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THE EFFECTS OF SHORT- AND LONG-TERM IRRADIATION ON THE FLAVONOID COMPLEMENT OF THE TERMINAL BUDS OF *PISUM SATIVUM* VAR. ALASKA*

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Abstract—Irradiation of etiolated seedlings leads to complex patterns of transitory changes in the concentration of the three major flavonol complexes in the terminal buds. Two categories of response are apparent: (*a*) a phytochrome-mediated rapid response of low magnitude, and (*b*) a longer term response of greater magnitude dependent on continuous irradiation. None of the changes can be simply correlated with changes in growth rate.

INTRODUCTION

THE IMPORTANCE of light in the regulation of flavonoid biosynthesis in higher plants has been well established.[1] In the terminal buds of etiolated pea seedlings, for example, very short irradiations with red light are sufficient to cause significant changes in flavonoid content, these changes being reversible by far-red light, indicating the involvement of phytochrome.[4-5] In several other species investigated, short irradiations with red light have little effect, whereas continuous illumination with light of various spectral regions elicits considerable, long-term increases in the levels of flavonoids or other phenolic compounds.[6-10] In recent years two opposing hypotheses have been developed concerning the manner in which these responses are mediated; (*a*) continuous illumination acts solely through the phytochrome system,[11] and (*b*) other photoreceptors than phytochrome are involved.[12]

In this investigation, the detailed kinetics of the changes in the major flavonoids of pea buds as induced by short- and long-term irradiation treatments have been analysed in an attempt to obtain information on this problem, and as a basis for subsequent biochemical investigations.

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

[1] H. W. SIEGELMAN, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 437, Academic Press (1964).
[2] M. FURUYA and R. G. THOMAS, *Plant Physiol.* **39**, 634 (1964).
[3] F. E. MUMFORD, D. H. SMITH and P. G. HEYTLER, *Biochem. J.* **91**, 517 (1964).
[4] W. BOTTOMLEY, H. SMITH and A. W. GALSTON, *Nature* **207**, 1311 (1965).
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[10] H. W. SIEGELMAN and H. B. HENDRICKS, *Plant Physiol.* **32**, 393 (1957).
[11] K. M. HARTMANN, *Photochem. Photobiol.* **5**, 349 (1966).
[12] G. ENGELSMA, *Planta* **77**, 49 (1967).

RESULTS

The Pea Flavonoids and Their Extraction

The major flavonoid components of pea leaves were shown by Furuya[13] to be kaempferol-3-triglucoside (KG), kaempferol-3-*p*-coumaroyltriglucoside (KGC), quercetin-3-triglucoside (QG), and quercetin-3-*p*-coumaroyl-triglucoside (QGC) (Fig. 1). The methods previously used for the separation of these very similar compounds involved either two-dimensional paper chromatography,[2,3] or selective adsorption on columns of borate-buffered silica gel.[4,5] The paper-chromatographic methods suffer from the disadvantage that only small amounts can be readily separated, whereas the selective adsorption method could only

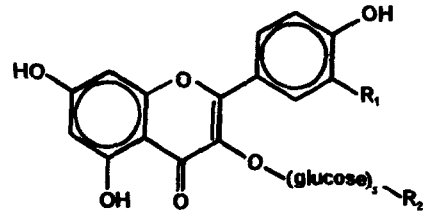

FIG. 1. THE PRESUMED STRUCTURES OF THE FOUR MAJOR FLAVONOIDS FOUND IN PEA LEAVES.
KGC, $R_1 = H$, $R_2 = p$-coumaroyl; QGC, $R_1 = OH$, $R_2 = p$-coumaroyl; KG, $R_1 = R_2 = H$; QG, $R_1 = OH$, $R_2 = H$.

be satisfactorily used to separate and estimate KGC and QGC, and was, in any case, highly sensitive to temperature fluctuations. All of these methods are relatively time-consuming. In order to more easily obtain reliable data, a TLC method has been developed[14] in which all four components can be separated within 45 min on a single plate in sufficient quantities for subsequent assay by u.v. spectrophotometry.

The earlier reports[2-5] had indicated that the terminal buds of dark-grown seedlings contained only KG and KGC, the quercetin components being either absent, or present only in very small quantities. In this investigation, however, QGC was always present in dark-grown buds at a concentration similar to that of KGC. QG, however, was only detected in tissues given several hours of continuous illumination. Table 1 provides data for the concentrations of KG, KGC and QGC in the terminal buds of 6-day-old dark-grown peas. It is clear that there is considerable variation in the amount of flavonoids present,

TABLE 1. FRESH WEIGHT AND FLAVONOID CONTENT OF THE TERMINAL BUDS OF 6-day DARK-GROWN SEEDLINGS

Fresh wt. (mg/bud)	Flavonoid content (μmoles/g fresh wt.) KG	KGC	QGC	Total (mμmoles/bud)
1·94	3·60	1·82	2·01	14·0
1·56	3·64	3·19	2·91	15·0
2·80	2·11	1·74	1·60	15·0
2·63	1·79	2·12	1·81	15·0
3·11	2·00	1·41	1·30	14·6

[13] M. FURUYA, Ph.D. Thesis, Yale University (1962).
[14] D. B. HARPER and H. SMITH, *J. Chromatog.* **41**, 138 (1969).

most obvious in KG. Furthermore, there is considerable variation in the mean bud weight of these samples, probably due to differences in growth conditions, in particular, water availability. However, if the total flavonoid content per bud is calculated from this data (column 6 of Table 1) very little variability is observed. The variability in flavonoid pattern of dark-grown tissues appears, therefore, to depend on the physiological stage of development of the tissues, whilst total amount of flavonoid per bud seems not to be affected. It will be seen below that the response of the plants to illumination does not seem to be affected by variation in the initial pattern of flavonoid complement.

The Effects of Short Red-light Treatments

The effects of a 15 min irradiation with red light on flavonoid content are shown in Fig. 2. It is apparent that red light initiates a steady increase in total KGC and total QGC.

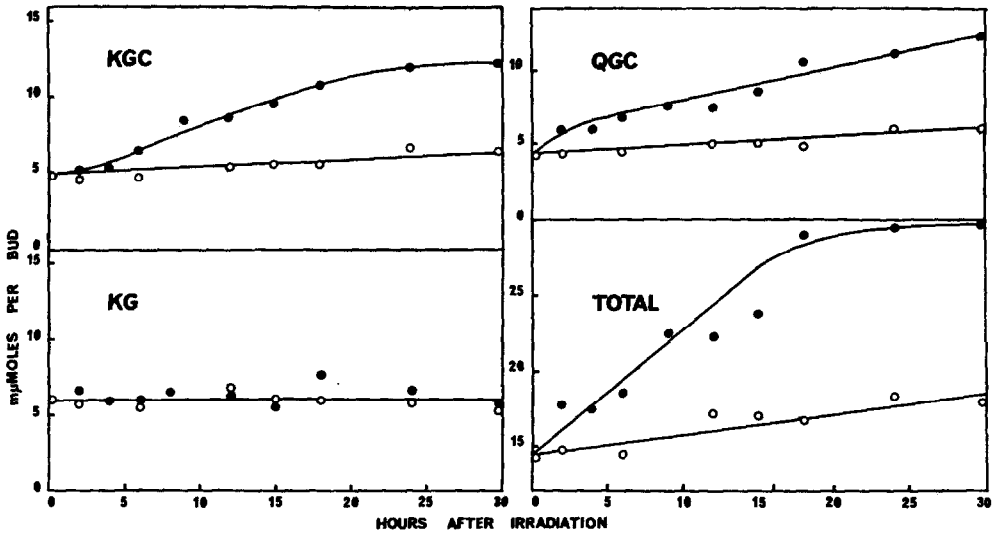

FIG. 2. CHANGES IN THE TOTAL AMOUNTS OF FLAVONOIDS PER BUD CAUSED BY IRRADIATING 6-day DARK-GROWN SEEDLINGS WITH 15 min RED LIGHT.

Open symbols, dark controls; solid symbols, red-light treated.

There are transient increases in concentration during the first 5–10 hr which appear to be due to the effect of red light on KGC and QGC levels being detected before any increase in bud weight. The total amount of KG does not change under the effect of red light; thus when expressed as μmoles/g fresh wt. there is an apparent reduction due to diluting out by bud growth.

The Effects of Continuous Illumination with White Light

When dark-grown pea plants are illuminated with continuous white light, the changes in flavonoid concentration and content of the terminal buds are considerably more complex than with short red-light irradiations. Figure 3 shows the growth of the buds under these conditions, and Fig. 4 shows the changes in total content of flavonoids of the buds. It is clear that continuous white light has marked effects on the concentrations of the three major flavonoids. The concentration of KG falls drastically under white light. However, within

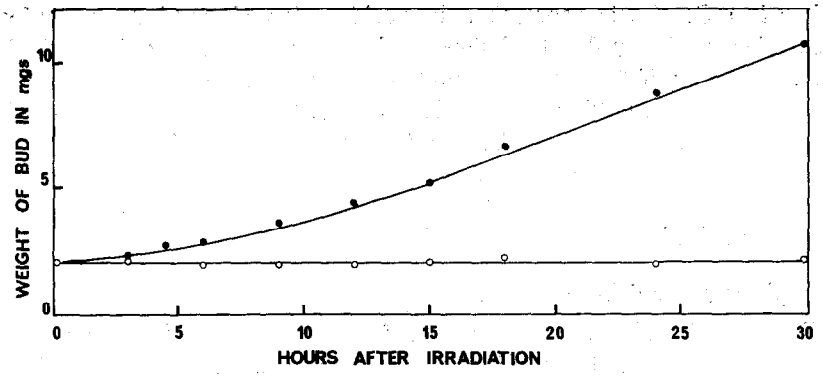

FIG. 3. THE GROWTH OF TERMINAL BUDS OF 6-day DARK-GROWN SEEDLINGS WHEN PLACED IN CONTINUOUS WHITE LIGHT (SOLID SYMBOLS) OR MAINTAINED IN DARKNESS (OPEN SYMBOLS).

limits this can again be accounted for in terms of diluting out by growth, as can be seen from Fig. 4. White light brings about two rises in QGC concentration; an early rise of low magnitude comparable with the red-light effect, and a much larger rise at 16–18 hr. KGC concentration also increases in the early stages but later undergoes a sudden decrease also

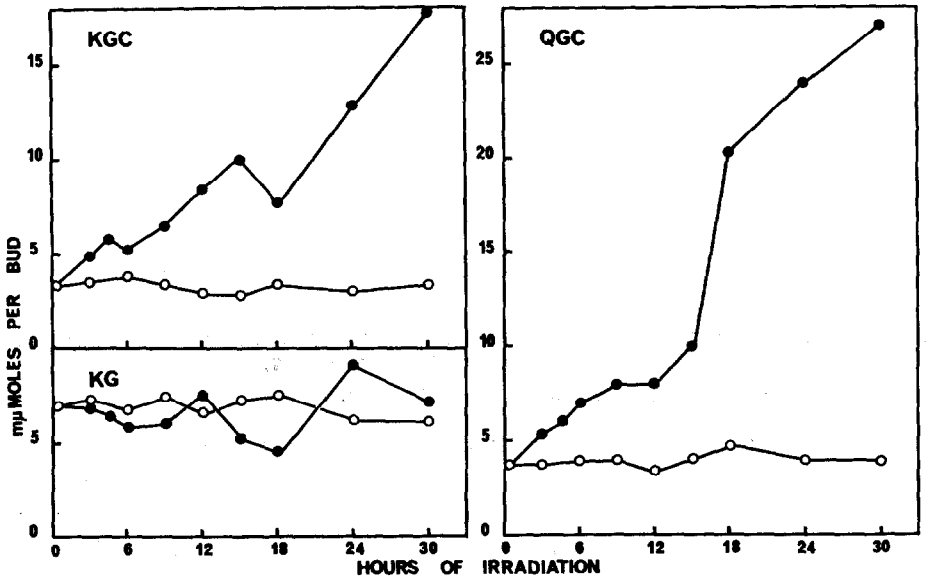

FIG. 4. CHANGES IN THE TOTAL AMOUNTS OF THE FLAVONOIDS IN TERMINAL BUDS OF 6-day DARK-GROWN SEEDLINGS UPON TRANSFERENCE TO CONTINUOUS WHITE LIGHT (SOLID SYMBOLS) OR MAINTENANCE IN DARKNESS (OPEN SYMBOLS).

between 16–18 hr. These changes clearly show up in the total amounts of each flavonoid in the bud (Fig. 4). From 18 hr onwards, it was possible to detect significant amounts of the other quercetin compound, QG, on the plates. However, this compound appears to be particularly susceptible to oxidation on the plate and, although it could be eluted, spectra good enough for assay could not be obtained.

The Effects of Continuous Illumination With Blue Light

The transference of dark-grown pea seedlings to continuous illumination with blue light brings about further complicated changes in fresh weight and in flavonoid concentration and content of the terminal buds (Figs. 5 & 6). In this case no significant early rises are apparent in the concentration of any of the three major flavonoids, whilst each undergoes a sudden and dramatic later rise. The presence of significant amounts of QG was again observed from 18 hr onward. If the curves for the increase in flavonoids in blue light (Fig. 6) are superimposed an apparent series of peaks is evident. The possible significance of this is discussed below.

The results described above have been substantially repeated and confirmed.

DISCUSSION

It is apparent from the results of this investigation that the irradiation of dark-grown pea tissues with various light sources leads to large and complex changes in the concentration of individual flavonoid components. The effects of short periods of red irradiation have been investigated previously. Furuya and Thomas[2] reported that red light treatment caused a marked increase in KGC concentration, whilst Bottomley *et al.*,[4, 5] using a different method of extraction and separation, claimed that KGC concentration remained unchanged whilst QGC concentration rose steadily for at least 36 hr after treatment. Under our conditions, and using yet another method, we have shown that the concentrations of both KGC and QGC increase after red-light treatment but the increases are slight and transient.

There are three possible reasons for the inconsistencies in these reports; (*a*) the quantitative methods used are unreliable, (*b*) the flavonoid concentrations are extremely sensitive to small differences in growth conditions, and (*c*) different batches of the same variety of pea respond very differently to light treatments. The method of flavonoid extraction and separation used in this investigation was aimed at complete extraction of the flavonoids and prevention of degradation during processing. Using crystalline KGC and QGC 92–97 per cent recovery was routinely achieved.[14] Furthermore, all the experiments reported here have been repeated with substantial confirmation of the results, the only variability being quantitative. Thus, it seems most probable that genetic differences in the plant material, and small differences in growth conditions are responsible for the anomalies observed. It should be noted here that the data of Table 1 illustrate the apparently random distribution of identical total amounts of flavonoid material between KG, KGC and QGC in bud tissues grown for identical periods under ostensibly identical conditions. We have not attempted to achieve any greater control over the already rigidly regulated growth conditions since we have observed that the responses to red-light treatment are always qualitatively similar, irrespective of the pattern of flavonoid complement at the beginning of the experiment.

When 6-day dark-grown peas are given a short treatment with red light, relatively rapid fluctuations in concentrations of KGC and QGC occur but, over a period of 30 hr, the concentrations resume very nearly the steady-state concentration existing before light treatment. Most interesting here is the fact that the rise in QGC concentration consistently occurs within 2 hr of light treatment, whereas the peak in KGC concentration is delayed until 8–12 hr. That is, the increase in flavonoids synthesized per bud is paralleled by the increase in bud fresh weight. Compared with the changes brought about by continuous illumination, these increases are relatively small; however, the rise in QGC represents one of the most rapid metabolic responses to phytochrome photoactivation yet reported.

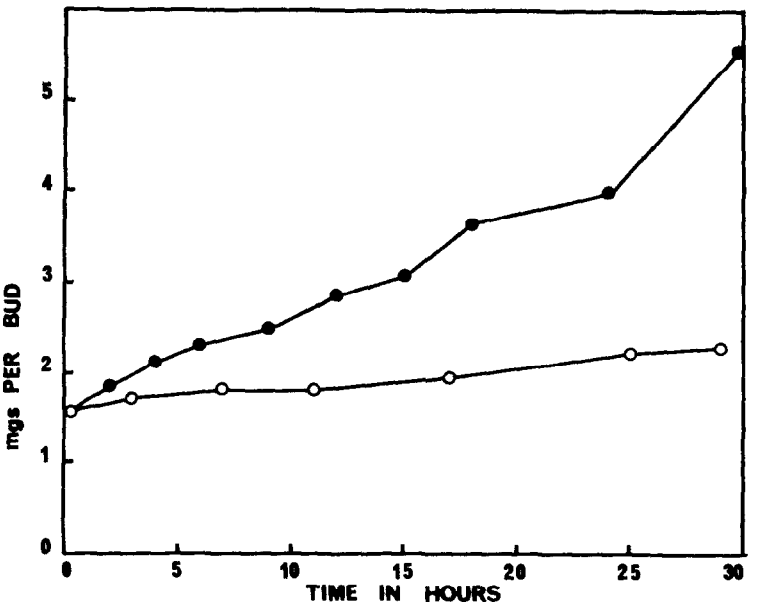

FIG. 5. THE GROWTH OF TERMINAL BUDS OF 6-day DARK-GROWN SEEDLINGS WHEN PLACED IN CONTINUOUS BLUE LIGHT (SOLID SYMBOLS) OR MAINTAINED IN DARKNESS (OPEN SYMBOLS).

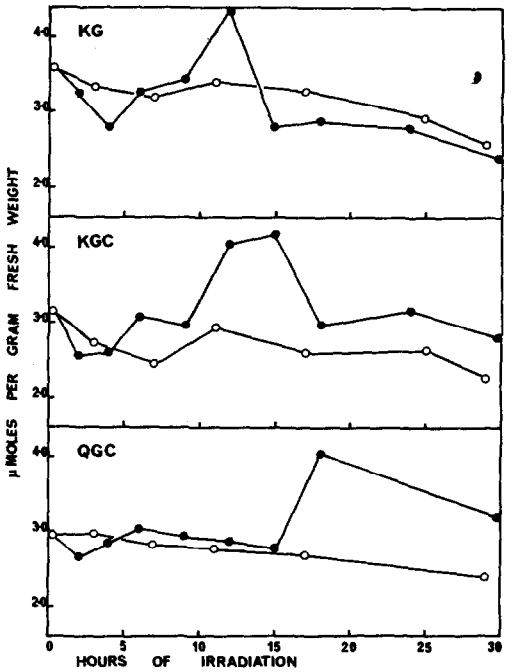

FIG. 6. CHANGES IN THE TOTAL AMOUNTS OF THE FLAVONOIDS IN TERMINAL BUDS OF 6-day DARK-GROWN SEEDLINGS UPON TRANSFERENCE TO CONTINUOUS BLUE LIGHT (SOLID SYMBOLS), OR MAINTENANCE IN DARKNESS (OPEN SYMBOLS).

The biosynthetic relationships of kaempferol, quercetin, their glycosides and their acylated glycosides are far from clear, but the scheme given in Fig. 7 is rational and fits in with recent hypotheses.[15] The possibility of interconversion of kaempferol and quercetin (and possibly of KG and QG, and of KGC and QGC) cannot be ruled out, and these interconversions are indicated by dotted arrows. The differences in kinetics of KG, KGC and QGC changes in relation to phytochrome photoactivation make it extremely difficult to envisage a single point of action of phytochrome in the biosynthesis of the flavonoids. (The question of the locus of phytochrome action in the biosynthetic pathway is discussed in a succeeding paper.[16])

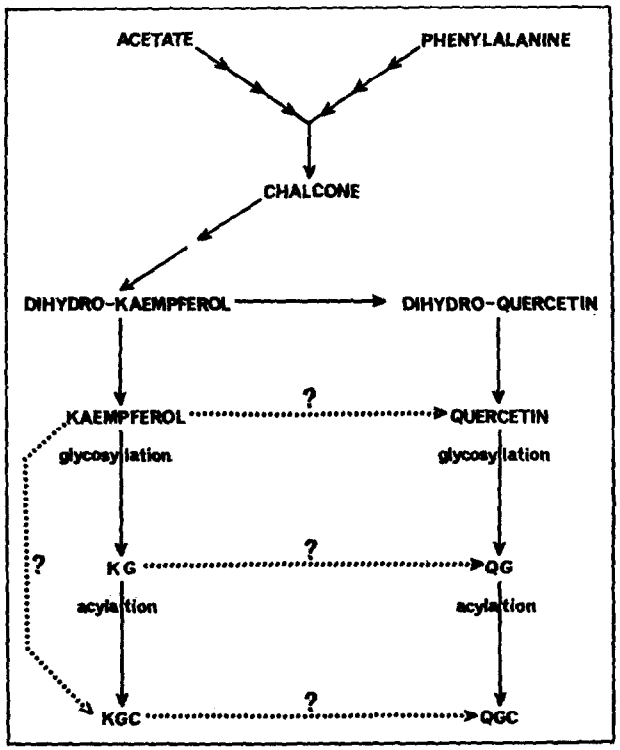

FIG. 7. THE PROBABLE PATHWAYS OF FLAVONOID SYNTHESIS, GLYCOSYLATION, AND ACYLATION.
The solid arrows represent currently accepted pathways, whilst the dotted lines indicate possible variations suggested by the results reported.

When the pea tissues are placed under continuous white fluorescent, or blue, light the changes in flavonoid complement are considerably more complex than after short red-light treatment. A major generalization, however, is that continuous illumination leads to large increases in KGC and QGC concentration, but that these increases do not occur until 12–20 hr after the beginning of irradiation. Under white light, small early increases in both KGC and QGC concentration occur, but these are almost identical, in both quantity and timing, with the responses due to short red light. Thus these early responses are probably due to phytochrome photoactivation (the white fluorescent source and the red light source both

[15] L. PATSCHKE and H. GRISEBACH, *Phytochem.* 7, 235 (1968).
[16] D. B. HARPER, D. J. AUSTIN and H. SMITH, *Phytochem.* 9, 497 (1970).

established the same proportion of phytochrome as Pfr, i.e. 80 per cent[17]). These early rises were not apparent under the blue illumination (the blue source established only 25 per cent Pfr).

The detailed differences in response to continuous blue and white light are difficult to rationalize, although it seems likely that white-light responses are an amalgam of the phytochrome mediated changes, and other changes that possibly involve a blue-absorbing photoreceptor. To resolve this problem it would be necessary to construct action spectra for the kinetic changes in each compound over a wide range of wavelengths and energies. Other workers, however, have concluded that blue-light-absorbing photoreceptors, other than phytochrome, must play a role in the continuous illumination effects on phenolic biosynthesis.[18]

On the biosynthetic problem, the continuous illumination experiments are consistent with a conversion of KGC to QGC, and, possibly, a conversion of KG to KGC. Thus in Fig. 4, the sudden increase in QGC concentration at 16–18 hr is paralleled by an almost equivalent decrease in KGC concentration. Furthermore, in Fig. 6, peaks of KG, KGC and QGC concentration of similar magnitude follow each other in an apparent conversion series. The position of KG, however, is somewhat anomalous. Under all treatments except continuous blue light, the concentration of KG decreases slowly in the dark and much more rapidly in the light. If the total KG per bud is determined, however, it is seen that this remains virtually constant (Figs. 2 and 4). Thus, it appears that KG is not being synthesized at rates sufficient to maintain steady-state concentration within each cell.

Finally, the point should be made that, with the exception of KG, the intracellular concentration of the flavonoids appears to return very nearly to the dark levels after about 30 hr of continuous illumination with either blue or white light. Thus, even in continuous illumination, it appears that the changes in flavonoid concentration are only transient. It is possible, furthermore, that the decrease in KG concentration may be counterbalanced by the appearance of QG, which was seen to develop between 18 and 30 hr.

One may conclude, therefore, that irradiation of dark-grown pea tissues with continuous white light brings into action at least two photoresponsive systems: (*a*) the phytochrome system, which mediates the early changes and (*b*) a further longer term system which, since it is evident under blue light, may involve a blue-absorbing photoreceptor. The transient nature of all the changes indicates that flavonoid synthesis is normally under relatively tight control, both in dark-grown and light-grown tissues, and changing from one set of conditions to another presumably temporarily releases these control mechanisms, leading to the observed perturbations in flavonoid complement.

It has been suggested that the effects of light on bud growth rate may be mediated through the change in flavonoid pattern by the effects of KGC and QGC on the enzymatic oxidation of IAA.[19] The results of this investigation do not support this hypothesis since it has not been possible to arrive at any significant correlations between flavonoid concentrations or flavonoid ratios and growth rate.

EXPERIMENTAL

Plant Materials and Culture Conditions

Seeds of *Pisum sativum* var. Alaska were obtained from Carters Seeds Ltd., Wimbledon, England, and soaked in running tap-water for 24 hr before sowing in vermiculite in seed trays. The seedlings were grown in a dark constant-temperature room at 25 ± 1° for 6 days before experimentation.

[17] H. SMITH and T. H. ATTRIDGE, *Phytochem.* **9**, 487 (1970).
[18] G. ENGELSMA, *Planta* **75**, 207 (1967).
[19] M. FURUYA, A. W. GALSTON and B. B. STOWE, *Nature* **193**, 456 (1962).

Light Sources and Treatments

Red light source. 3 × 18 in., 15 W Atlas Double-Life red fluorescent tubes filtered through one layer of Cinemoid (Strand Electrics, London) No. 1 Yellow and one layer of No. 14 Ruby. Cut-off at 600 nm and 700 nm and with an intensity of 700 erg cm^{-2} sec^{-1}. Plants were given 15 min irradiation with this source.

Blue light source. 6 × 40 W tropical daylight fluorescent bulbs filtered through 10 cm of running water and one layer of No. 20 Deep Blue (Primary) Cinemoid. 90% of the radiant energy was between 400 and 515 nm; incident energy at plant height was 250 erg cm^{-2} sec^{-1}.

White light source. 5 × 40 W daylight fluorescent bulbs, with an intensity at plant height of *ca.* 5000 erg cm^{-2} sec^{-1}.

Green safe-light. 1 × 8 W daylight fluorescent tube filtered through three layers of No. 39 Primary Green Cinemoid.

Extraction of the Flavonoids

For each determination, 100–200 apical buds were excised at the point of insertion of the first true leaf (i.e. the third node) and extracted by the method previously reported.[14] The separation of the flavonoids was also performed by the thin-layer chromatographic method reported previously.[14] The flavonoids were quantitized spectrophotometrically on the basis of extinction coefficients determined from crystalline extracted samples: QGC, log E_{315} in absolute ethanol, 4·3765; KGC, log E_{315} in absolute ethanol, 4·4689; KG, log E_{350} in water, 4·1059.

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