the color reactions were identical. Spectra of compound A and standard luteolin are shown in figure 6. Agreement is good with methanol alone and with added NaOH. The curves do not coincide at the long wavelength peaks after the addition of $AlCl_3$, but the differences could be attributed to only a partial shift in the case of compound A due to impurities affecting pH or some other factor. After acid hydrolysis, the aglycone was easily extracted into ether or ethyl acetate. The glycoside, if present, has not yet been adequately separated from the aglycone chromatographically with the solvents used, and the 5-glucoside would migrate to a position similar to that of the aglycone in BAW and 10 % A (10).

The possibility of small amounts of other flavonoids has not been ruled out. Occasionally, traces of other pigments have been observed in the region of the monoglycosides A- and L-2. Heat treatment of acidified extracts or tissues has never given any indication of the presence of leucoanthocyanins in the first internodes.

Two-dimensional paper chromatography was necessary to separate these flavonoids in crude extracts (C or E). The most useful combination was 10 % A-HCl followed by BAW-HCl, although the pairs A-1 and 2 and L-1 and 2 were inadequately separated. In addition, the first solvent was effectively combined with the formic acid mixture (F-W-HCl). Thin layer chromatography with the ethyl acetate system was the most rapid method of identification with partially purified fractions when only the apigeninidin and luteolinidin compounds were present. This technique was less satisfactory with cyanidin derivatives because of their instability, but was the only one that adequately separated the 4 compounds, A-1 and 3 and L-1 and 3.

When C^{14} labelled DL-phenylalanine-3-C^{14} was fed to excised seed-internodes under light of low intensity for 24 hours, all of the above flavonoids became labelled as detected by the use of X-ray film and the determination of radioactivity of eluted samples dried in planchets. No significant difference was observed in the amount of label incorporated into the various flavonoids.

Presence of These Flavonoids in Other Plant Parts. All stages of growth of *Sorghum* have not been examined, but some of the flavonoids described here are not peculiar to the first internode. The cyanidin type of anthocyanin was produced in the coleoptiles and roots as well as the internodes (3). The yellow and orange pigments, apigeninidin and luteolinidin, were found in large amounts in the glumes and to a much smaller extent in the tan seed coats and in the roots. Most of these pigments were in the form of the aglycones A- and L-1, but the unidentified forms A- and L-3 were also present. Only trace amounts of the glycosides were detected. In addition, there was a large amount of a red-brown pigment in the glumes which was unstable during chromatography, although a major portion remained at the origin in both the 10 % A and formic mixtures. The spectrum of the HCl-methanol soluble portion was quite broad in the long wavelength region, including significant absorption out to 550 mμ, but with a major peak in the luteolinidin area of about 495 mμ. It is possibly a polymeric form. If luteolin was present in the extracts of the glumes, it is found in only trace amounts.

Experimental Conditions Affecting the Formation of These Flavonoids: Dark Grown Controls. The zero time controls in these studies were 4-day-old completely dark grown first internodes, the same stage used by Downs and Siegelman in their study of the photocontrol of anthocyanin biosynthesis (3). The internodes were white except for a slight yellow tinge at the tip just below the coleoptilar node.

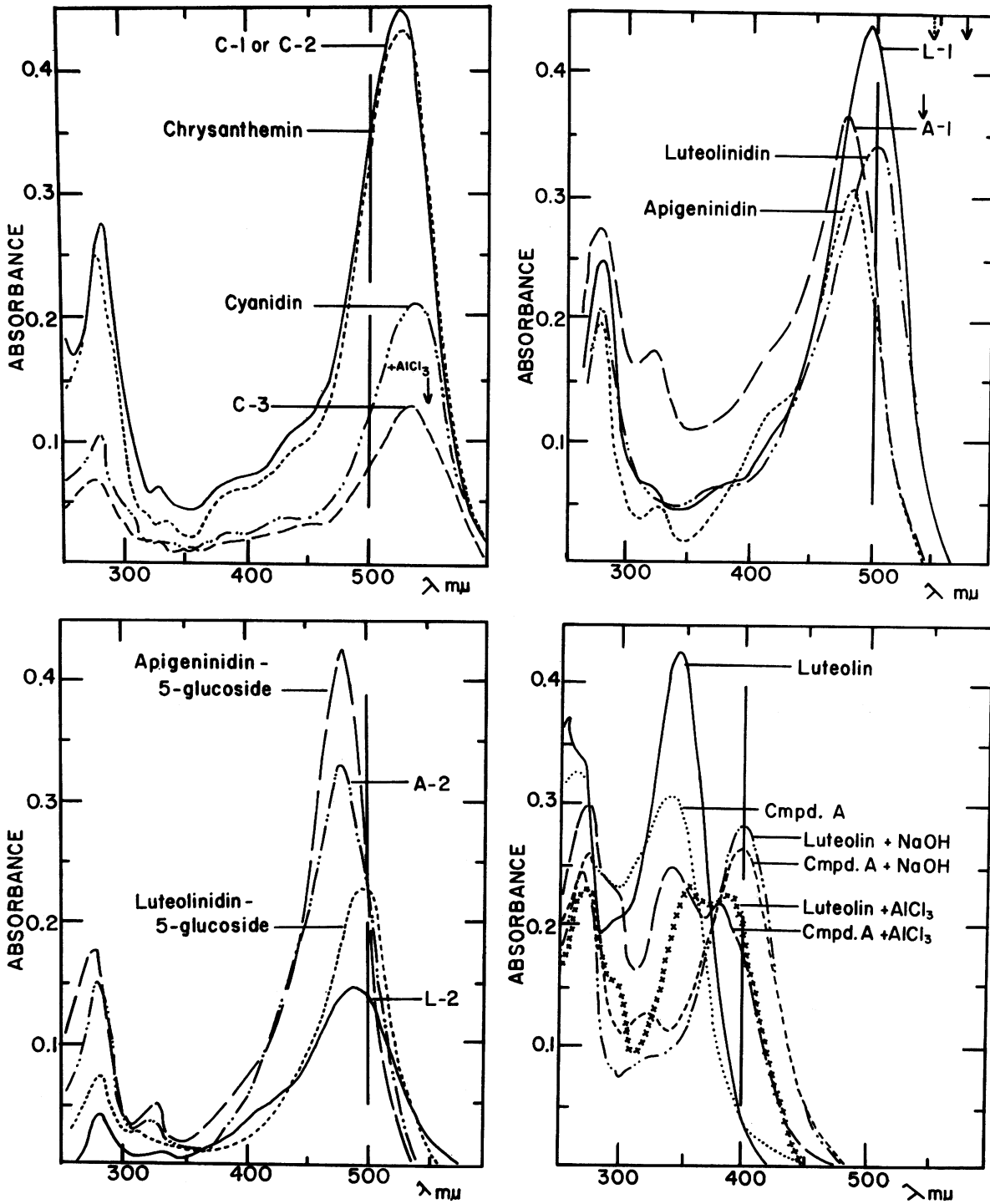


FIG. 3 (*upper left*). Spectra of anthocyanins containing cyanidin and standards in 0.01 % HCl in methanol. The spectra of the acylated form before (C-1) and after alkaline hydrolysis [C-2, which cochromatographs with cyanidin-3-glucoside (chrysanthemin)] are similar. The spectrum after acid hydrolysis (C-3) is similar to that obtained with known cyanidin; both gave similar spectral shifts with $AlCl_3$. FIG. 4 (*upper right*). Spectra of presumed luteolinidin (L-1) and apigeninidin (A-1) with standards in 0.01 % HCl in methanol. Arrows indicate shifts in the peak at long wavelengths upon addition of NaOH (solid arrow) and $AlCl_3$ (broken arrows). FIG. 5 (*lower left*). Spectra of glycosides A-2 and L-2 eluted in 0.01 % HCl in methanol with standards. The data for the curve for luteolinidin-5-glucoside was obtained from J. B. Harborne. FIG. 6 (*lower right*). Spectra of presumed luteolin (compound A) in methanol alone, with added NaOH and with $AlCl_3$, compared with a known sample of luteolin.

When an aliquot of extract C or E equivalent to 10 to 50 mg dry weight of internodes was applied to paper for 2-dimensional chromatography, flavonoids were not detectable or were present in only trace amounts. Traces of a spot which cochromatographed with gesnerin, the 5-glucoside of apigeninidin, were found, but the concentration of this material was so low that it could be detected only by its characteristic pink fluorescence when the area was fumed with NH_3 or sprayed with Na_2CO_3. No evidence of other flavonoids was found, but there were unidentified phenolic compounds migrating to high R_F values in both 10% A, BAW or BeAW (2: 1) solvents. Dhurrin, a cyanogenic glycoside (12), was the major phenolic compound in unhydrolyzed extracts (C or E). It could be detected at a level of less than 1 mg of tissue per spot with sprays such as Na_2CO_3 as a yellow spot after 24 hours or with 2,4-dinitrophenylhydrazine plus Na_2CO_3 as a slow-forming purple spot. Besides a similarity in R_F characteristics, the dhurrin isolated from internodes produced the same acid and alkaline degradation products as a known sample of dhurrin. Both standard and presumed dhurrin exhibited spectra with sharp peaks at 274 mμ. Free amino acids could also be detected as ninhydrin spots at similar levels.

After alkaline hydrolysis, and in some cases acid hydrolysis, a variety of C_9 hydroxycinnamic acids were detected chromatographically in the ether soluble fraction F (or H) by means of color reactions in white and UV light before and after spraying with Fast Red and Na_2CO_3. At the level of 10 to 50 mg dry weight of tissue per spot, both sinapic and ferulic acids were present in greatest amounts, with smaller amounts of *p*-hydroxycinnamic acid and possible traces of caffeic acid. In both acid and alkaline hydrolyzates, there were other unidentified phenolic compounds detected with the $FeCl_3$-$K_3(FeCN_6)$ reagent, and nonphenolic compounds observed as UV absorbing or fluorescing spots. Solvent systems used in 2-dimensional chromatography were either of the benzene-acetic acid-water mixtures, followed by 2%, 10% acetic or butanol-ammonia-water. When it was necessary to separate ferulic and sinapic acids, the sodium formate-formic acid-water mixture was used.

Effect of Continued Darkness. If the seedlings were kept intact for a further period of 48 hours in the dark, the internodes doubled in length and all 3 forms of apigeninidin and luteolinidin were produced. Significant amounts of the same anthocyanins were also detected in internodes excised in the dark and kept on moist filter paper for a further 48 hours in the dark. Under these conditions, there was little further elongation. These pigments were found mainly in the lower portions of the internode. No cyanidin was ever detected in these completely dark grown plants (table II).

Effect of Low Light Intensity. While the above experiments indicated that light was not an obligatory requirement for the production of apigeninidin and luteolinidin, white light of a low intensity of 1 to 12 hours duration did speed up the rate of production of these pigments so that total incubation periods of 12 to 24 hours were sufficient to detect these compounds chromatographically or by spectrophotometric assay of the crude HCl-methanolic extracts C or E (fig 7). Treatment with white light also inhibited further elongation of the intact internode. There was an indication that the apigeninidin pigments predominated in the dark with a shift toward the production of a greater amount of luteolinidin in the light treated seedlings. These shifts could be detected in the spectra of crude extracts (fig 7). All 3 compounds of each were detected, but the aglycones A-1 and L-1 were always the major forms.

If the treatment with white light of low intensity was continued for 8 to 18 hours, with a subsequent dark period for a total of 24 hours, the red cyanidin was detected along with comparable amounts of apigeninidin and luteolinidin (table II and fig 7). In addition, the yellow flavone, luteolin, could sometimes now be detected. All 3 forms of apigeninidin and luteolinidin were found, but only the acylated form

Table II. *Relative Amounts of Flavonoid Pigments Produced in Internodes after 48 Hours in Darkness and in Light*
The pigments were estimated after chromatography of aliquots of extracts C equivalent to 3 to 5 mg dry weight of tissue.

	Apigeninidin Compounds	Luteolinidin Compounds	Cyanidin Compounds	Luteolin Compound A
Darkness–4 days				
0′ control	Trace	0	0	0
+ 48 hr dark	++	+	0	0
+ 1 hr low light + 47 hr dark	++	+	0	0
+ 12–24 hr low light + dark for total of 48 hr	+	+++	++	+
+ 12–24 hr high light + dark for total of 48 hr	Trace	++	++++ ++++	+

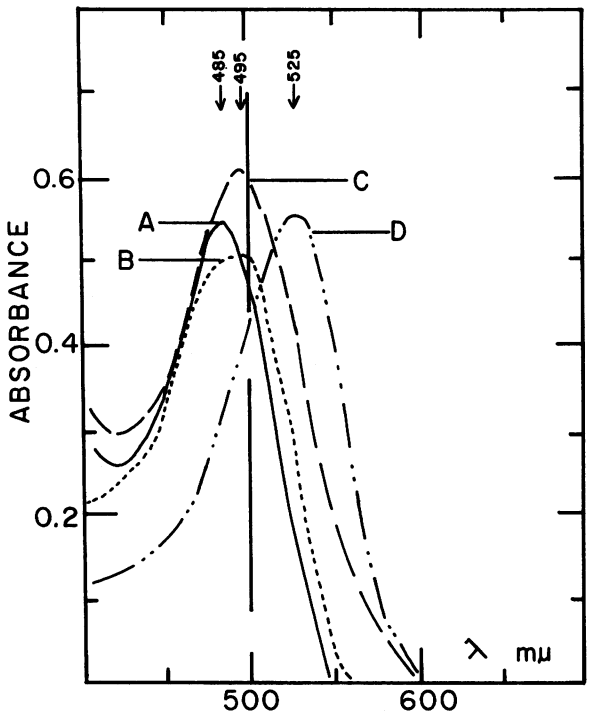


FIG. 7. Spectra of HCl-methanol extracts (C) of excised 4-day-old dark grown internodes after 48 hours incubation under the following conditions: Curve A, excised and incubated in complete darkness; B after 30 to 60 minutes of laboratory light of about 300 ft-c with the remainder of 48 hours in the dark; C after about 8 hours daily of white light of about 300 ft-c with dark periods for a total of 48 hours; D, as in C but about 1000 ft-c of white light. Absorbance per mg dry weight per ml in the cuvette was about 0.1 for A, B, and C, and 0.3 for D. Two-dimensional chromatography indicated the presence in extracts A and B of all 3 forms of apigeninidin and luteolinidin, but slightly more of the latter types in B. C extracts contained the same, but less of the apigeninidin types, and in addition the acylated cyanidin glycoside (C-1). D contained predominantly the cyanidin C-1, but traces of A-2, L-1 and L-2.

of the cyanidin glucoside was detected in nonhydrolyzed extracts.

Effect of High Light Intensity. With white light at about 1000 ft-c, the predominance of the acylated cyanidin studied by Downs and Siegelman (3) was striking. This red anthocyanin could be visually detected on chromatograms at less than 1 mg dry weight of tissue per spot applied, a dilution at which other phenolic compounds except dhurrin were undetectable. The spectrum of the crude extract was typical of the cyanidin derivative only (fig 7). In the excised internode or the seed-internode system in bright light, the yellow and especially the orange anthocyanins were still detectable, but were at a lower concentration than after the treatment with light of low intensity. Their presence was easily obscured by the predominant red anthocyanin, and repeated chromatographic separations sometimes were necessary to detect them. The easiest way to detect them was in fraction I after acid hydrolysis which tended to destroy more of the cyanidin type. Under all light conditions tested, more pigments and a shift toward those of the cyanidin type were produced in intact seed-shoots or seed-internodes than in excised internodes.

At high light intensities for 16 to 24 hours, the internodes generally became completely red, except at the very tip just below the coleoptilar node. Initially, the color first appeared in the epidermal layers, but ultimately the red color extended through the internode. At lower light intensities, scattered red and orange-red patches were visible, the latter generally beginning at the base of the internode. In complete darkness, randomly scattered orange-brown patches were visible in intact internodes. With completely excised internodes in the dark or with low light intensity, the base became completely brown. Some of the brown color was not extracted in the HCl-methanol and the final residue was tan with dark brown dots in contrast to the white residue of the 4-day-old controls.

In addition to those participating in the browning reaction, other phenolic products were observed. These diffused out of the internodes or other cut surfaces such as the root stump on to the filter paper. These spots varied in color from an initial yellow or orange and changed to purple or red upon drying. They gave a strong blue color with the $FeCl_3$-K_3 ($FeCN_6$) spray. Some of these products were soluble in HCl-methanol and apigeninidin and luteolinidin have been identified. Other products were insoluble in organic reagents, but could be dissolved in dilute alkali.

Discussion and Conclusions

Flavonoids were not detected in the actively growing parts of the first internode. Upon cessation of growth, C_{15} compounds accumulated. Growth of the internode ceased upon excision even in the dark, or in the intact seedling after light treatment. In internodes capable of further elongation at the tip, the flavonoids present were localized in the basal or oldest half, and were never found in the extreme tip even after growth had ceased.

Light was not an obligatory requirement for the condensation of C_2 and C_9 units to produce the C_{15} flavonoid skeleton, or for the formation of the flavylium cation peculiar to anthocyanins, although light may have accelerated 1 or more of the early steps. Since some step in the synthesis is affected by red light of low intensity (3), and a part of the inhibition of the first internode of grasses is also under control of a red sensitive system (6), a common reaction step is possible. Because cessation of growth occurs rapidly with white light and at least 6 hours was necessary for detectable pigment production, it is not likely that an anthocyanin inhibitor of growth

is involved, although an earlier C_{15} intermediate might be. The 2 processes could also be competing for the same C_2 or other units. A similar relationship between the control by red light of flavonoid biosynthesis and growth of the hypocotyl in *Fagopyrum* (buckwheat) is discussed by Mohr (12, 14).

Light treated plants tended to have more of the flavonoids with the B-ring substituted with hydroxyl groups at both 3′ and 4′ positions found in cyanidin, luteolinidin and luteolin, rather than just the 4′ position as in apigeninidin (fig 2). Therefore, this second hydroxylating step, either at the level of C_9 or C_{15} intermediates, may be accelerated by a low intensity of light, possibly via the red light or phytochrome effect. This would be consistent with data reported in peas where kampferol glycosides accumulated in etiolated peas and quercetin glycosides in light grown peas (4).

White light of a relatively high intensity was an obligatory requirement for the synthesis of the red cyanidin. This may be the blue light effect of Downs and Siegelman (3). Since cyanidin differs from apigeninidin and luteolinidin in the presence of an hydroxyl group at the 3 position (fig 2), this step could be a leading contender for the site of the action of the light in *Sorghum*. But in other plants, the step controlled by bright light was not associated just with the occurrence of an hydroxyl group in the 3 position, but with a combination of this and the flavylium cation peculiar to anthocyanins. For instance, a facultative requirement for light in the synthesis of the flavonol, quercetin, and an obligatory one for that of the anthocyanidin, cyanidin, have been demonstrated in *Fagopyrum* (12, 14).

The significance of the presence of only the acylated glycoside of the cyanidin and the multiple forms of the apigeninidin and luteolinidin is not clear. The aglycone was the dominant form even in unhydrolyzed extracts. Glucosidation in the 5 position may be slow in comparison with the production of the aglycone. If the spot tentatively identified as luteolin is present also in the glycosidated form, R_F values would indicate that the sugar was in the 5 position (10). The cyanidin, on the other hand, is glucosidated in the 3 position in chrysanthemin (fig 2). Although definite evidence of an acylated form of the A and L types has not been found, it is possible that the unidentified A and L-3 forms are acylated, but that they or the monoglycosides formed are unstable in alkali so that a stepwise breakdown is not detected. Since these forms are still found after acid hydrolysis in 2 N HCl for 2 hours, the linkages are highly acid stable.

Prior to this study, apigeninidin and luteolinidin have been identified only in 2 dicot families, the *Gesneriaceae* and *Sterculiaceae* (11); their distribution may be more widespread than formerly thought.

The nongrowing first internodes have a potential for flavonoid production which is expressed in various ways. With sufficient light and precursors, the pathway leading to cyanidin is preferred; without high light intensity, apigeninidin and luteolinidin accumulate. Further work needs to be done to determine what factors other than light may affect these syntheses. Preliminary experiments indicate that over 10 times as much of the total yellow and orange pigments can be produced if the internodes are infiltrated with a solution of 0.05 M KH_2PO_4.

The data presented do not clarify the problem of C_{15} intermediates in anthocyanin biosynthesis. Synthesis was at least partially de novo, because radioactivity from C^{14}-phenylalanine was incorporated into the final products. Translocation of C_{15} units from the seed was ruled out, since excised internodes could synthesize flavonoids. It is possible that none of the 4 pigments described here is an intermediate in the synthesis of any of the other pigments, but that all are end products branching from the main flavonoid pathway at various points. Although all 3 anthocyanidin nuclei were sometimes present in extracts of internodes, they may not have occurred in the same cell, as the macroscopically visible pattern of orange-brown and of red patches of pigments did not always coincide. The concentration of C_{15} intermediates could, of course, be too low for detection. But it is also possible that true intermediates in flavonoid biosynthesis may exist only on the surface of an enzyme complex capable of catalyzing a sequence of chemical events leading to the various aglycones and their glycosides. Isolation of the cell-free enzymes concerned is vital to the study of the intermediate steps in flavonoid biosynthesis.

Summary

Four-day-old first internodes of *Sorghum vulgare* var. Wheatland milo, grown in complete darkness, contained little or no detectable flavonoids, although C_9 phenolic compounds and dhurrin were relatively abundant. After subsequent dark or light treatment, 4 flavonoids could be identified in the nongrowing portions of either intact or excised internodes. Evidence was based on solubility characteristics, color reactions, R_F values and spectrophotometric data. Three were forms of anthocyanidins, the acylated cyanidin-3-glucoside studied by previous workers, and apigeninidin and luteolinidin which existed as the aglycone, the 5-glucoside, and an unidentified acid stable form. The fourth was probably the flavone, luteolin. All pigments became labelled when tissues were incubated with DL-phenylalanine-3-C^{14}.

White light of high intensity was obligatory for the production after 12 to 24 hours of the red cyanidin glycoside in 4-day-old intact or excised internodes from dark grown seedlings. But only an incubation in the dark for 48 hours in addition to the initial 4 days of growth in the dark was necessary for the synthesis of all 3 forms of apigeninidin and luteolinidin. Light, therefore, was not required for the synthesis of C_{15} compounds or for the flavylium cation, although it may have accelerated their formation. There was an indication that light favored the syn-

thesis of the B-ring substituted with hydroxyl groups in both the 3′ and 4′ positions, because the production of luteolinidin in light was greater than that of apigeninidin, and luteolin has been detected only after treatment with light of at least a low intensity.

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Changes in Calcium Levels in Cell Walls during Elongation of Oat Coleoptile Sections [1, 2]

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The calcium bridge hypothesis was proposed by Bennet-Clark (1) in 1955 to explain the mode of action of IAA in controlling elongation of oat coleoptile sections. He found that the chelating agent ethylenediaminetetraacetic acid (EDTA) promotes growth of oat coleoptile sections. He assumed that the chelating agent stimulated growth by removing calcium from the cell wall and believed that IAA acted in a similar manner. Cleland (4) recently tested this hypothesis by determining the effect of IAA upon the redistribution of Ca^{45} within the cell wall and loss of calcium from the wall. He found that the loss of calcium from *Avena* coleoptile and maize mesocotyl cell walls is not enhanced by auxin and that auxin does not cause a redistribution of calcium between pectin and protopectin. On the basis of these findings he suggested that the removal of calcium cross-linkages is not brought about by auxin and that the calcium bridge hypothesis is incorrect.

If IAA has no effect on the redistribution of calcium, the calcium levels might be expected to be similar in the walls of sections incubated in inhibitory concentrations of calcium both in the presence and absence of added IAA. A search of the literature has not revealed data that either supports or refutes the assumption that the inhibition of cell elongation in the presence of supraoptimal levels of calcium is due to an increase of calcium in the cell wall. Therefore, the current study was undertaken to determine the relationship between the absolute calcium levels in

[1] Received June 22, 1964.

[2] This paper is based on a thesis submitted by the senior author to Dartmouth College in partial fulfillment of the requirements for the M.A. degree. This work was supported in part by a research grant from the National Science Foundation (GB705).

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