Phytochemistry, 1970, Vol. 9, pp. 497 to 505. Pergamon Press. Printed in England.

THE PHOTOCONTROL OF PRECURSOR INCORPORATION INTO THE *PISUM SATIVUM* FLAVONOIDS*

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(*Received* 12 *July* 1969)

Abstract—The incorporation of ^{14}C-phenylalanine into the flavonoids is markedly stimulated by red light in a phytochrome-type response pattern. The incorporation of ^{14}C-cinnamate, ^{14}C-*p*-coumarate and ^{14}C-malonate is either inhibited or not affected by the light treatment. The results suggest that phytochrome photoactivation triggers the conversion of phenylalanine into precursors of the flavonol nucleus. The precursors of the *p*-coumaroyl acylating-moiety, on the other hand, appear to be formed from phenylalanine in the absence of light treatment. This may indicate that the pathways of flavonol synthesis and of acylating-moiety synthesis, from phenylalanine may be different, or may occur in separate compartments.

INTRODUCTION

IT HAS been demonstrated[1-5] that the biosynthesis of the flavonoid glycosides and their acylated derivatives in etiolated seedlings of *Pisum sativum* is controlled by light operating, at least in the early stages, through the phytochrome system. Other investigations have shown that the enzyme which catalyses the conversion of phenylalanine to cinnamic acid (phenylalanine ammonia-lyase) is increased in level or activity by the photoactivation of phytochrome. These observations have led to the hypothesis[6,7] that phytochrome controls flavonoid biosynthesis through controlling the enzymatic conversion of phenylalanine to cinnamate, the first step in the biosynthesis of the flavonoid B-ring.

In order to obtain more direct evidence on this hypothesis we have investigated the effects of various light treatments on the incorporation of B-ring precursors into the flavonol nucleus and into the *p*-coumarate acyl group. Intensive investigation of A-ring precursors has not yet been attempted, although one experiment with a possible A-ring precursor is included. The results of these investigations are consistent with phenylalanine ammonia-lyase having an important regulatory function, although they pose further problems for the understanding of the details of flavonol synthesis and acylation.

* Part III of the series "Biochemical Studies on the Photocontrol of Flavonoid Biosynthesis", for Part II, see *Phytochem.* **9**, 487 (1970).

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[1] M. FURUYA and R. G. THOMAS, *Plant Physiol.* **39**, 634 (1964).
[2] F. E. MUMFORD, D. H. SMITH and P. G. HEYTLER, *Biochem. J.* **91**, 517 (1964).
[3] W. H. BOTTOMLEY, H. SMITH and A. W. GALSTON, *Nature* **207**, 1311 (1965).
[4] W. H. BOTTOMLEY, H. SMITH and D. W. GALSTON, *Phytochem.* **5**, 117 (1966).
[5] H. SMITH and D. B. HARPER, *Phytochem.* **9**, 477 (1970).
[6] T. H. ATTRIDGE and H. SMITH, *Biochim. Biophys. Acta* **148**, 805 (1967).
[7] H. SMITH and T. H. ATTRIDGE, *Phytochem.* **9**, 487 (1970).

RESULTS

Uptake and Transport of the Precursors

In order to introduce labelled precursors into the probable site of flavonoid synthesis, the terminal buds, it is necessary to excise the bud and epicotyl from the cotyledons and roots and to place them in solution containing the precursor. The growth of the terminal buds and their responses to light treatment depend on certain unknown factors translocated from the stems[8] and thus it is necessary to allow as much epicotyl as possible to remain attached to the buds. This implies that the precursor must be transported to the site of synthesis, a distance of some 10–15 cm, before incorporation can proceed. As a check that the excised system behaved in a similar manner to the intact plant, the increase in the fresh weight of the terminal buds over a period of 24 hr after a 15 min red light treatment was estimated on several separate occasions (Table 1). It can be seen that the red/far-red photoreversible increase in bud size characteristic of intact plants is present. In preliminary experiments it was observed that the capacity of the buds to respond to red light by increased growth diminished with time after excision, until at 5–7 hr no increase was obtainable.

TABLE 1. THE EFFECT OF RED AND FAR-RED LIGHT ON THE GROWTH OF TERMINAL BUDS OF SEEDLINGS GROWN IN THE DARK FOR 6 days, EXCISED ABOVE THE COTYLEDONARY NODE AND PLACED IN BUFFERED, 0·1 M SUCROSE

Fresh wt. of buds 24 hr after light treatment				
Dark (mg)	Red (mg)	R/FR (mg)	FR/R (mg)	$\frac{R}{D} \times 100$ (per cent)
1·48	1·76	1·50	1·70	119
1·67	2·13	1·89	1·98	128
1·75	2·00	1·77	1·89	114
1·70	2·45			144
1·86	2·42			130

Figure 1 illustrates the increase in radioactivity extractable from the terminal buds in methanol as a function of time after exposure to ^{14}C-phenylalanine in the basal solution. The uptake and transport of the precursor to the buds reaches a peak at about 6 hr, followed by a decline and a further rise reaching a maximum at 20–22 hr. It is presumed that the first rise represents the accumulation of the precursor into the buds, whilst the second rise is caused by the accumulation of metabolic products of the precursor. Consequently in the following experiments, excised epicotyls were allowed to take up the precursors for 2 hr before the light treatments were given, and the terminal buds were harvested and extracted 15 hr later. This experimental procedure was a compromise to achieve the maximum uptake of precursor into the buds with the minimum complication from conversion products.

A further factor important in the design of the experiments is the effect of light treatment on the transport of the precursors. Goren and Galston[8] have previously shown that red light treatment leads to increased rates of transport of ^{14}C-sucrose into the terminal buds of peas grown in a similar manner. Table 2 demonstrates that this also is true of some of the precursors used in these experiments, and that the response pattern indicates phytochrome control. It has been necessary, therefore, to correct for transport differences in the subsequent experiments, by expressing the results as percentages of the methanol-extractable radioactivity. In some experiments specific activity data are also provided.

[8] R. GOREN and A. W. GALSTON, *Plant Physiol.* **41**, 1055 (1966).

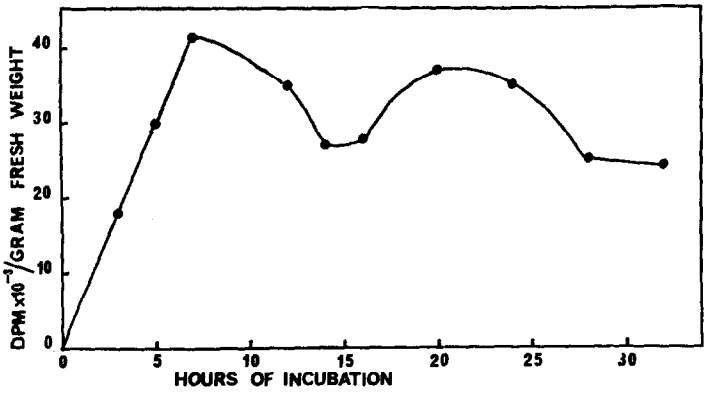

FIG. 1. THE KINETICS OF UPTAKE OF ^{14}C-PHENYLALANINE INTO THE TERMINAL BUDS.
6-day dark-grown seedlings were excised above the cotyledons and the shoots placed in 4 ml of 0·1 M sucrose, 0·015 M phosphate buffer, pH 6·4, containing 1 μc L-phenylalanine-^{14}C(U). Buds were excised at intervals, extracted in methanol and the absolute activity of the soluble fraction determined.

TABLE 2. THE EFFECT OF RED AND FAR-RED LIGHT ON THE TRANSPORT OF ^{14}C-PHENYLALANINE AND ^{14}C-CINNAMATE FROM THE BASE OF THE EPICOTYL INTO THE TERMINAL BUDS

	Activity of methanol-extract of buds in dpm/100 mg fresh wt.			
^{14}C-Precursor	Dark	Red	R/FR	FR/R
Phenylalanine	2057	3718	2893	4164
Cinnamate	287	785	241	836

Incorporation into total flavonoids. In a preliminary attempt to determine which general region of the pathway would be most fruitful for intensive investigation, the incorporation (over a 24 hr period) of ^{14}C-phenylalanine, ^{14}C-cinnamate, ^{14}C-*p*-coumarate and ^{14}C-malonate into total flavonoids as affected by light treatment was analysed. The bulk flavonoids were separated from other components by two-dimensional paper chromatography (see Methods) in which they ran together as an easily discernible u.v.-absorbing spot. The radioactivity eluted from this region is expressed in Table 3 as a percentage of the total radioactivity extracted from the buds in methanol. It is clear that the only precursor whose

TABLE 3. EFFECT OF RED LIGHT ALONE, AND FOLLOWED BY FAR-RED LIGHT, ON THE INCORPORATION OF ISOTOPIC PRECURSORS INTO THE TOTAL FLAVONOIDS OF THE TERMINAL BUDS

	Radioactivity in total flavonoids as % total, methanol-extractable radioactivity		
^{14}C-Precursor	Dark	Red	R/FR
Phenylalanine	5·9	21·4	7·2
Cinnamate	4·2	1·7	2·5
p-Coumarate	2·2	3·5	1·9
Malonate	13·7	4·9	10·2

incorporation into the flavonoids is stimulated by red light is phenylalanine and this stimulation is far-red reversible. Little effect is apparent on the incorporation of ^{14}C from cinnamate and *p*-coumarate whilst that from malonate is clearly inhibited, again in a photoreversible manner. It was decided, therefore, to concentrate subsequent experimentation on the effects on ^{14}C-phenylalanine and ^{14}C-cinnamate incorporation.

The effect of light on ^{14}C-phenylalanine incorporation into the individual flavonoids. In order to analyse the detailed effects of red light on the incorporation of ^{14}C-phenylalanine and ^{14}C-cinnamate into the individual flavonoids, the thin-layer separation method of Harper and Smith[9] was employed. This method enables the determination of flavonoid quantity and flavonoid radioactivity to be made on the same extract.

The effects of red and far-red light treatments on the incorporation of ^{14}C-phenylalanine into kaempferol-3-triglucoside (KG), kaempferol-3-*p*-coumaroyltriglucoside (KGC), and quercetin-3-*p*-coumaroyltriglucoside (QGC), are presented in Tables 4 and 5. Table 4

TABLE 4. SPECIFIC ACTIVITY OF THE TERMINAL-BUD FLAVONOIDS EXTRACTED 17 hr AFTER FEEDING WITH 1 μc ^{14}C-PHENYLALANINE

	dpm/μmole			
Flavonoids	Dark	Red	R/FR	FR/R
KGC	306	7160	992	7530
QGC	64	342	116	320
KG	226	356	278	440

TABLE 5. THE INCORPORATION OF ^{14}C-PHENYLALANINE INTO THE TERMINAL-BUD FLAVONOIDS EXPRESSED AS A PERCENTAGE OF THE TOTAL RADIOACTIVITY EXTRACTABLE IN METHANOL

	Percent bud soluble			
Flavonoids	Dark	Red	R/FR	FR/R
KGC	4·8	66·9	10·8	54·6
QGC	0·8	3·0	1·3	3·1
KG	3·8	2·9	3·1	3·1

gives the specific activity data, whilst Table 5 gives the incorporation expressed as percentages of the total extractable radioactivity, to take account of the effects on transport. The most striking data is the very large increase in ^{14}C-incorporation into KGC as a result of red light treatment, an effect which is very nearly completely photoreversible. A similar pattern of lower magnitude is apparent for incorporation into QGC whereas there is no discernible effect on incorporation into KG when the transport effects are taken into account.

The effect of light on ^{14}C-cinnamate incorporation into the individual flavonoids. Tables 6 and 7 provide data for the effects of red and far-red light on the incorporation of ^{14}C from cinnamate into the individual flavonoids. There is no obvious effect of light on the specific activity of the flavonoids, although when transport changes are taken into consideration, a phytochrome-mediated inhibition of incorporation becomes apparent.

[9] D. B. HARPER and H. SMITH, *J. Chromatog.* **41**, 138 (1969).

TABLE 6. SPECIFIC ACTIVITY OF THE TERMINAL-BUD FLAVONOIDS EXTRACTED 17 hr AFTER FEEDING WITH 1 μc ^{14}C-CINNAMIC ACID

	dpm/μmole			
Flavonoids	Dark	Red	R/FR	FR/R
KGC	90·8	106·9	98·6	72·9
QGC	106·6	67·9	77·4	73·7
KG	84·8	132·6	96·8	128·3

TABLE 7. THE INCORPORATION OF ^{14}C-CINNAMIC ACID INTO THE TERMINAL-BUD FLAVONOIDS EXPRESSED AS A PERCENTAGE OF THE TOTAL RADIOACTIVITY EXTRACTABLE IN METHANOL

	Percent bud soluble			
Flavonoids	Dark	Red	R/FR	FR/R
KGC	8·6	4·0	11·3	2·4
QGC	10·0	2·8	9·5	2·8
KG	11·8	4·5	12·9	4·5

The incorporation of ^{14}C-phenylalanine into the flavonol nucleus and acyl groups of KGC and QGC. The fact that two of the major flavonoids in pea seedlings are esterified at the terminal glucose residue with *p*-coumaric acid[10] raises considerable difficulties in the interpretation of the above results as they stand, since both the flavonol nucleus and the acylating moiety are thought to be synthesized along a common pathway.[11] In order to obtain information on the relative contributions of the acyl groups and the flavonol nuclei to the overall radioactivity of the molecules, an experiment was performed with ^{14}C-phenylalanine in which the two residues were separated by alkaline hydrolysis. The specific activity data for the two parts of the molecule are given in Table 8. The significance of these results for the role of phytochrome and for the pathways of flavonol synthesis and acylation are considered below.

TABLE 8. INCORPORATION OF ^{14}C-PHENYLALANINE INTO THE FLAVONOL NUCLEUS AND THE ACYL PORTION OF KGC AND QGC UNDER THE INFLUENCE OF RED AND FAR-RED LIGHT TREATMENTS

		dpm/μmole			
Flavonoid	Portion of molecule	Dark	Red	R/FR	FR/R
KGC	Flavonol	0·6	5950	471	6130
	Acyl	305·6	1220	520	1400
QGC	Flavonol	14·1	220	71·0	188
	Acyl	50·2	122	44·8	132

DISCUSSION

The experiments reported here demonstrate that the photoactivation of phytochrome could influence flavonoid synthesis in several ways. The continued synthesis of flavonoids in the terminal buds of etiolated seedlings is presumably dependent on the translocation of

[10] M. FURUYA, A. W. GALSTON and B. B. STOWE, *Nature* **193**, 456 (1962).
[11] A. C. NEISH, in *Biochemistry of Phenolic Compounds* (edited by H. B. HARBORNE), p. 295, Academic Press, London (1964).

precursors from the storage tissues (i.e. the cotyledons). These substances are normally likely to be carbohydrates (e.g. sucrose)[8] and possibly amino acids.[12] The effects of red light on the translocation of phenylalanine and cinnamate to the buds reported here, together with the observations on sucrose transport reported by Goren and Galston,[8] and of translocation of K^+ and Ca^{2+} by Köhler,[13] suggest that phytochrome could control flavonoid synthesis through effects on the availability of precursors. These effects are relatively large (Table 2) and it is quite possible that the phytochrome control of translocation is very important in the normal responses of the plant. It has been shown in the previous paper in this series that phenylalanine ammonia-lyase, the first enzyme in the pathway of B-ring synthesis, is present in dark-grown tissues.[7] It seems likely, therefore, that the biochemical apparatus necessary for flavonoid synthesis is present in dark-grown tissues, and an increased availability of precursors would therefore, presumably, lead to increased synthesis. There is also the possibility that the increased levels of phenylalanine ammonia-lyase found after phytochrome photoactivation[7] may be due to substrate induction by phenylalanine transported from the cotyledons. However, attempts to demonstrate phenylalanine-induction of phenylalanine ammonia-lyase in the pea seedling have so far proved negative (Attridge and Smith, unpublished results). Even so, when looking for the locus of phytochrome action in a specific biosynthetic sequence, the possibility of a phytochrome-mediated change in the intercellular distribution of metabolites should not be disregarded.

Even when the above proviso is taken fully into consideration, however, it is quite clear that phytochrome photoactivation very strikingly stimulates the incorporation of ^{14}C-phenylalanine into the flavonoids (Tables 3 and 5). It should be noted that the data in these tables is expressed as percentages of the radioactivity extractable from the buds in methanol, thus eliminating any errors due to uptake differences. Furthermore, when the detailed incorporation into the three flavonoids is considered (Table 5), it is seen that red light stimulates incorporation into KGC by a factor of approximately 14. Thus, a specific effect of phytochrome on the conversion of phenylalanine to KGC over and above that due to increased precursor availability seems unequivocal. Red light also increases phenylalanine incorporation into QGC, but less strikingly. Table 4 also shows that the absolute activity data for QGC is considerably lower than for KGC, suggesting that QGC is either synthesized more slowly, or is further along the biosynthetic pathway, than KGC. Red light apparently has no effect on the conversion of phenylalanine to KG, although the absolute activity data of Table 4 suggests that considerable KG synthesis is occurring. The rather anomalous biosynthetic position of KG is considered below.

The incorporation of the other precursors into the flavonoids is either not affected, or is inhibited by red light (Tables 3 and 7). This is taken to indicate that the major biosynthetic effect of red-light treatment is to stimulate the conversion of phenylalanine into the subsequent intermediate (presumably cinnamate[14]) and that effects later in the pathway are of little importance. However, the apparent inhibition of incorporation of ^{14}C-cinnamate and ^{14}C-malonate into the flavonoids (Tables 7 and 3 respectively) remain to be explained. It is quite possible that the very large rise in endogenous cinnamate synthesis brought about by red-light treatment could lead to the production of an unusually large pool size of cinnamate thus diluting the added ^{14}C-cinnamate relative to the untreated controls. This would give rise to the apparent decrease in incorporation into the flavonoids. Such an

[12] J. S. PATE and W. WALLACE, *Ann. Bot.* **28**, 83 (1964).
[13] D. KÖHLER, *Planta* **84**, 158 (1969).
[14] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 537 (1959).

argument, however, cannot be used to explain the inhibition of ^{14}C-malonate incorporation, unless the supply of precursors of the A-ring, e.g. malonyl-CoA, is also phytochrome controlled, a hypothesis that has in fact previously been proposed.[15]

The results taken as they stand, therefore, indicate that phytochrome controls the conversion of phenylalanine into precursors of the flavonoids. The acylated nature of the pea flavonoids, however, raises difficulties for the simple interpretation of these results since it is normally thought that the flavonoid B-ring is synthesized along the same pathway as is the *p*-coumaroyl moiety. The results in Table 8 show, in fact, that red light not only increases the incorporation of ^{14}C-phenylalanine into total KGC and QGC, but that it also drastically changes the relative rates of incorporation into the two parts of the molecules. In the dark,

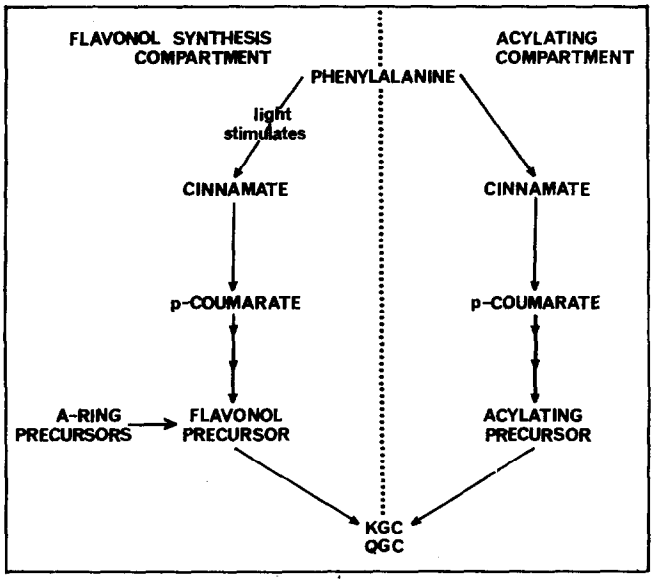

FIG. 2. SCHEME SHOWING THE SEPARATION OF THE BIOSYNTHETIC PATHWAYS OF THE *p*-COUMAROYL PRECURSORS OF THE FLAVONOID NUCLEUS, AND OF THE ACYL GROUP, AS SUGGESTED BY THE RESULTS.

the radioactivity is found predominantly in the acylated portion of the molecule, whereas after red light it occurs predominantly in the flavonol nucleus. It is true that incorporation into both parts is increased by red light, but incorporation into the flavonol nucleus is very much more strikingly increased than that into the *p*-coumarate. It seems to the authors that there are only two ways in which these results can be reconciled; (*a*) that the conversion of *p*-coumarate to the flavonol nucleus is subject to a further photocontrol whilst its conversion to an acylating precursor is not, and (*b*) that the synthesis of the flavonol precursors takes place in a separate compartment, or by a separate pathway from that of the acyl moiety. Alternative (*a*) would explain the effects of light on the distribution of label within the molecule, but would require that the incorporation of exogenous ^{14}C-cinnamate and ^{14}C-*p*-coumarate into the whole molecule would be increased, a prediction that is not realized. Alternative (*b*), however, could explain all the results if it was assumed that the conversion of phenylalanine into the flavonol synthesis precursors was strongly dependent on red light

[15] H. W. SIEGELMAN, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 437, Academic Press, London (1964).

whilst its conversion to the acylating moiety was virtually independent of light. The compartmentalization could be either physical, i.e. in separate parts of the cell or chemical, e.g. the flavonol precursors could be coenzyme-A esters whilst the acylating precursors could be glucose-esters. It is not possible to decide definitely between these possibilities at present, especially in view of our lack of knowledge of possible photocontrol of A-ring synthesis. The evidence of this communication, however, favours alternative (*b*) (Fig. 2).

The non-acylated flavonoid, KG, is rather anomalous from the biosynthetic point of view. Its total amount per bud remains quite constant under all conditions,[5] yet it appears to incorporate label from both ^{14}C-phenylalanine and ^{14}C-cinnamate in both light and darkness. There is, however, no effect of light on its synthesis in bud tissues. The logical position of KG as a precursor of KGC is by no means established, and the radioactivity data of these experiments argue against such a role. There is, however, a possible rationalization. Russell and Galston[16] have shown that red-light treatment brings about a large increase in KG levels in the stems of Alaska peas, organs which contain only traces of KGC. It is possible therefore, that the site of KG synthesis is within the stem, and that an equilibrium between KG in the stem and KG in the bud is maintained by translocation. This could explain the appearance of label in the KG of the bud under conditions when net synthesis was apparently not occurring.

In conclusion it can be said that phytochrome probably controls flavonoid synthesis at several levels. One control point is almost certainly the enzyme phenylalanine ammonia-lyase, although it is extremely unlikely to be the only regulatory step. Effects on the translocation of precursors are obviously important and it is possible that the translocation of the products is also involved. In order to fully explain the phytochrome regulation of flavonoid synthesis, it may ultimately be necessary to invoke multiple control points and compartmentalization of biosynthetic sequences.

EXPERIMENTAL

Plant Materials and Growth Conditions

Seeds of *Pisum sativum* var. Alaska were obtained from Carters, Wimbledon, England and the growth conditions were as described earlier.[5]

Light Sources and Treatments

The sources of red and far-red light were as reported previously.[5,7] Plants were exposed to red light for 15 min, or far-red light for 15 min, or to sequences of the above treatments. All manipulations were carried out under a dim green safe-light.

Isotopic Precursors

L-β-Phenylalanine-^{14}C(U) spec. act. 495 mc/mmole, and sodium malonate-2-^{14}C, spec. act. 18·3 mc/mmole, were obtained from the Radiochemical Centre, Amersham, England. The *trans*-2-^{14}C-cinnamic acid and *trans*-2-^{14}C-*p*-coumaric acid used in the experiment reported in Table 3 were prepared from the malonate-2-^{14}C and the appropriate benzaldehyde by methods reported in the literature.[17,18] Radiochemical purity was assayed by thin-layer chromatography. The *trans*-2-^{14}C-cinnamic acid used in other experiments had a spec. act. of 1·68 mc/mmole and was obtained from Tracerlab, Waltham, Mass., U.S.A.

Precursor Incorporation and Flavonoid Extractions

6-day dark-grown seedlings were excised just above the cotyledons and fifty shoots placed in a 3 cm diameter glass vial with their bases dipping in 4·0 ml of solution of 0·1 M sucrose, 0·015 M phosphate buffer, pH 6·4, and containing 1 μc of the isotopic precursor under investigation. Each treatment (i.e. red-light treatment, dark control, etc.) consisted of 200 shoots (four vials), each batch being preincubated for 2 hr before

[16] D. W. RUSSELL and A. W. GALSTON, *Phytochem.* 6, 791 (1967).
[17] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* 33, 908 (1955).
[18] D. J. AUSTIN and M. B. MEYERS, *Phytochem.* 4, 245 (1965).

light treatment. After light treatment the vials were returned to darkness for 15 hr, whereupon the buds were excised at the third node and weighed. The buds from each treatment were pooled and homogenized in 30 ml MeOH in a Griffiths tube. The extract and 2 × 30 ml MeOH washings were poured into a flask and boiled for 3 min, filtered and the residue washed with MeOH. The combined filtrate and washings were evaporated to dryness and taken up in 20 ml of MeOH. 1·0 ml of this was taken for assay of total bud-soluble activity. The remainder of the extract was evaporated to dryness and taken up in 2·0 ml. This extract was then separated either by the TLC method of Harper and Smith[9] for separation of the individual flavonoids, or by paper chromatography for bulk separation of total flavonoids from other materials. In the thin-layer separation the whole 2·0 ml extract was loaded quantitatively onto a prepared plate in a stripwise manner. In the paper chromatographic separation a known quantity of the extract was spot loaded onto several 20 × 20 cm papers which were then developed in ascending two-dimensional fashion in (*a*) *n*-BuOH, acetic acid, H_2O, 5:1:2·2; and (*b*) 5% acetic acid. In this method the three major flavonoids run together near the centre of the chromatogram and are distinctly visible as an u.v.-absorbing spot.

Elution, Estimation and Radioassay of Flavonoids

The bulked paper chromatogram spots were placed in a scintillation vial containing 10·0 ml MeOH, and acidified with 2 drops of 6 N HCl. The vials were left, with occasional shaking, for 6 hr and the papers removed and washed. The washings were bulked in the scintillation vial, dried down and taken up in 2·0 ml of scintillation fluid and assayed as below. No data for quantities of flavonoids was possible with the paper chromatographic method. The zones on the thin-layer chromatograms were examined under u.v. light and the powder scraped off and placed in separate 100-ml flasks. 20 ml of EtOH acidified with 2 drops 6 N HCl were added and the suspension stirred thoroughly. The suspensions were then centrifuged and the clear supernatant solutions decanted and made up to a standard volume. KGC and QGC concentrations were then determined in a Unicam SP 800 spectrophotometer: KGC, $\log_{10} E_{315} = 4{\cdot}4689$; QGC, $\log_{10} E_{315} = 4{\cdot}3765$ both in EtOH. For KG, 4·0 ml of the EtOH solution was evaporated to dryness and taken up in 4·0 ml H_2O and the concentration determined using $\log_{10} E_{350} = 4{\cdot}1059$ or $\log_{10} E_{267} = 4{\cdot}3553$ in H_2O. For radioassay, 8·0 ml of the EtOH solutions of KGC, QGC and KG were evaporated to dryness and taken up in 2·0 ml of MeOH. 10·0 ml of a scintillation fluid (5·0 g 2,5-diphenyloxazole in 1 l. toluene) were added and thoroughly shaken. Radioactivity determinations were made in a Beckman LS-100 scintillation counter with quench correction by the external standard method.

Hydrolysis of KGC and QGC

8·0-ml samples of the ethanolic KGC and QGC eluates were dried down, taken up in 5 ml of N NaOH and allowed to stand at room temperature for 6 hr. The solution was acidified (2 N HCl) and extracted (3 × 10 ml) with Et_2O. The Et_2O extracts were bulked, and the *p*-coumarate estimated spectrophotometrically ($\log_{10} E_{309} = 4{\cdot}3118$ in Et_2O). 20·0 ml of the Et_2O extract was dried down, taken up in 2·0 ml of MeOH, and the radioactivity of the *p*-coumarate determined as above. Control experiments showed that this procedure gave complete hydrolysis of both KG and QGC to *p*-coumarate, and to KG and QG respectively. The specific activities of the KG and QGC produced were estimated by difference.

Acknowledgements—The authors are grateful for the support of research grants from the Science Research Council and the University of London Central Research Fund, and for encouragement from Professor E. A. Bevan.