resulted in increased indole disappearance. The omission of pyridoxal phosphate also markedly reduced the extent of indole disappearance.

L-Serine rather than D-serine appeared to be involved in the coupling reaction. Glycine, threonine, homoserine, cysteine, alanine and glycolate were all unable to substitute for serine. pH dependence was studied in 3 different buffer systems. The overall results suggest low activity below pH 7, a sharp increase starting somewhere between 6.5 and 7, and a leveling-off somewhere near 8 or 8.5. Indole disappearance progressed at a constantly decreasing rate during the 100 minute incubation period studied.

Expanded pea leaves also yielded active extracts. Preparations from stems, root tips, pods, and developing seeds, as well as plants other than the pea, did not promote serine-dependent indole disappearance. The results with pea cotyledons were uncertain. The tryptophan synthetase of pea buds appears to be very labile, and it is believed that the observed lack of activity in extracts of other plants may be partly attributable to enzyme lability.

Chromatographic evidence suggests that in the pea bud system, the product of serine-dependent indole disappearance is tryptophan. It is concluded that pea bud extracts contain an enzyme analogous to tryptophan synthetase in microorganisms, and that tryptophan synthesis in the pea plant probably follows the same pathway as in microorganisms.

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IDENTIFICATION OF THE ANTHOCYANINS IN PETALS OF TULIP VARIETIES SMILING QUEEN AND PRIDE OF HAARLEM [1]

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Willstatter and Bolton (9) were the first to identify the pigments from petals of *Tulipa gesneriana*. They found that the scarlet-red color of some varieties was due to a mixture of cyanidin diglucoside (cyanin) and carotenoids. Robinson and Robinson (6) found that garden tulips contained either a mixture of cyanidin and pelargonidin biosides or cyanidin bioside and delphinidin diglucoside. They stated that "the examination of the anthocyanins of the tulip is much more difficult than that of most other plants." Recently the anthocyanin from the tulip variety Queen of the Night was identified by Shibata (7) as delphinidin rhamnoglucoside and Shibata and Sakai (8) identified the anthocyanin of the variety Eclipse as cyanidin rhamnoglucoside. There is a wide range of complexity in the anthocyanin constitution of tulips ranging from the simple one component found by Shibata (7) and Shibata and Sakai (8) to the more complex multiple components found in the varieties examined by Robinson and Robinson (6).

The purpose of the present investigation was to isolate and identify by chromatographic and spectrophotometric methods the anthocyanins from the petals

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of the tulip varieties Smiling Queen and Pride of Haarlem. These varieties were chosen because a preliminary examination had indicated that they contain several pigments.

Methods and Materials

Smiling Queen: Petals were dried at 50° C in a forced-draft oven and then ground in a Wiley mill to pass a 20-mesh screen. The ground tissue was extracted in cold methanol containing 1 % hydrochloric acid by blending for 5 minutes. The extract was filtered and the anthocyanins were precipitated with neutral lead acetate. The supernatant was discarded, and the lead salt was washed with distilled water and then air-dried at room temperature. The anthocyanins were recovered from the lead salt by blending with methanol containing 2 % hydrochloric acid for 5 minutes. The lead chloride precipitate was removed by filtration. Excess lead in the methanolic anthocyanin extract was precipitated by passing hydrogen sulfide through the solution and it was removed by centrifugation. The methanolic anthocyanins solution was then concentrated under reduced pressure at 40° C, and the anthocyanins were precipitated with ether. The anthocyanins precipitate was dissolved in a minimum volume of methanol containing 1 % hydrochloric acid and placed at 4° C for 3 days. In the cold a brown-yellow precipitate formed and was removed by filtration and discarded. The methanolic anthocyanins solution was then taken to dryness under reduced pressure at 4° C and the residue dissolved in a minimum volume of 1 % aqueous hydrochloric acid. The aqueous anthocyanins solution was shaken several times with ethyl acetate to remove yellow flavonoid pigments and the clear red solution containing the anthocyanins was subjected to a paper chromatographic separation.

Chromatograms were prepared by streaking the red solution in a band approximately 1 cm wide across the narrow width of Whatman no. 3 MM paper (18 × 22 inches). The papers were irrigated in a chromatographic cabinet by the descending method in a solvent of 1-butanol : acetic acid : water (6 : 1 : 2 v/v). The anthocyanins separated into 3 bands and each band was cut out and eluted with methanol containing 5 % acetic acid. The separated anthocyanins were rechromatographed, as previously described by using 15 % acetic acid as the solvent. The 1st band separated with the 1-butanol : acetic acid : water (6 : 1 : 2 v/v) solvent resolved into 2 components in 15 % acetic acid. The 4 anthocyanins obtained were further purified by rechromatographing with 1-butanol : acetic acid : water (6 : 1 : 2 v/v) on water-washed Whatman no. 3 MM filter paper.

A 1 ml portion of the purified, concentrated anthocyanin was hydrolyzed with 1 ml of 2 *N* hydrochloric acid by heating at 100° C under reflux for 45 minutes. The anthocyanidin fraction was extracted with a minimum volume of *iso*-amyl alcohol and the aqueous phase was examined for sugars as described by Asen et al (1).

R_f values for the anthocyanins and anthocyanidins were determined by ascending chromatography. A suitable portion of each extract was applied at 1 inch intervals along the starting line on 15 × 16 inch sheets of Whatman no. 1 filter paper rolled into cylinders as described by Irreverre and Martin (5) and irrigated with solvents listed in table I and II. Detection of the anthocyanins and anthocyanidins was

Table I

R_f Values and Wave Lengths of Maximum Absorption for the Anthocyanins of Tulip var. Smiling Queen

Compounds	R_f values in indicated solvent							λ Max in ethanol containing 0.01 *N* HCl (Mμ)
	a	b	c	d	e	f	g	
Tulip anthocyanins:								
Band no. 1	.27	.35	.46	.49	.38	.41	.25	537
Band no. 2	.32	.33	.34	.45	.32	.56	.39	539
Band no. 3	.36	.47	.62	.74	.40	.59	.32	517
Band no. 4	.42	.46	.67	.75	.48	.46	.40	517
Authentic anthocyanins:								
Cyanidin-3-glucoside	.33	.34	.43	.51	.35	.43	.24	538
Cyanidin-3-galoctoside	.32	.32	.51	.61	.32	.44	.25	538
Cyanidin-3-rhamnoglucoside	.29	.32	.35	.47	.34	.56	.35	539
Pelargonidin-3-glucoside	.44	.43	.67	.79	.51	.45	.32	518
Pelargonidin-3-rhamnoglucoside	.30	.45	.65	.77	.43	.57	.41	517
Delphinidin-3-glucoside	.12	.21	.17	.30	.21	.29	.19	552

Solvent:

a. 1-Butanol : acetic acid : water (6 : 1 : 2 v/v)
b. 1-Butanol : 2 *N* hydrochloric acid (1 : 1 v/v)
c. *m*-Cresol : acetic acid : water (50 : 2 : 48 v/v)
d. Phenol : water (73 : 27 w/w)
e. Ethyl acetate : *t*-Butyl alcohol : acetic acid : water (5 : 4 : 1 : 3)
f. Acetic acid (15 %)
g. Acetic acid (5%)

made by co-chromatography with authentic compounds.

The absorption spectra of the anthocyanins and anthocyanidins were determined by using an ethanolic solvent containing 0.01 *N* hydrochloric acid.

PRIDE OF HAARLEM: A 15 g sample of dried ground petals was extracted in 500 ml of methanol containing 1 % hydrochloric acid for 20 hours at room temperature. The extract was filtered and the anthocyanins precipitated with neutral lead acetate. Only a portion of the anthocyanins precipitated as the lead salt and the lead salt precipitate was removed by filtration. A considerable amount of the pigment remained in solution even when excess neutral lead acetate was added. The color of the solution indicated that it contained principally pelargonidin glycosides. Excess lead was precipitated by passing hydrogen sulfide gas through the solution and was removed by centrifugation. The solution was taken to dryness under reduced pressure at 40° C and the residue was dissolved in a minimum volume of methanol. The anthocyanins were precipitated with ether and kept at −5° C for 24 hours. The ether-precipitated anthocyanins were recovered by filtration and dissolved in a minimum volume of methanol. The anthocyanins precipitated by neutral lead acetate were recovered by blending for 5 minutes in methanol containing 2 % hydrochloric acid and filtering. The anthocyanin extracts from the ether precipitate and the lead precipitate were combined and further purified by reprecipitating from ether 4 more times. The anthocyanins from the last ether precipitation were reconstituted in water and extracted 5 times with ethyl acetate to remove other flavonoid pigments. The aqueous anthocyanins solution was taken to dryness under reduced pressure at 40° C and the residue dissolved in a minimum volume of methanol, precipitated with ether and kept at −5° C for 3 days.

The isolation and purification of the anthocyanins were accomplished by column chromatography since Harborne (4) found that arabinose could be produced as an artifact during the purification of anthocyanin on Whatman no. 3 filter paper. Columns of Whatman cellulose powder 40 by 500 mm were prepared for each solvent used to resolve the anthocyanins. The columns were packed in approximately 5 cm bands by pouring a suitable volume of a suspension of the cellulose powder in the solvent into a 10 cm column of the solvent and allowing the cellulose powder to settle completely before pouring the next portion.

The anthocyanin was dissolved in a minimum volume of 1-butanol : acetic acid : water (6 : 1 : 2 v/v), placed on the top of the cellulose powder column and developed with the same solvent. The anthocyanin resolved into 3 bands, which were collected automatically in 5 ml fractions. The resolved bands were contaminated with yellow pigments and compounds which fluoresced in ultra-violet light. The 1st and 2nd band resolved by 1-butanol : acetic acid : water (6 : 1 : 2 v/v) were further purified by re-chromatographing with 15 % acetic acid. In this solvent the 2nd band separated into 2 components. The 3rd band resolved by 1-butanol : acetic acid : water (6 : 1 : 2 v/v) was further purified by developing with 2 *N* hydrochloric acid : 1-butanol (1 : 1 v/v) followed by 15 % acetic acid. In the 15 % acetic acid solvent, the 3rd band separated into 2 components. The resolved 5 pigments were identified by methods previously described.

RESULTS

SMILING QUEEN: The R_f values and the wave length of maximum absorption for the 4 anthocyanins resolved indicated that 2 are cyanidin glycosides (bands 1 and 2) and that 2 are pelargonidin glycosides (bands 3 and 4) (table I). The 2 cyanidin glycosides

TABLE II

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR ANTHOCYANIDINS FROM ACID HYDROLYSIS OF THE ANTHOCYANINS IN TULIP VAR. SMILING QUEEN

COMPOUND	R_f VALUES IN INDICATED SOLVENT			λ MAX IN ETHANOL CONTAINING 0.01 *N* HCl (Mμ)
	a	b	c	
Tulip anthocyanidins:				
Band no. 1	.56	.76	.65	544
Band no. 2	.56	.75	.64	544
Band no. 3	.75	.84	.84	529
Band no. 4	.75	.84	.85	530
Authentic anthocyanidins:				
Cyanidin	.56	.76	.65	545
Pelargonidin	.76	.84	.85	530
Delphinidin	.37	.46	.33	555
Petunidin	.53	.55	.48	557

Solvents:

a. Acetic acid : hydrochloric acid : water (30 : 3 : 10 v/v)
b. 1-Butanol : 2 *N* hydrochloric acid (1 : 1 v/v)
c. *iso*-Amyl alcohol : hydrochloric acid : water (5 : 1 : 1 v/v)

were best separated by *m*-cresol : acetic acid : water (50 : 2 : 48 v/v), 15 % acetic acid and 5 % acetic acid, while the pelargonidin glycosides were best resolved by ethyl acetate : *t*-butyl alcohol : acetic acid : water (5 : 4 : 1 : 3), 15 % acetic acid, and 5 % acetic acid.

To identify the anthocyanins further, the 4 pigments were acid-hydrolyzed and the products were identified by paper chromatographic and spectrophotometric methods. The anthocyanidin produced from bands 1 and 2 was cyanidin and from bands 3 and 4 pelargonidin (table II). Hydrolysis of bands 1 and

TABLE III

R_g VALUES AND COLOR WITH ANILINE HYDROGEN PHTHALATE OF SUGARS FROM ACID HYDROLYSIS OF ANTHOCYANINS IN PETALS OF TULIP VAR. SMILING QUEEN

COMPOUND	R_g VALUES IN INDICATED SOLVENT			COLOR WITH ANILINE HYDROGEN PHTHALATE
	a	b	c	
Sugars from acid hydrolysis of tulip anthocyanins:				
Band no. 1	1.00	1.00	1.00	Brown
Band no. 2	1.01	1.00	1.01	Brown
	1.68	1.62	1.53	Brown
Band no. 3	1.00	0.99	1.00	Brown
	1.69	1.65	1.52	Brown
Band no. 4	1.00	1.00	1.00	Brown
Authentic sugars:				
Glucose	1.00	1.00	1.00	Brown
Galactose	0.91	0.95	1.10	Brown
Lyxose	1.40	1.27	1.28	Red
Arabinose	1.17	1.16	1.33	Red
Xylose	1.39	1.31	1.21	Red
Rhamnose	1.69	1.63	1.53	Brown

Solvents:
a. Ethyl acetate : pyridine : water (8 : 2 : 1 v/v)
b. 1-Butanol : ethanol : water (40 : 11 : 19 v/v)
c. Phenol : water (73 : 27 w/w)

TABLE IV

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR THE ANTHOCYANINS OF TULIP VAR. PRIDE OF HAARLEM

COMPOUND	R_f VALUES IN INDICATED SOLVENT						λ MAX IN ETHANOL CONTAINING 0.01 *N* HCl (Mμ)
	a	b	c	d	e	f	
Tulip anthocyanins:							
Band no. 1	.15	.13	.35	.44	.25	.44	553
Band no. 2	.25	.30	.34	.55	.38	.53	539
Band no. 3	.30	.32	.44	.57	.36	.43	538
Band no. 4	.34	.45	.60	.72	.47	.55	518
Band no. 5	.43	.42	.63	.78	.50	.43	518
Authentic anthocyanins:							
Cyanidin-3-glucoside	.30	.31	.46	.61	.38	.41	538
Cyanidin-3-galoctoside	.28	.30	.48	.70	.36	.43	538
Cyanidin-3-rhamnoglucoside	.27	.33	.34	.56	.40	.53	539
Pelargonidin-3-glucoside	.41	.45	.68	.80	.51	.42	518
Pelargonidin-3-rhamnoglucoside	.35	.42	.60	.73	.50	.55	518
Delphinidin-3-glucoside	.19	.16	.17	.45	.25	.27	552
Delphinidin-3-rhamnoglucoside	.16	.16	.36	.44	.24	.44	552

Solvents:
a. 1-Butanol : acetic acid : water (6 : 1 : 2 v/v)
b. 1-Butanol : 2 *N* hydrochloric acid (1 : 1 v/v)
c. *m*-Cresol : acetic acid : water (50 : 2 : 48 v/v)
d. Phenol : water (73 : 27 w/w)
e. Ethyl acetate : 3-Butanol : acetic acid : water (5 : 4 : 1 : 3 v/v)
f. Acetic acid (15 %)

4 yielded 1 sugar identified as glucose and that of bands 2 and 3, 2 sugars, identified as glucose and rhamnose, (table III). The 4 anthocyanins in the petals of the tulip variety Smiling Queen are cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin), and pelargonidin-3-rhamnoglucoside.

PRIDE OF HAARLEM: The R_f values and the wave length of maximum absorption for the 5 anthocyanins resolved indicate that there is 1 delphinidin glycoside (band 1), 2 cyanidin glycosides (bands 2 and 3) and 2 pelargonidin glycosides (bands 4 and 5) (table IV).

Acid hydrolysis of the anthocyanins yielded delphinidin from band 1, cyanidin from bands 2 and 3 and pelargonidin from bands 4 and 5 (table V). The sugar moiety of the anthocyanins yielded glucose for bands 3 and 5 and glucose and rhamnose for bands 1, 2, and 4 (table VI). The 5 anthocyanins in the petals of the tulip variety Pride of Haarlem are delphinidin-3-rhamnoglucoside, cyanidin-3-rhamnoglucoside (an-

TABLE V

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR ANTHOCYANIDINS FROM ACID HYDROLYSIS OF THE ANTHOCYANINS IN TULIP VAR. PRIDE OF HAARLEM

COMPOUND	R_f VALUES IN INDICATED SOLVENT			λ MAX IN ETHANOL CONTAINING 0.01 *N* HCl (Mμ)
	a	b	c	
Tulip anthocyanidins:				
Band no. 1	.38	.43	.34	555
Band no. 2	.56	.72	.70	545
Band no. 3	.56	.73	.70	544
Band no. 4	.77	.78	.82	530
Band no. 5	.77	.79	.82	529
Authentic anthocyanidins:				
Cyanidin	.55	.73	.70	545
Pelargonidin	.77	.79	.82	530
Delphinidin	.37	.41	.34	555
Petunidin	.54	.52	.47	557

Solvents:
a. Acetic acid: hydrochloric acid: water (30: 3: 10 v/v)
b. 1-Butanol: 2 *N* hydrochloric acid (1: 1 v/v)
c. *iso*-Amyl alcohol: hydrochloric acid: water (5: 1: 1 v/v)

TABLE VI

R_g VALUES AND COLOR WITH ANILINE HYDROGEN PHTHALATE OF SUGARS FROM ACID HYDROLYSIS OF ANTHOCYANINS IN PETALS OF TULIP VAR. PRIDE OF HAARLEM

COMPOUND	R_g VALUES IN SOLVENT			COLOR WITH ANILINE HYDROGEN PHTHALATE
	a	b	c	
Sugars from acid hydrolysis of tulip anthocyanins:				
Band no. 1	1.00	0.98	1.01	Brown
	1.86	1.56	1.62	Brown
Band no. 2	1.00	0.99	1.00	Brown
	1.92	1.61	1.59	Brown
Band no. 3	1.01	0.98	1.00	Brown
Band no. 4	1.00	1.00	0.98	Brown
	1.94	1.59	1.60	Brown
Band no. 5	1.00	0.98	1.00	Brown
Authentic sugars:				
Glucose	1.00	1.00	1.00	Brown
Galactose	0.89	0.95	1.09	Brown
Lyxose	1.55	1.32	1.28	Red
Arabinose	1.30	1.20	1.34	Red
Xylose	1.37	1.30	1.22	Red
Rhamnose	1.91	1.58	1.60	Brown

Solvents:
a. Ethyl acetate: pyridine: water (8: 2: 1 v/v)
b. 1-Butanol: ethanol: water (40: 11: 19 v/v)
c. Phenol: water (73: 27 w/w)

tirrhinin), cyanidin-3-glucoside (chrysanthemin), pelargonidin-3-rhamnoglucoside and pelargonidin-3-glucoside (callistephin).

Discussion

Acid hydrolysis of anthocyanins purified on Whatman no. 3 filter paper may produce arabinose as an artifact (4). This was eliminated by the use of cellulose powder columns for the resolution of the anthocyanins in the petals of the tulip variety Pride of Haarlem. Separation of the anthocyanins on the cellulose powder columns was usually accomplished within 4 hours and also offered the advantage of being a simple means of purifying a large amount of pigment. The cellulose powder columns had to be remade frequently because of the accumulation of air bubbles. The resolution of the anthocyanins on these columns was not quite as distinct as with filter paper presumably because of the loose packing.

Hall (3) and Beal et al (2) studied the anthocyanins of most species of the genus Tulipa and found that the 2 sub-genera differed in their anthocyanin constitution. Only pelargonidin and cyanidin derivatives were found in the Leiostemones and only cyanidin and delphinidin derivatives in the Eriostemones. The anthocyanin in all cases was a pentoseglycoside. Robinson and Robinson (6) examined 34 varieties of tulips and separated them into 2 groups. They stated that there was 1 group "in which pelargonidin and cyanidin occur as 3-biosides and then apparently with a rather sharp separation a second group containing delphinidin derivatives" sometimes with cyanidins but "they are free from pelargonidins." Apparently a 3rd group can be added: those varieties which contain derivatives of delphinidin, cyanidin, and pelargonidin as found in the variety Pride of Haarlem. Robinson and Robinson (6) also stated that "All [the anthocyanins of the tulip] are diglycosides." On the basis of quantity of pigment in the 2 varieties tested in the present work, the major components were diglycosides, the rhamnoglucosides of cyanidin and pelargonidin. But by the use of chromatographic methods the monoglucosides of cyanidin and pelargonidin were also identified as minor components.

Summary

The anthocyanins in petals of the tulip varieties Smiling Queen and Pride of Haarlem were isolated and identified by chromatographic and spectrophotometric methods. The petal tissue of the variety Smiling Queen contained cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin) and pelargonidin-3-rhamnoglucoside. The petals of the variety Pride of Haarlem contained the 4 anthocyanins found in the petals of the variety Smiling Queen and delphinidin-3-rhamnoglucoside.

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