Phytochemistry, 1965, Vol. 4, pp. 285 to 296. Pergamon Press Ltd. Printed in England

FLAVONOID COMPLEXES IN *PISUM SATIVUM* L.—I

NATURE AND DISTRIBUTION OF THE MAJOR COMPONENTS*

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(*Received* 21 *June* 1964)

Abstract—The four major flavonoids of the pea plant have been characterized as kaempferol-3-triglucoside (KG), its *p*-coumaric acid ester (KGC), quercetin-3-triglucoside (QG) and its *p*-coumaric acid ester (QGC). The spectral and chromatographic properties of these compounds and of their hydrolysis products are reported. Etiolated plants contain mainly KGC and KG with only traces of QGC and QG; in green plants, QGC and QG equal or surpass KGC and KG in concentration. KGC is present only in leaf tissue; KG in leaf and stem; QGC in leaf, petiole, tendril and stem; QG in leaf, petiole and tendril. No flavonoids could be detected in dark-grown roots.

INTRODUCTION

IN RECENT years, several flavonol glucosides and their acylated derivatives have been isolated from etiolated and green pea seedlings[1] and red-light pretreated seedlings[2] and have been implicated as major natural compounds controlling indoleacetic acid (IAA) oxidase activity in an *in vitro* assay. This is interesting in view of the fact that while the occurrence of acylated glycosides of anthocyanins in higher plants has been well established,[3–7] comparatively little has been reported about similar derivatives of flavonols.[8,9]

We have reported earlier that, in peas, etiolated tissues yield mainly kaempferol derivatives while plants grown in high-intensity light contain considerable quantities of quercetin complexes as well. We have now studied in further detail the structures of these acylated flavonols, and have discovered some new facts regarding their localization and biosynthesis. It appears that each organ of the pea seedling has its own specific flavonoid pattern, and that the patterns of flavonoid distribution may be modified by light.[10]

* The work described in this paper forms part of a dissertation presented by M. Furuya to the Faculty of the Graduate School of Yale University in 1962, in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

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[1] M. FURUYA, A. W. GALSTON and B. B. STOWE, *Nature* **193**, 456 (1962).
[2] F. E. MUMFORD, D. H. SMITH, and J. R. CASTLE, *Plant Physiol.* **36**, 752 (1961).
[3] P. KARRER and R. WIDMER, *Helv. Chim. Acta* **11**, 837 (1928).
[4] J. B. HARBORNE, *Biochem. J.* **63**, 30P (1956).
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[6] J. B. HARBORNE, *Biochem. J.* **70**, 22 (1958).
[7] N. ISHIKAWA and K. HAYASHI, *Botan. Mag. Tokyo* **75**, 28 (1962); K. TAKEDA and K. HAYASHI, *Botan. Mag. Tokyo* **76**, 206 (1963).
[8] L. HÖRHAMMER, L. STICH and H. WAGNER, *Naturwiss.* **46**, 358 (1959).
[9] F. E. MUMFORD, D. H. SMITH and P. G. HEYTLER, *Biochem. J.* **91**, 517 (1964).
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RESULTS

Chromatographic and Spectral Properties of Pea Flavonoid Complexes

Four distinct flavonoids can be detected on chromatograms of extracts from pea leaves, irrespective of the light conditions under which they are grown. Typical chromatographic and spectral data are presented in Table 1. All four are extremely soluble in water, and also in methanol, ethanol, and 1-butanol, but not in ethyl ether, petroleum ether, ethyl acetate,

TABLE 1. R_f VALUES AND U.V. ABSORPTION MAXIMA OF PEA FLAVONOID COMPLEXES

Compound	Source	Average R_f			λ_{max}	
		BAW*	5% AcA†	IPA‡	EtOH	+ $AlCl_3$
KGC	Green leaves & etiolated plumules	0·42	0·46	0·04	267·5, 316	276, 320, 395
KG	Green leaves & etiolated plumules	0·34	0·58	0·02	266, 350	272, 342, 385
QGC	Green leaves	0·39	0·42	0·03	257, 267, 319	274, 315, 390
QG	Green leaves	0·32	0·55	0·02	257, 355	274, 394

* BAW; *n*-butanol:acetic acid:water, 4·1:2·2 v/v.
† 5% AcA; 5% aqueous acetic acid solution.
‡ IPA; iso-propanol:ammonia (0·88):water 20:1·2 v/v.

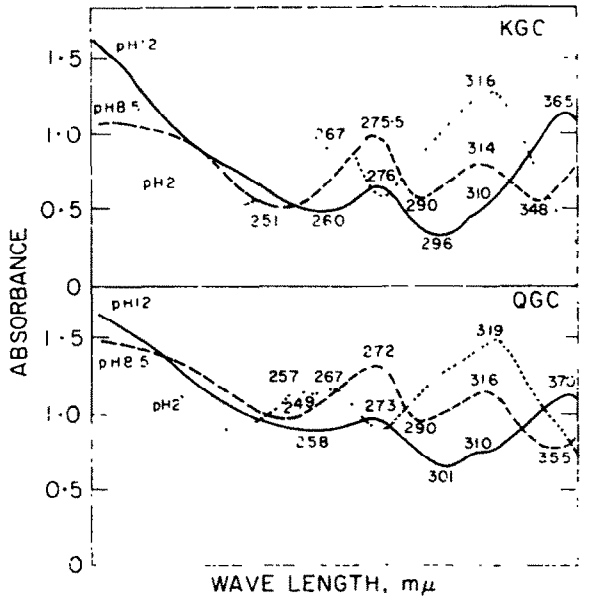


FIG. 1. ULTRAVIOLET ABSORPTION SPECTRA OF KGC AND QGC AT DIFFERENT pH VALUES IN AQUEOUS SOLUTION.

The maximum for KGC was found to be 267 mμ at a range of pH from 2–7; this peak shifts to 273 mμ above pH 7 and to 276 mμ at pH 12. Another peak of this compound located at 316 mμ in neutral or an acidic solution moves to 365 mμ at or above pH 9. These shifts of absorption maxima are reversible with KGC but not with QGC in both directions by titration with dilute acid or alkali.

chloroform and benzene and respond to various colour reactions for phenolics and related compounds.[11] All four flavonoids showed distinct (u.v.) absorption maxima in ethanolic solution which were shifted by aluminium chloride (Table 1). The u.v. spectra of KGC and QGC were also found to vary markedly with change in the pH of the solution (Fig. 1).

Analysis of Hydrolysis Products

The precipitate and the supernatant fraction of the acid hydrolysates of KGC and QGC were found to have characteristic u.v. absorption maxima in ethanol (Fig. 2). The aqueous

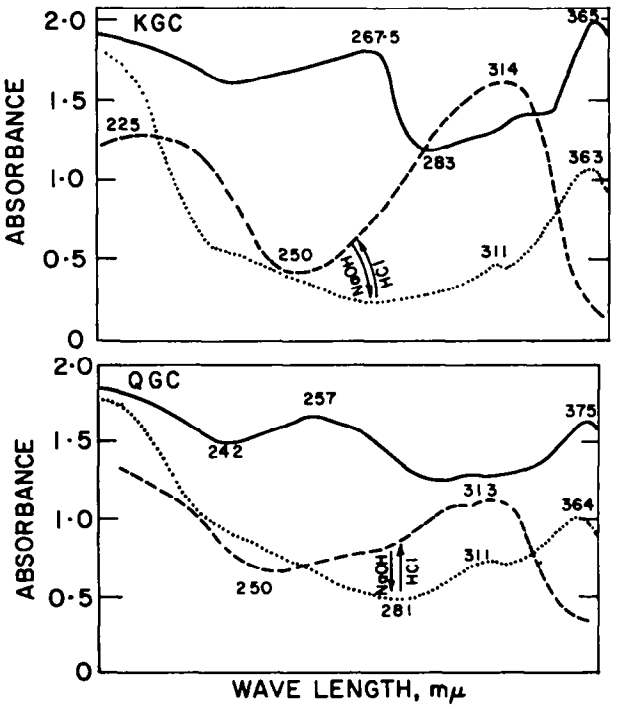


FIG. 2. THE U.V. ABSORPTION SPECTRA OF ACID HYDROLYSATES OF KGC AND QGC.

The precipitate in ethanol (——), the supernatant fraction in HCl-ethanol (– – – –), in NaOH-ethanol (. . . .).

supernatant fractions of KG and QG after acid hydrolysis did not show any u.v. peaks, but the solutions of the precipitates had sharp u.v. peaks similar to those of hydrolysed KGC and QGC respectively. After alkaline hydrolysis of KGC and QGC, the reaction mixtures, showing u.v. peaks at 280 mμ and 335 mμ in basic solution and at 267 mμ and 312 mμ in acidic solution, were extracted with ethyl acetate and with *n*-butanol. The u.v. absorption maxima of these extracts are shown in Fig. 3. The acid and alkaline hydrolysates of KGC, KG, QGC, and QG were chromatographed on paper (Table 2); KGC and QGC yield at least three spots after acid hydrolysis and two after alkaline hydrolysis. However, KG and QG yield two spots after acid hydrolysis and only one after treatment with alkali.

[11] I. SMITH, *Chromatographic and electrophoretic techniques*, Vols. I and II, Heinemann, London (1960).

The experimental evidence in terms of R_f values and melting points (Table 2), u.v. spectra (Figs. 2 and 3), properties of methylated and acetylated derivatives (see next section), and spectrophotofluorometry (Table 3) supports the view that the aglycone from KGC and KG is kaempferol, and that from QGC and QG is quercetin.

All flavonoids yielded a reducing sugar after acid hydrolysis as shown by Nelson's reagent.[12] To determine the nature of this sugar, the supernatant fractions of the acid hydrolyse were concentrated, and two-way chromatography was applied to the mixtures of the unknowns plus various authentic sugars. The results showed clearly that the sugar moiety of KGC, KG, QGC and QG was glucose.

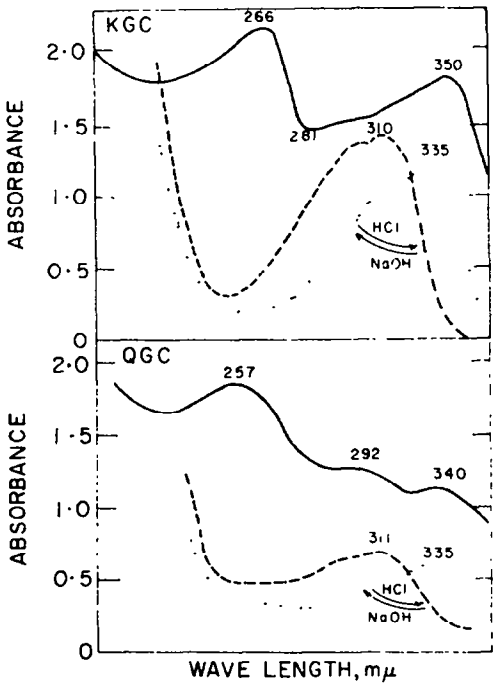


FIG. 3. THE U.V. ABSORPTION SPECTRA OF ALKALINE HYDROLYSATES OF KGC AND QGC AFTER EXTRACTION BY ETHYL ACETATE AND *n*-BUTANOL.

n-Butanol extract (——); Ethylacetate extract in basic solution (. . . .); and in acidic solution (- - -).

In addition to flavonol and sugar, the acid and alkaline hydrolysis of KGC and QGC yielded several other products. Two showed u.v. absorption maxima near 311 mμ (Table 2) and positive responses to sulphanilic acid reagent[11] and nitroaniline reagent.[11] The products of acid hydrolysis were water-soluble but not extractable by ethyl acetate from aqueous solution, and showed lower R_f values in the butanolic solvent and a large spectral shift as a result of pH changes (~ 50 mμ, see Fig. 2), whereas the products of alkaline hydrolysis were readily extracted by ethyl acetate, showed a higher R_f in the butanolic solvent, and a small spectral shift as a result of the pH change (~ 24 mμ; see Fig. 3). The release of these various products by acid and alkaline hydrolysis indicates that there are different types of linkage present in the substances involved.

[12] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).

TABLE 2. SPECTRAL AND CHROMATOGRAPHIC PROPERTIES OF ACID AND ALKALINE HYDROLYSATES OF PEA FLAVONOID COMPLEXES

Compound	Type of hydrolysis	R_f1*	u.v. spectra (in ethanol) max. (mμ)	Colour† reaction: $FeCl_3$ ferricyanide	$AlCl_3$ v.	$AlCl_3$ u.v.	Anthrone reagent
KGC	none	0·53	267·6, 316	GB	fY	Y	+
	acid	0·18¶	—	—	—	—	+
		0·40**	315	fB	—	—	+
		0·84‡	268, 365	B	fY	BG	−
	alkali	0·42	266, 350	fB	Y	YG	+
		0·88	284				
KG	none	0·44	266, 350	B	Y	Y	+
	acid	0·18¶	—	—	—	—	+
		0·84§	267·5, 365	B	Y	G	−
QGC	none	0·48	258, 266, 318	B	Y	OY	+
	acid	0·18¶	—	—	—	—	+
		0·33	314	fB	—	—	+
		0·70‖	257, 370	B	fY	BG	−
	alkali	0·39	263, 355	fB	fY	OY	+
		0·81	broad 267–280	B	—	—	+
QG	none	0·37	265, 335	B	Y	OY	+
	acid	0·18¶	—	—	—	—	+
		0·70‖	257, 373	B	fY	BG	−

* Descending paper chromatograms, using 4:1: 2·2 *n*-butanol:acetic acid:water.
† +, positive reaction obtained; −, negative; B, blue; f, faint; G, green; O, orange; Y, yellow.
‡ Melting point of aglycone from KGC, 275–276°.
§ That from KG, 275–277°.
‖ That from QGC, and QG, 314–315°.
¶ Positive reaction with Nelson's reagent[21].
** In spectro-photofluorometry, the maximum fluorescence wavelength was observed to be 460 mμ when activated by the maximally active excitation wavelength, 395 mμ, in sodium borate buffer (pH 10).

TABLE 3. THE MAXIMUM ACTIVATION AND FLUORESCENCE WAVELENGTHS OF PEA FLAVONOIDS, AGLYCONES OF KGC AND QGC, AND AUTHENTIC FLAVONOLS

Compound	Activation maximum wavelength (mμ)	Fluorescence maximum wavelength (mμ)	pH*
KGC	430	525	10
QGC	415	504	10
Aglycone from KGC	420	480	2
	470	555	10
	355	565	12
Aglycone from QGC	430	500	2
	290	555	12
Kaempferol	350	567	12
Morin	495	560	12
Quercetin	430	510	2
	290	560	12
Rutin	480	538	12

* Buffers used: pH 2, glycine-HCl, pH 10, borate; pH 12, glycine.

To gain additional information on this question, the products of acid hydrolysis of KGC and of QGC were further hydrolysed by alkali and were found to yield glucose and phenylpropanoids whose u.v. absorption and chromatographic characteristics were identical with the products of direct alkaline hydrolysis of KGC and QGC. These products were not altered by further acid hydrolysis. It was evident that the phenylpropanoid residues were bound to sugar by an ester link, but were not directly bound to the flavonol moiety. Jurd, Geissman, and Seikel[13] reported that methyl *p*-coumarate shows a u.v. absorption maximum at 312 mμ in aqueous acid but at 367 mμ in alkali, whereas *p*-coumaric acid shows a peak at 310 mμ in acid but at 335 mμ in alkali. The former corresponds to the product of acid hydrolysis of KGC, and the latter to that of alkaline hydrolysis.

On the basis of the u.v. spectra described above and comparative spectrophotofluorometry, the product of KGC alkaline hydrolysis was identified as *p*-coumaric acid, and that of acid hydrolysis as glucosyl-*p*-coumarate. The identity of the phenyl-propanoid from QGC remains to be established with certainty, but it sufficiently resembles that from KGC to suggest that the two are identical

TABLE 4. THE MOLAR RATIO OF KAEMPFEROL, QUERCETIN, GLUCOSE, AND *p*-COUMARIC ACID IN PEA FLAVONOL COMPLEXES

Compound	Amounts of compounds liberated on hydrolysis (μ moles)				Ratio of	
	Kaempferol (K)	Quercetin (Q)	Glucose (G)	*p*-Coumaric acid (C)	G/K or Q	C/K or Q
KG from etiolated buds	0·30		0·86	-	2·87	-
KGC from etiolated buds*	0·45		1·31	0·50	2·91	1·11
QG from green leaves		1·26	3·95	-	3·14	
QGC from green leaves	-	0·83	2·44	0·97	2·93	1·17

* 5 mg of KGC yielded 1·35 mg of kaempferol, 0·75 mg of *p*-coumaric acid, and 2·35 mg of glucose after acid hydrolysis; that is 89 per cent of the KGC used was recovered. The 11 per cent unrecovered was presumably due to the presence of water of crystallization in KGC.

The molar ratios of flavonol to sugar to *p*-coumaric acid were determined in the four pea flavonoid compounds and the results are summarized in Table 4.

Methylation and Acetylation Studies

Methylated KGC was obtained as light yellow needles (m.p. 214–219°, λ_{max} 310 and 265 mμ) and was soluble in ethanol but not in water. After acid hydrolysis, it was converted to a product melting at 151° and with λ_{max} at 254 and 355 mμ. This product appears to be 3-hydroxy-4′, 5,7-trimethoxyflavone. In addition, the hydroxyl group of the *p*-hydroxycinnamic acid moiety was found, by means of its u.v. spectrum (Fig. 4A) and its R_f values, to be methylated by the above treatment.

Similarly, acid hydrolysis of methylated QGC and QG gave a product, m.p. 191–193° and λ_{max} at 251·5 and 358 mμ. This corresponds to 3-hydroxy-3′,4′,5-7- tetramethoxyflavone. The spectra of these various products are presented in Fig. 4.

[13] L. JURD, T. GEISSMAN and M. K. SEIKEL, *Arch. Biochem. Biophys.* 67, 284 (1957).

It is well known that acetylation abolishes the bathochromic effect hydroxyl groups have on the u.v. absorption maximum of the flavone molecule.[14] This suggests that, if complete acetylation and acetylation of all but the 5-hydroxyl group were carried out with QGC,

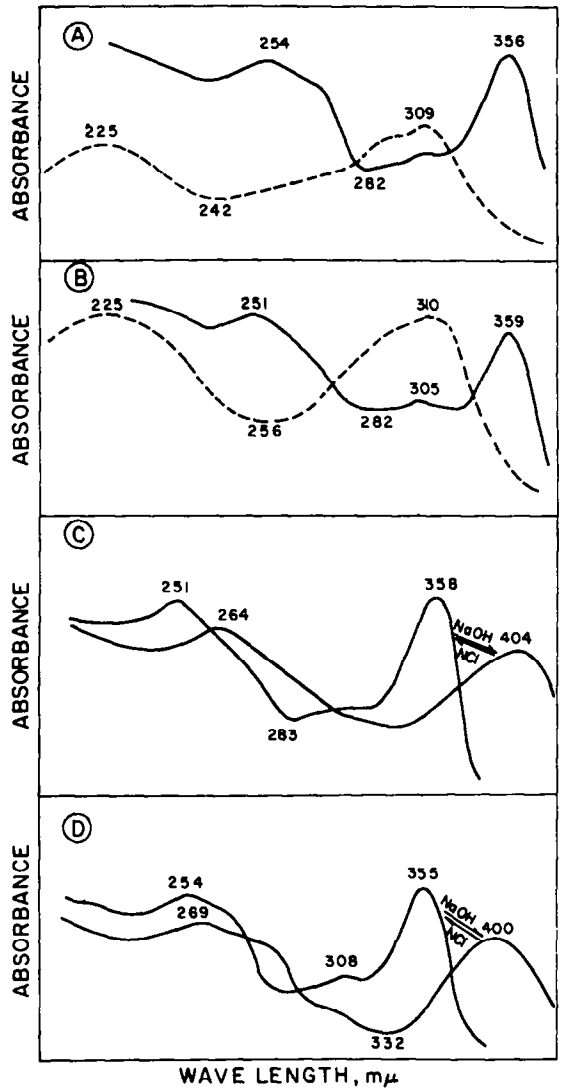


FIG. 4. THE U.V. ABSORPTION SPECTRA OF ACID HYDROLYSATES OF THE METHYL ETHERS OF KGC (A), QGC (B), QG (C), AND KG (D).

Methyl ether of flavonol (——), and methylated phenylpropanoid (– – – –) in ethanol.

and authentic quercetin and rutin, a comparison of the u.v. absorption spectra of these derivatives might tell whether the 5-position of the quercetin moiety of QGC is free or not. The results thus obtained are summarized in Table 5 and show that the 5-hydroxyl group of

[14] Y. SHIBATA and K. KIMOTSUKI, *Acta Phytochim.* **1**, 91 (1923).

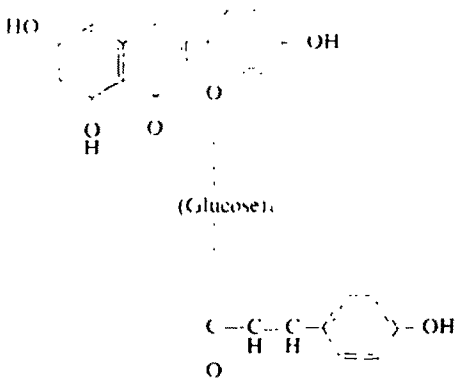
QGC is indeed free. This result agrees with the conclusions from the above methylation experiments.

TABLE 5. ULTRAVIOLET ABSORPTION CHARACTERISTICS OF ACETATES OF QGC, QUERCETIN AND RUTIN

Compound	mμ in ethanol	
	Maximum	Minimum
Quercetin	256·5, 372	238, 286
Rutin	258, 365	238·5, 285
QGC	257, 318	245, 279
Quercetin pentacetate	251, 298	238·5, 270
Rutin, fully acetylated	251, 300	240, 272
QGC, fully acetylated	257, 292	242, 270
Tetracetylquercetin	267, 335	242, 321
Rutin, partly acetylated	268, 302, 342	242, 290, 324
QGC, partly acetylated	268, 302, 345	243, 328

Structures Proposed for the Pea Flavonoid Complexes

Hydrolysis experiments show that the flavonol moiety in KGC and QGC is not bound to the phenylpropanoid moiety, but that flavonol and phenylpropanoid moieties are both bound to sugar. The bond between phenylpropanoid and sugar is hydrolysed by alkali, suggesting an ester linkage. Methylation experiments show that only the 3-position of kaempferol and quercetin is available for the sugar linkage, and quantitative hydrolysis shows that 1 mole of flavonol, 3 moles of sugar, and 1 mole of phenylpropanoid are present in the complex. These facts suggest that three molecules of glucose are all attached at the 3-position. The fact that emulsin does not hydrolyse the intact compounds further suggests that the phenylpropanoid moiety is bound to the terminal glucose. A possible structure for KGC is, therefore, kaempferol-3-(*p*-coumaryltriglucoside) (I). QGC has a structure very similar to that of KGC, but its phenylpropanoid moiety has not been definitely identified. KG and QG are kaempferol 3-triglucoside, and quercetin 3-triglucoside, respectively.



FORMULA I. PROPOSED STRUCTURE OF KGC.

Distribution of Pea Flavonoid Complexes

The distribution pattern of flavonoid complexes in pea plants was investigated by means of two-way paper chromatography. Fresh plant material (10–100 mg) was applied to paper as a methanolic extract and the results shown in Table 6 were obtained. In addition to the Alaska pea, the plumules of etiolated seedlings of the cultivars Hundred, Progress and American Wonder were found to contain KGC and KG.

TABLE 6. THE DISTRIBUTION OF FLAVONOIDS IN *Alaska* PEA TISSUES GROWN UNDER VARIOUS LIGHT CONDITIONS

Organ tested		Lamina			Petiole or tendril	Stem			Root
Light condition		D	R	L	L	D	R	L	D
Compound detected	KGC	+	+	+	−	−	−	−	−
	KG	+	+	+	−	+	+	+	−
	QGC	+	+	+	+	−	−	+	−
	QG	+	+	+	+	−	−	−	−

+, detected; −, not detected on paper chromatograms. D, materials grown in total darkness; R, materials grown in dark, but exposed to low intensity red light for a short period of time, 16 hours prior to harvest; L, materials grown under continuous high intensity white light (ca. 1200 ft-candles).

DISCUSSION

It appears from this work that in the pea plant, the flavonols kaempferol and quercetin occur mainly as the triglucoside or as the *p*-coumaroyl ester of the triglucoside. Acylated flavonols of a similar type have been described in recent years.[15,16] Tiliroside, for example, also yields flavonol, pehnylpropanoid and sugar after hydrolysis.[8] Its structure has recently been amended to kaempferol (3-*p*-coumaroylglucoside) and it now conforms with the pattern in *Pisum*.[17] Sophorose units have been obtained from *Pisum* acylated flavonoids,[16] and a kaempferol hexaglucoside has been reported in red light pretreated pea seedlings.[9] We have found no evidence for the existence of this latter compound.

Although no quantitative data are available, it is clear (Table 6) that kaempferol is the major flavonol in etiolated pea tissues, and that quercetin is more abundant in light-grown tissues. Thus, the relative amounts of the different pea flavonoids appear to be controlled by light. The exact nature of the action of light is unknown, though red light is suspected of controlling precursors of the A-ring of flavonoids[18] and of directing acyl units into flavonoids rather than into the formation of volatiles.[19] It should be noted that pea leaves always produce the same four flavonoids irrespective of light conditions. Similarly in *Spirodela*,[20] light is without qualitative effect on pattern of hydroxylation of flavonoids.

[15] L. BIRKOFER and C. KAISER, *Z. Naturforsch.* **17b**, 359 (1962).
[16] J. B. HARBORNE, *Experienta* **19**, 7 (1963).
[17] J. B. HARBORNE, *Phytochem.* **3**, 151 (1964).
[18] H. GRISEBACH, *Z. Naturforsch.* **12b**, 227 (1957).
[19] H. W. SIEGELMAN and S. B. HENDRICKS, *Plant Physiol.* **33**, 409 (1958).
[20] M. FURUYA and K. V. THIMANN, *Arch. Biochem. Biophys.* **108**, 109 (1964).

It is premature to speak of the physiological role of the flavonoids. They may act as inhibitors of the enzyme indoleacetic acid oxidase,[1] as dormancy inducers,[21,22] as uncouplers of oxidative phosphorylation,[23] as stimulators of plant growth,[24,25] and as inhibitors of flowering. Though knowledge of the physiology of the flavonoids is incomplete and uncertain, our understanding of the biosynthesis of these compounds is advancing rapidly.[18,26,29] Our own studies provide data which may contribute further to a clarification of both kinds of problems.

EXPERIMENTAL

Plant Materials. The seeds of *Pisum sativum* L., cv. Alaska, were used throughout.

Etiolated seedlings: Seven-day-old seedlings were grown as previously described.[30] Where red-light exposed peas were needed, 6-day-old etiolated seedlings were exposed to a red fluorescent tube wrapped with two layers of DuPont red cellophane for 15 min; returned to darkness for 16 hr, then harvested.

Light-grown seedlings: Three-week-old light grown seedlings, cultured as previously described[30] were harvested, and were immediately extracted; occasionally they were kept in a deep freeze for several days until extraction was carried out.

Isolation of Flavonoid Complexes

Tissues were extracted repeatedly with hot methanol until the extracts were colorless. The methanolic extract was flash-evaporated at 40° until the methanol had been removed, and the aqueous residue extracted by shaking successively with several portions of petroleum ether, ethyl acetate, and *n*-butanol. The *n*-butanol fraction was flash-evaporated, and further purified by column chromatography. Whatman cellulose powder (standard grade) was packed in a column, which was then washed thoroughly with the solvent subsequently employed in the elution, i.e. *n*-butanol:water, 86:14 (by vol.). To locate the flavonoids, the u.v. absorption spectra of portions of the eluted fractions were measured on a "Spectracord" recording spectrophotometer. Fractions were combined as appropriate and concentrated *in vacuo* at 40°. Each residue was repeatedly purified by column chromatography, various other solvent systems being used. The flavonoids were thus obtained in crystalline or powder form.

Paper chromatography was used for testing the purity of these isolated materials and for their subsequent identification. To produce a spot of a constant diameter, an alcoholic solution of an authentic or unknown sample was first pipetted onto a disc (5 mm in diameter)

[1] C. H. HENDERSHOTT and D. R. WALKER, *Proc. Am. Soc. Hort. Sci* **74**, 121 (1959).
[22] I. D. J. PHILLIPS, *Nature* **192**, 240 (1961).
[23] G. STENLID, *Physiol. Plant.* **14**, 659 (1961).
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[25] F. C. STEWARD and E. M. SHANTZ, in *The chemistry and mode of action of plant growth substances* (Edited by R. L. WAIN and F. WIGHTMAN) p. 165. Butterworths Scientific Publications, London (1956).
[26] T. A. GEISSMAN and E. HINRINER, *Bot. Rev.* **18**, 77 (1952).
[27] A. C. NEISH, *Ann. Rev. Plant Physiol.* **11**, 55 (1960).
[28] J. B. PRIDHAM, *Phenolics in plants in health and disease*, Pergamon Press, Oxford (1960).
[29] W. D. OLLIS, *Recent developments in the chemistry of natural phenolic compounds*, Pergamon Press, Oxford (1961).
[30] A. W. GALSTON and R. KAUR, in *Light and Life* (Edited by W. D. MCELROY and B. GLASS) p. 687. Johns Hopkins Press, Baltimore (1961).

of Whatman No. 1 paper, which, after drying, was applied to the origin of chromatogram by being inserted through two parallel slits 1·5 mm apart.

Acid and Alkaline Hydrolysis

For acid hydrolysis, the aqueous solution of a compound in 2% HCl was heated at 100° for 60 min. After the reaction mixture had been kept in a refrigerator overnight, the precipitate of the aglycone was collected by filtration. For quantitative estimations, 2 ml of a methanol solution containing the sample and 2 ml of 2 N HCl or H_2SO_4 were heated at 80° for 60 min. After cooling, the reaction mixture was made up to 10 ml with methanol, and the concentration of aglycone was determined spectrophotometrically.[15] After removal of methanol from the reaction mixture by a flash-evaporator at 40°, the residue was made up to 5 ml with water, and the sugar concentration was measured by the anthrone method.

Sodium hydroxide (2 N) was used for the alkaline hydrolysis. The mixture was allowed to stand for 2 hr at room temperature, after which it was acidified to pH 2 with HCl.

Quantitative Hydrolysis

The molar ratio of kaempferol, *p*-coumaric acid and glucose in the KGC molecule was determined as follows.[31] 5 mg KGC were dissolved in 4 ml of methanol, to which 4 ml of 2 N H_2SO_4 were added. This mixture was refluxed for 90 min at 80°, and after cooling, made up to 10 ml with methanol. The u.v. spectrum of a diluted solution of this mixture (50 μg/ml equivalent) was measured. It showed three peaks, at 269 mμ (A = 1·04), 317 mμ (A = 1·38), and 365 mμ (A = 0·95). When the methanol was removed *in vacuo* and the aqueous residue was kept in a refrigerator, an orange-yellow precipitate appeared. The precipitate was filtered off and the supernatant made up to 10 ml by addition of water. The precipitate obtained was also dissolved in 10 ml of methanol. The u.v. spectrum of the supernatant (50 μg/ml equivalent) showed peaks at 229 mμ (A = 0·52) and 314 mμ (A = 0·96) due to the glycosyl-*p*-coumarate, and the precipitate showed peaks at 267·5 mμ (A = 0·69) and 365 mμ (A = 0·86). As shown in Fig. 2, the glycosyl-*p*-coumarate had low absorbance at 365 mμ (A = 0·11). Therefore, the optical density 0·95 at 365 mμ of the methanolic reaction mixture was calculated to be 0·84 (0·95–0·11) for the kaempferol moiety. This amount agrees well with the value measured directly on the precipitate of kaempferol (O.D. = 0·86). The amount of kaempferol in the sample was determined from a standard curve; (an A of 0·84 corresponds to 13·5 μg/ml of kaempferol).

As *p*-coumaric acid is known to have a log. molar absorptivity of 4·38 at 307 mμ in ethanol, the amount of this compound could also be determined by means of spectrophotometry. The absorptivity of the supernatant fraction containing the glycosyl *p*-coumarate (50 μg/ml equivalent) was 0·96. After alkaline hydrolysis, the *p*-coumaric acid moiety of the given material was determined to be 0·75 mg per 5 mg of KGC.

The amount of sugar in the sample was determined in variously diluted aqueous solutions of the supernatant fraction by the anthrone method. Two milliliters of freshly prepared reagent, consisting of 0·2% anthrone solution in 96% H_2SO_4, were added to 1 ml of the sample solution, and after 15 min the absorption of the reaction mixture was measured in a Klett photoelectric colorimeter equipped with a green filter. The supernatant fraction of the above was found to contain 2·35 mg per 5 mg of the KGC preparation.

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Methylation and Acetylation

For complete methylation[32,33] a 250 mg sample of each flavonoid was methylated in 25 ml of acetone with 7·5 g of K_2CO_3 and 5 ml of dimethyl sulfate by heating for 3 hr at 100°. The reaction mixture was cooled and poured into 50 ml of water and the product dissolved in 25 ml of ethanol and 0·5 g conc. H_2SO_4 and hydrolysed by heating for 3 hr at 100°. After removal of the ethanol, a precipitate was obtained and this was repeatedly recrystallized from ethanol.

Complete acetylation was performed as follows:[34] 100 mg of flavonol was refluxed with 20 ml of acetic anhydride and 1 ml of pyridine for 2 hr. The product was washed with water and recrystallized from ethanol. 3,3′,4′,7-Tetracetyl quercetin and related flavonoids were synthesized as follows; 300 mg of air-dried flavonol was put into a 5-ml test tube, mixed with 1 ml of acetic anhydride and 2–3 drops of pyridine, and then stirred with a glass rod at room temperature for 5–10 min. The reaction mixture was washed with water, and recrystallized from ethanol.

Acknowledgements—M.F. is indebted to Yale University for University fellowships awarded from 1958–1962, and to the Yale University Committee on Atypical Growth for the award of summer fellowships which were provided by American Cancer Society Institutional Grant for the summers of 1959–61. This work was partially supported by the National Science Foundation under a grant to one of us (A.W.G.).

We wish to thank Dr. Bruce B. Stowe for his many valuable suggestions and criticisms during this work, and Dr. W. Bottomley for his helpful discussion during the preparation of the manuscript.

Dr. Bruce B. Stowe and Mr J. F. Schilke advised and aided in manipulation of the spectrophotofluorometer.

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