complex schemes with the aid of computers (Chance, 1960; Chance, Garfinkel, Higgins & Hess, 1960). The theoretical study of simple model multi-enzyme systems will surely play an important role in guiding biochemical thought on the behaviour of living systems.

SUMMARY

1. The variation of the rate of flux with the concentrations of the enzymes in multi-enzyme systems is considered.

2. For linear sequences (when the concentrations of the linking intermediates are relatively low) the reciprocal rate is given by a formula in which each term involves the reciprocal of the concentration of one of the enzymes.

3. Interaction occurs in branched systems, and the relative rate of competing reactions is not

proportional to the ratio of the amounts of the enzymes, unless the reactions are irreversible.

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The Effect of Red Light on the Flavonoid Composition of Etiolated Pea Plumules

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(Received 21 May 1963)

Hillman & Galston (1957) reported that the indolylacetate-oxidase activity of the buds of etiolated pea plants is inhibited by red light (640 m μ) and that the effect is reversed by near-infrared light (730 m μ). In following up this report, Mumford, Smith & Castle (1961) found that pea buds from plants treated with red light possess increased amounts of an indolylacetate-oxidase-inhibiting flavonoid, R_F 0.14 in 20% (w/v) potassium chloride, which yielded kaempferol, glucose and 4-hydroxycinnamic acid on acid hydrolysis. This flavonoid was also present in smaller amounts in extracts of pea buds from plants completely grown in the dark, but the major flavonoid from tissue from plants grown in the dark had R_F 0.28 in 20% potassium chloride. Investigations on the structure of these flavonoids, now reported, show that the compound isolated from plants treated with red light is 3-(4-hydroxycinnamoyl-triglucosyl)kaempferol and that from plants grown in the dark is probably 3-(hexaglucoyl)-kaempferol.

EXPERIMENTAL

Flavonoid from pea plumules from plants treated with red light

Acid hydrolysis. The flavonoid, R_F 0.14 in 20% KCl, from pea buds from plants treated with red light was obtained as the dihydrate (mol.wt. 954), as described by Mumford *et al.* (1961). The flavonoid (0.5 mg.; 0.523 μ mole) was dissolved in methanol (2 ml.), and the solution heated on a steam bath under reflux with 2N-H₂SO₄ (2 ml.) for 2 hr. The hydrolysate was diluted to 10 ml. with methanol, and the resulting solution assayed quantitatively for kaempferol and glucose by the method of Hörhammer (1956). The results are shown in Table 1.

Alkaline hydrolysis. Flavonoid (10.4 mg.) from pea buds from plants treated with red light was heated with 20% (w/v) NaOH (2 ml.) on a steam bath for 1 hr. The solution was cooled and adjusted to pH 6.6 with dilute HCl. The solution was extracted three times with portions (10 ml.) of ethyl acetate. The ethyl acetate extracts were dried over anhydrous Na₂SO₄, and the ethyl acetate was removed *in vacuo*. The ultraviolet-absorption spectrum of the residue dissolved in methanol (4 ml.) was almost identical with

that of a solution containing 90 $\mu\text{g.}$ of an authentic sample of 4-hydroxycinnamic acid/ml. The yield of 4-hydroxycinnamic acid, 0.36 mg., was 20 % of the theoretical if the kaempferol:glucose:4-hydroxycinnamic acid molar proportions in the flavonoid are assumed to be 1:3:1 (cf. Table 1).

The aqueous phase from the ethyl acetate extraction was further acidified to pH 2.2 and extracted again with ethyl acetate. Paper-chromatographic examination and spectral studies showed that this extract also contained a substantial amount of 4-hydroxycinnamic acid.

Paper-chromatographic examination of the pH 2.2 aqueous phase showed that it contained a kaempferol glucoside with R_F 0.28 in 20 % KCl and R_F 0.54 in water. This was not the original glycoside, which has R_F 0.14 in 20 % KCl (Mumford *et al.* 1961), nor was it identical with the hexaglucoide isolated from pea buds from plants grown in the dark, since this has R_F 0.28 in 20 % KCl but R_F 0.43 in water.

The glycoside was purified by chromatography on a cellulose column with butan-1-ol saturated with water as developing solvent, and the kaempferol:glucose molar ratio in the purified material was determined as above. The results are shown in Table 1.

Methylation. Flavonoid (32 mg.) from pea buds from plants treated with red light, acetonitrile (25 ml.), dimethyl sulphate (0.2 ml.) and NaHCO_3 (3.5 g.) were heated together with stirring for 3 days. The slurry was cooled and filtered, and the filtrate was evaporated to dryness. The residue (37.3 mg.) was chromatographed on an LKB size 4 pressurized paper-chromatographic column (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) with butan-1-ol-acetic acid-water (4:1:5, by vol.) as solvent. The flow rate was 0.75 ml./min.; 100 fractions were collected at 20 min. intervals. Fractions 60–74 contained the flavonoid as judged by their yellow colour. The ultraviolet-absorption spectrum of fraction 63 (diluted 1:4, v/v, with butan-1-ol) showed λ_{max} at 316 $m\mu$ (E 0.53) and at 269 $m\mu$ (E 0.32). Fractions 62–70 had similar spectra. These fractions were combined and evaporated to dryness.

The methylated aglycone was obtained by hydrolysing the residue with 1 % (v/v) H_2SO_4 (10 ml.) followed by extraction of the hydrolysate with ethyl acetate. The washed and dried ethyl acetate extracts yielded crude

methylated aglycone (16.7 mg.) on evaporation. The aglycone was purified by paper chromatography in an ethyl acetate solvent system in which it had R_F 0.9. The purified methylated kaempferol derivative showed λ_{max} at 361 and 257 $m\mu$ (E_{257}/E_{361} ratio 1.63).

Flavonoids from pea buds from plants completely grown in the dark

Isolation. Terminal buds (89 g.) of 8-day-old etiolated pea plants were extracted with cold ethanol, as described by Mumford *et al.* (1961), to yield a residue (3.3 g.) after evaporation of the ethanol. This residue was dissolved in 10 ml. of propan-1-ol-acetic acid-water (20:1:10, by vol.) and applied to an LKB size 4 pressurized paper-chromatographic column. The column was eluted with the same solvent at a flow rate of 1 ml./min. Fractions were collected at 15 min. intervals. Elution was discontinued after 150 fractions had been obtained and the extinctions of the fractions at 317 $m\mu$ were determined. The results obtained suggested that the extract contained two different flavonoid materials, one of which was present in fractions 61–83, the other in fractions 84–104. Fractions 61–83 were evaporated to dryness at 50° to give a tan tacky residue (760 mg.); fractions 84–104 also yielded a residue (1.15 g.). These residues were fractionated further on the pressurized paper column, first with propan-2-ol-water (4:1, v/v) and then water-saturated butan-1-ol (Mumford *et al.* 1961). The residue from fractions 61–83 gave a purified flavonoid (25.8 mg.), R_F 0.14 in 20 % KCl. This flavonoid had the same properties as that isolated (see above) from buds from etiolated pea plants treated with red light.

The residue from fractions 84–104 gave a second flavonoid (70.4 mg.). This material was judged to be fairly pure since it yielded a single spot on paper chromatography in several solvent systems. The material had R_F 0.28 in 20 % KCl and R_F 0.43 in water; R_F 0.28 in 20 % KCl was reported by Mumford *et al.* (1961) for the major flavonoid in extracts of peas grown in the dark. The ultraviolet-absorption spectrum of the compound is shown in Fig. 1.

Acid hydrolysis. A portion (2 ml.) of a methanolic solution containing 1.1 mg. (about 0.8 μmole) of the flavonoid (R_F 0.28 in 20 % KCl) from pea buds from plants grown in

Table 1. *Kaempferol and glucose liberated on hydrolysis of flavonoid*

Analyses were made by the method of Hörhammer (1956). Other details are given in the text.

Material hydrolysed	Amount taken		Kaempferol liberated		Glucose liberated		Aglycone/ sugar ratio	4-Hydroxycinnamic acid liberated	
	mg.	μmoles	mg.	μmoles	mg.	μmoles		mg.	μmole
Flavonoid* from pea buds from plants treated with red light	0.5	~0.523	0.147	0.514	0.267	1.48	1:2.89	0.086†	0.525
Flavonoid glycoside liberated on alkaline hydrolysis of flavonoid from pea buds from plants treated with red light	1.2	1.5	0.41	1.44	0.864	4.8	1:3.33	0	—
Flavonoid† from pea buds from plants grown in the dark	1.1	0.8	0.208	0.73	0.810	4.5	1:6.16	0	—

* Flavonoid isolated as dihydrate [mol.wt. 954 (Mumford *et al.* 1961); R_F 0.14 in 20 % KCl].

† R_F 0.28 in 20 % KCl, R_F 0.43 in water.

† Quantity obtained by difference; quantitative yields of kaempferol (0.523 μmole) and glucose (1.57 μmoles) and uptake of 2.09 μmoles of water in the hydrolysis assumed.

the dark was added to 2N-H₂SO₄ (2 ml.), and the mixture was heated for 2 hr. on a steam bath. The solution was cooled and diluted to 10 ml. with methanol. Paper-chromatographic analysis showed that it contained a flavonol that had the same *R_F* values as kaempferol in several solvent systems. However, 4-hydroxycinnamic acid was not present in the hydrolysate. The ultraviolet-absorption spectrum confirmed the presence of kaempferol in the solution. Quantitative assay for kaempferol and glucose by the method of Hörhammer (1956) gave the results shown in Table 1.

Assay for hydroxyl substitution. The method of Jurd (1956, 1957) was used to determine whether the 3-, 7- and 4'-hydroxyl groups of the flavonoid nucleus were substituted. A model 4000-K Spectracord spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn., U.S.A.) was used in these studies.

Paper-chromatographic detection of a 4-hydroxycinnamic acid glucose ester in extracts of pea buds from etiolated plants. Pea buds (10 g.) from etiolated plants were extracted with 50% (v/v) ethanol by the general procedure described by Mumford *et al.* (1961). The extract was filtered and the filtrate (10 μ l.) spotted on Whatman no. 1 paper. After ascending development of the chromatograms in various solvent systems (Table 2) the chromatograms were dried and examined under ultraviolet light (model SL 3660 long-wave ultraviolet lamp; Ultra-Violet Products Inc., South Pasadena, Calif., U.S.A.) alone and also under ultraviolet light in the presence of ammonia vapour. 4-Hydroxy-

cinnamic acid and its esters are characteristically colourless under ultraviolet light alone, but yield a bluish-fluorescent spot under ultraviolet light and ammonia (Birkhofer & Kaiser, 1960, 1961; Harborne & Corner, 1961). The *R_F* values of the bluish spots that appeared on the chromatograms under ultraviolet light in the presence of ammonia vapour are shown in Table 2.

Assay of indolylacetate oxidase. Indolylacetate-oxidase assays were carried out with bean-root indolylacetate oxidase by the procedure described by Mumford *et al.* (1961). The assay cups, which were incubated in a Dubnoff metabolic shaker at 26°, contained indolylacetic acid (350 μ g., 2 μ moles), MnCl₂ (126 μ g., 1 μ mole), bean-root indolylacetate-oxidase preparation (1 ml.), inhibitor or cofactor solution or both (0–5 ml.), and 0.01M-potassium phosphate buffer, pH 6.1, to give a total volume of 10 ml. The results are shown in Table 3.

RESULTS

Previous work established that the flavonoid, *R_F* 0.14 in 20% potassium chloride, from pea buds from plants treated with red light yielded kaempferol, glucose and 4-hydroxycinnamic acid on acid hydrolysis (Mumford *et al.* 1961). The present investigation shows that these substances are present in the flavonoid in 1:3:1 molar proportions respectively. Further, since alkaline hydrolysis cleaved the flavonoid into a kaempferol triglucoside and 4-hydroxycinnamic acid, the glucose residues must all be attached to the kaempferol nucleus; any other sequence would yield a glucosyl-4-hydroxycinnamic acid fragment under alkaline conditions.

The spectral shifts that occurred in the unhydrolysed flavonoid from pea buds from plants treated with red light, the aglycone liberated on acid hydrolysis of this flavonoid, and the aglycone liberated on acid hydrolysis of this methylated flavonoid are shown in Table 4. These results indicate (cf. Jurd, 1957) that the original flavonoid has a substituent in either the 3- or 4'-position, since it was not decomposed in the presence of

Fig. 1. Ultraviolet-absorption spectrum of major flavonoid (62 μ g./ml. in methanol) in extracts of peas grown in the dark.

Table 2. Chromatography of extracts of pea buds from etiolated plants

The *R_F* values are for spots colourless to ultraviolet light alone, but blue in the presence of ultraviolet light and ammonia.

Solvent system	<i>R_F</i>	
	Found	Value for 4-hydroxycinnamic acid glucose derivative (Harborne & Corner, 1961)
Butan-1-ol-2N-ammonia (1:1, v/v)	0.0	0.0
Butan-1-ol-acetic acid-water (4:1:5, by vol.), upper phase	0.65	0.63
Water	0.89	0.90
Butan-1-ol-ethanol-water (20:5:11, by vol.)	0.25	0.27
20% (w/v) Potassium chloride	0.70	Not reported

2 mM-sodium ethoxide. Also, the bathochromic shift of about 9 $m\mu$ in the 268 $m\mu$ maximum caused by sodium acetate suggests a free 7-hydroxyl group (Jurd, 1956). The aglycone obtained on acid hydrolysis of the methylated flavonoid did not undergo decomposition in the presence of sodium ethoxide and, accordingly, either the 3- or the 4'-hydroxyl group was methylated during the methylation reaction. As the flavonoid obtained from pea buds from plants treated with red light is substituted in the 3-position before methylation (Mumford *et al.* 1961) it follows that the 4'-hydroxyl group must have been free and available for methylation. These spectral studies show, then, that in the flavonoid from pea buds from plants treated with red light the 7- and 4'-hydroxyl groups are free, and since it forms yellow lakes with lead and aluminium salts the 5-hydroxyl group must also be free. Therefore, by elimination, the only hydroxyl

group substituted on the kaempferol nucleus of this flavonoid is the one at the 3-position. Accordingly the flavonoid isolated from pea buds from plants treated with red light must be 3-(4-hydroxycinnamoyltrigluco-syl)kaempferol (I). Furuya, Galston & Stowe (1962) have also obtained two different flavonoids on fractionation of extracts of terminal buds of pea plants completely grown in the dark. The first of these, R_F 0.14 in 20 % potassium chloride, was identical with the 3-(4-hydroxycinnamoyltrigluco-syl)kaempferol isolated by us in previous work from tissues from plants treated with red light. The buds from plants completely grown in the dark yielded 0.29 mg. of this flavonoid/g. fresh wt. of tissue, and buds from plants treated with red light gave 0.82 mg. of flavonoid/g. fresh wt. of tissue (Mumford *et al.* 1961). Thus irradiation with red light appears to cause a 2-3-fold increase in the concentration of this flavonoid in pea plumules from etiolated plants.

The other flavonoid, R_F 0.28 in 20 % potassium chloride, isolated from pea plants grown in the dark was present in higher concentration than the first, 0.8 mg. being obtained/g. fresh wt. of buds extracted. This flavonoid yielded kaempferol and glucose in a 1:6 molar ratio on acid hydrolysis (Table 1). No 4-hydroxycinnamic acid was detected on paper-chromatographic examination of the hydrolysate. The flavonoid formed lakes with lead and aluminium salts, suggesting that the 5-hydroxyl group is free. The 7-hydroxyl group must also be unsubstituted, since a bathochromic shift of 11 $m\mu$ in the 268 $m\mu$ maximum was produced by sodium acetate (Table 4). Thus the sugar residues must be attached to one or both of the 3- and 4'-hydroxyl groups of the kaempferol ring. This compound is thought to be 3-hexagluco-sylkaem-

Table 3. *Effect of pea-bud flavonoids on activity of indolylacetate oxidase*

Experimental details are given in the text.		
Additions to assay system		
Flavonoid	2,4-Dichlorophenol (μ M)	Indolylacetic acid destroyed in 60 min. (%)
None	None	4
None	50	68
0.42 mM-3-(4-Hydroxycinnamoyltrigluco-syl)kaempferol	50	2
0.21 mM-3-(4-Hydroxycinnamoyltrigluco-syl)kaempferol	50	62
0.16 mM-Kaempferol hexagluco-side	50	0
0.016 mM-Kaempferol hexagluco-side	50	30

Table 4. *Effect of sodium ethoxide and sodium acetate on ultraviolet-absorption maxima of pea-bud flavonoids*

As in the procedures of Jurd (1956, 1957), spectra were examined in ethanol saturated with sodium acetate, or in ethanol containing sodium ethoxide (2 mM).

Flavonoid	λ_{max} ($m\mu$) in test solution		
	Ethanol	Ethanol-sodium acetate	Ethanol-sodium ethoxide
Flavonoid* from pea buds from plants treated with red light	317	315	Spectrum indicated no flavonoid decomposition
Aglycone liberated on acid hydrolysis of flavonoid from pea buds from plants treated with red light	367	370	Spectrum indicated flavonol decomposition
	267	277	
Aglycone liberated on acid hydrolysis of methylated flavonoid from pea buds from plants treated with red light	361	361	Spectrum indicated no flavonol decomposition
	257	257	
Flavonoid† from pea buds from plants grown in the dark	345	345	Spectrum indicated no flavonoid decomposition
	266	277	

* R_F 0.14 in 20 % KCl.

† R_F 0.28 in 20 % KCl.

(I)

pferol. However, flavonoids containing more than three sugar residues linked together have not been previously reported (Karrer, 1958) and the exact structure of the glucoside is uncertain.

The effects of 3-(4-hydroxycinnamoyltrigluco-3-O-4-hydroxycinnamoyl)kaempferol and the kaempferol hexagluco-3-O-4-hydroxycinnamoyl on the activity of indolylacetate oxidase are shown in Table 3. The kaempferol hexagluco-3-O-4-hydroxycinnamoyl completely inhibited indolylacetate oxidase at 0.16 mM and caused over 50% inhibition at 0.016 mM. At 0.21 mM 3-(4-hydroxycinnamoyltrigluco-3-O-4-hydroxycinnamoyl)kaempferol had little effect on indolylacetate oxidase, but it did cause complete inhibition at a concentration of 0.42 mM. Of the two flavonoids, then, the hexagluco-3-O-4-hydroxycinnamoyl isolated from pea buds from plants grown in the dark is the considerably better inhibitor of indolylacetate oxidase.

DISCUSSION

In their study on the effects of red light on indolylacetate-oxidase activity in pea buds from etiolated plants Hillman & Galston (1957) reported that the addition of increasing amounts of extract from pea buds from plants grown in the dark to a standard indolylacetate-oxidase assay system increased indolylacetate-oxidase activity, whereas the addition of increasing amounts of extract of buds from plants treated with red light led to inhibition. These results are most readily explained on the basis that the indolylacetate-oxidase cofactor-inhibitor balance in buds from plants grown in the dark is in favour of cofactor, but in tissue from plants treated with red light is in favour of inhibitor. The nature of the indolylacetate-oxidase cofactor in peas is not definitely known. However, paper-chromatographic examination of extracts of pea buds from plants grown in the dark has revealed spots that have a bluish fluorescence in the presence of ultraviolet light and ammonia vapour, but that are colourless under ultraviolet light alone. Such a reaction is characteristic of 4-hydroxycinnamic acid and its sugar esters (Birkofer & Kaiser, 1960, 1961; Harborne & Corner, 1961); these compounds are known to be excellent indolylacetate-oxidase cofactors (Gortner, Kent & Sutherland, 1958). It

seems possible, therefore, that a relatively high concentration of this glucose derivative of 4-hydroxycinnamic acid in extracts of pea buds from plants grown in the dark gives rise to the net cofactor activity observed. Further, since the predominant flavonoid in pea buds from plants grown in the dark is thought to be 3-hexagluco-3-O-4-hydroxycinnamoylkaempferol, whereas that in buds from plants treated with red light is 3-(4-hydroxycinnamoyltrigluco-3-O-4-hydroxycinnamoyl)kaempferol, in some manner red light may trigger an exchange reaction between the 4-hydroxycinnamic acid ester and the kaempferol hexagluco-3-O-4-hydroxycinnamoyl. Such a reaction would result in a lowered cofactor concentration in the pea bud and an increase in the concentration of the indolylacetate-oxidase inhibitor, 3-(4-hydroxycinnamoyltrigluco-3-O-4-hydroxycinnamoyl)kaempferol. As observed by Hillman & Galston (1957), then, tissue from plants treated with red light would possess increased concentrations of a diffusible indolylacetate-oxidase inhibitor.

The suggested conjugation of a flavonol glucoside with a sugar ester of 4-hydroxycinnamic acid is of special interest as it may represent a biochemical reaction that is under the control of the phytochrome pigment system. However, whether flavonoids and 4-hydroxycinnamic acid influence plant growth through their effect on the indolylacetate-oxidase system, or through some other mechanism, or not at all, has yet to be established.

SUMMARY

1. The indolylacetate-oxidase inhibitor previously isolated from pea plumules from plants treated with red light was shown to be 3-(4-hydroxycinnamoyltrigluco-3-O-4-hydroxycinnamoyl)kaempferol. The compound is 2-3 times as abundant in plumules of plants treated with red light as it is in plumules of plants completely grown in the dark.

2. The major flavonoid present in pea buds from plants completely grown in the dark appears to be a kaempferol hexagluco-3-O-4-hydroxycinnamoyl. Paper-chromatographic examination of extracts of pea buds from plants completely grown in the dark indicates that they also contain substantial amounts of a glucose derivative of 4-hydroxycinnamic acid.

3. It is suggested that red light somehow triggers an exchange reaction between the 4-hydroxycinnamic acid glucose derivative and the kaempferol hexagluconide, with the resultant formation of 3-(4-hydroxycinnamoyltrigluconyl)kaempferol.

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Biochem. J. (1964) **91**, 522

Intracellular Components Associated with Protein Synthesis in Developing Wheat Endosperm

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(Received 29 August 1963)

Almost all plant seeds accumulate storage proteins which are utilized by the developing embryo on germination. In wheat grain, the protein is formed predominantly in the endosperm tissue. Jennings & Morton (1963*a, b*) and Graham, Morton & Simmonds (1963*a*) have described the changes in the protein, carbohydrate, nucleic acids and other components during development of the endosperm of several varieties of wheat (*Triticum* spp.) grown under field conditions in South Australia.

During development of the endosperm from about 10 days after flowering until maturity at about 40 days there is rapid synthesis of protein; in variety Dural, for example, the protein nitrogen increased from 54 µg./grain at day 12 to 558 µg./grain at day 26 after flowering (Graham *et al.* 1963*a*). There is very little cell division in the endosperm during this period, but there is considerable cell expansion (Jennings & Morton, 1963*b*). The proteins formed during endosperm development may be fractionated by differential extraction into components soluble in 0.01M-sodium pyrophosphate, pH 7.4, in 0.05N-acetic acid and in 0.1N-sodium hydroxide respectively

(Graham *et al.* 1963*a*). From about day 15, relative to the other protein components those soluble in acetic acid increase at the greatest rate, and thus represent the greater portion of the storage proteins of the grain. These storage proteins accumulate in distinctive granules called 'protein bodies' which have been isolated from homogenates of developing endosperm (Graham, Jennings, Morton, Palk & Raison, 1962; Graham, Morton & Raison, 1963*b*). The protein components soluble in pyrophosphate buffer remain in the supernatant fraction on sedimentation of the intracellular granules.

This and the succeeding papers (Morton & Raison, 1964; Morton, Raison & Smeaton, 1964) describe the fine structure and the enzymic features of intracellular components that incorporate amino acids into endosperm proteins.

MATERIALS AND METHODS

Wheat endosperm. This was obtained from developing grain of *Triticum vulgare* (cultivar. Gabo, Insignia, or Kashmir no. 5) or of *T. durum* (cultivar. Dural) grown in plots at Adelaide. Endosperm used for isolation of protein-body and supernatant preparations was obtained by the rolling method (Graham *et al.* 1963*b*) and that used for electron microscopy was isolated by dissection by hand.

Electron microscopy. Sections of intact endosperm were cut by hand with a razor blade and fixed in 1% osmium

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