Phytochemistry, 1967, Vol. 6, pp. 791 to 797. Pergamon Press Ltd. Printed in England

FLAVONOID COMPLEXES IN *PISUM SATIVUM*—IV.

THE EFFECT OF RED LIGHT ON SYNTHESIS OF KAEMPFEROL COMPLEXES AND ON GROWTH IN SUB-APICAL INTERNODE TISSUES*

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(*Received* 27 *September* 1966)

Abstract—Internode tissues of dark-grown peas contain kaempferol-3-triglucoside (KG) as the major flavonoid and smaller amounts of 3-*p*-coumaroyltriglucosyl-kaempferol (KGC). Tissues harvested 24 hr after irradiation with weak red light showed a 200–300 per cent increase in KG, and also increases in KGC. No quercetin compounds were detectable. The increases in kaempferol compounds were reversed by far-red light treatment given immediately after red irradiation. Kinetic studies showed that the first detectable effect of red light on growth preceded significant red-light-induced increases in both kaempferol complexes.

INTRODUCTION

IN THE apical buds of dark-grown peas (*Pisum sativum* cv. *Alaska*) 3-*p*-coumaroyltriglucosyl-kaempferol (KGC) is the major flavonoid present; kaempferol-3-triglucoside (KG) is also found in these tissues.[1] However, in high-intensity light considerable amounts of the comparable quercetin derivatives QGC and QG are present in addition.[1]

Brief irradiation with weak red light induces an increase in the synthesis of flavonoids[2] and specifically in the synthesis of QGC.[3] These are reversible by far-red light. The changes in QGC appear to parallel or precede light-induced promotion of bud growth.[4]

By contrast weak red light inhibits internode growth in dark-grown seedlings of beans[5] and peas.[6] We have examined sub-apical internode tissues of the dwarf pea (cv. Progress No. 9) for flavonoids and, using the techniques of Bottomley *et al.*,[4] were unable to detect any quercetin compounds in extracts from more than 3·0 g (fresh weight) of either dark-grown or red-light-treated tissues. However, two kaempferol complexes occurred and were identified as KG and KGC. The concentration of KG was much higher than that of KGC. Red light increased the synthesis of both compounds and the effects were reversible by far-red light.

The magnitude and time relations of the light-induced concentration changes have been nvestigated and compared with the light-induced inhibition of internode elongation to determine whether any causal relationship may be considered to exist between the two.

* This work represents a part of a dissertation presented for the Ph.D. degree in Yale University by D. W. R.

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[1] M. FURUYA, Ph.D. Thesis, Yale University (1962).
[2] M. FURUYA and R. G. THOMAS, *Plant Physiol.* **39**, 634 (1964).
[3] W. BOTTOMLEY, H. SMITH and A. W. GALSTON, *Nature* **207**, 1311 (1965).
[4] W. BOTTOMLEY, H. SMITH and A. W. GALSTON, *Phytochem.* **5**, 117 (1966).
[5] R. J. DOWNS, *Plant Physiol.* **30**, 468 (1955).
[6] M. W. PARKER, S. B. HENDRICKS, H. A. BORTHWICK and F. W. WENT, *Am. J. Botany* **36**, 194 (1949).

RESULTS

The effect of red light on the levels of flavonoids 24 hr after treatment is shown in Fig. 1. It is evident that red light induced a 250 per cent increase in the concentration of KG and an increase of about 700 per cent in KGC. However, the absolute concentrations of KGC are comparatively low even in red-light-treated tissue and only trace amounts are present in control tissues. The effects of red light on the synthesis of both compounds are reversible with far-red light.

Red light treatment induced an inhibition of about 50 per cent in elongation of similar tissues measured 24 hr after light treatment. The effect was reversible with far-red light (Fig. 2).

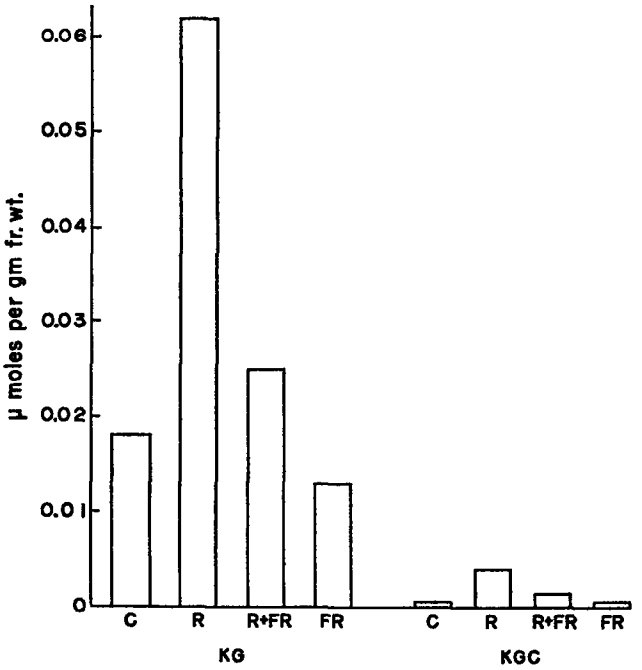

FIG. 1. THE EFFECTS OF RED AND FAR-RED LIGHT ON THE LEVELS OF KG AND KGC IN INTERNODE TISSUES DERIVED FROM 5 MM SUBAPICAL SEGMENTS OF INTACT ETIOLATED PROGRESS SEEDLINGS. THE SEGMENTS WERE MARKED OFF, TREATED AT ZERO TIME AND HARVESTED 24 HR LATER.

C=control; R=red; FR=far red.

Since there is a 50 per cent inhibition of growth and an increase of more than 200 per cent in flavonoid concentrations, the concentration increase cannot be due to a relative decrease in concentration in the controls through dilution by elongation growth.

Time course studies were carried out over the first 12 hr after irradiation to determine the relationship of the flavonoid increases to the growth response. Changes in absolute concentrations of the flavonoids (Fig. 3) show that in both red-light-treated and control tissues KG concentration decreased with time and with distance down the stem, since the distance of the marked internode segment from the apex increased with time. However, comparative increases occurred in red-light-treated tissues from about the third hour and thereafter. Although the relative increase in KG is linear from the third hour, the increase over the control at 3 hr after red light treatment is less than 10 per cent.

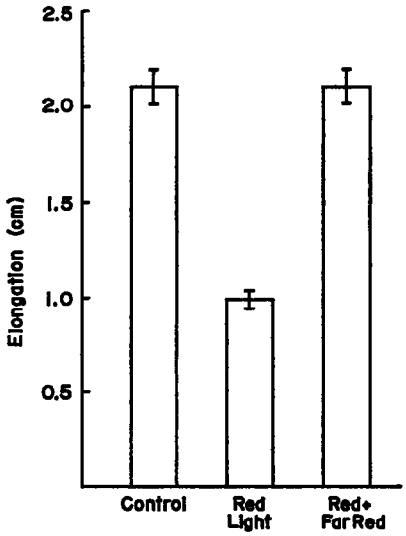

FIG. 2. THE EFFECT OF RED AND FAR-RED LIGHT ON GROWTH OF INTACT 10 MM SEGMENTS OF INTERNODES OF ETIOLATED PROGRESS SEEDLINGS. SMALL LINES ATOP BARS INDICATE STANDARD ERRORS.

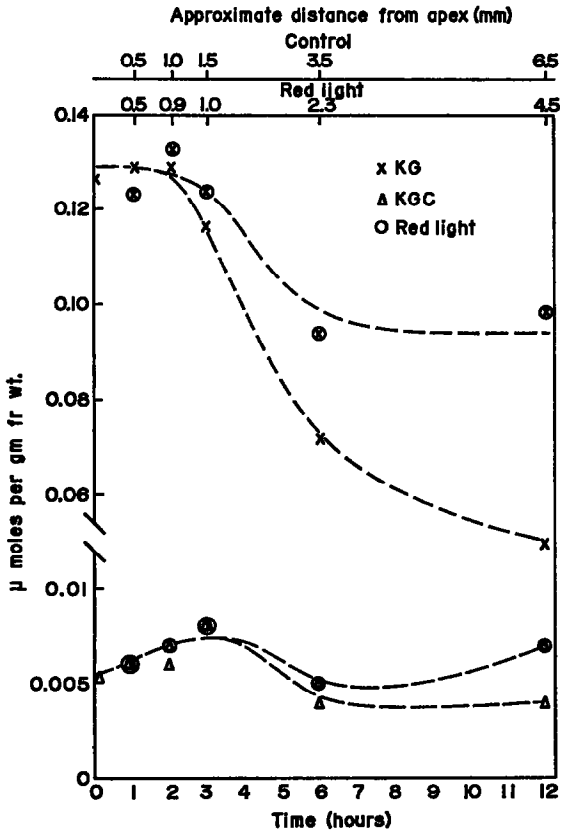

FIG. 3. THE LEVELS OF KG AND KGC IN INTERNODE TISSUES DERIVED FROM 10 MM SUBAPICAL SEGMENTS OF INTACT ETIOLATED PROGRESS SEEDLINGS. THE SEGMENTS WERE MARKED OFF, TREATED AT ZERO TIME AND HARVESTED AFTER VARIOUS TIME INTERVALS.

While the percentage change in KGC were large, the absolute levels (Fig. 3) in both controls and red-light-treated tissues were very low compared with those of KG. It is evident that decreases in KG with time and distance down the stem do not result in concomitant rises in KGC, nor does red light induce a decrease in KG with a corresponding rise in KGC as has been suggested for other tissues.[7]

The decrease in KG concentration down the stem from regions subjacent to the apex appears to indicate that the glycoside is present in higher concentrations in regions of greater metabolic activity and declines with increase in age and decrease in metabolic activity.

The kinetics of elongation growth (Fig. 4) show that there is no light effect on internode elongation 1 hr after red light treatment. However some inhibition is detectable at 2 hr, followed by comparatively severe inhibition between hour two and three, and partial recovery after hour three to a new steady growth rate about 50 per cent of the controls.

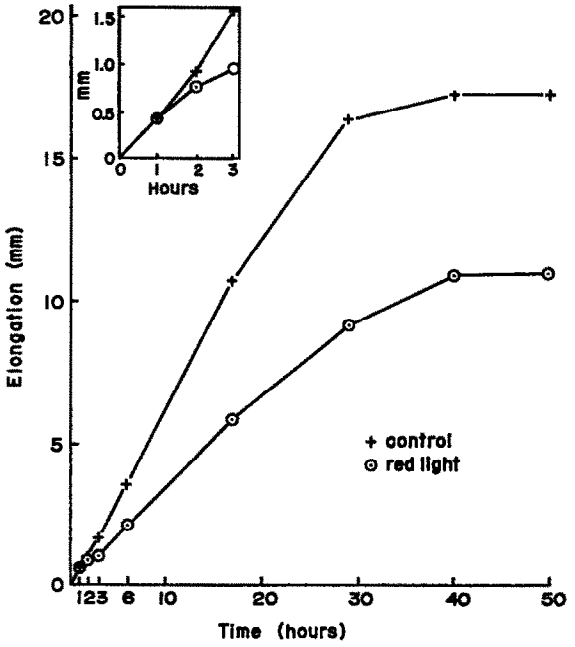

FIG. 4. KINETICS OF GROWTH IN 10 MM SUBAPICAL SEGMENTS OF INTACT INTERNODES OF ETIOLATED PROGRESS SEEDLINGS.

The time at which light inhibition of growth commences, as indicated by the growth kinetics, is corroborated by the time course for failure of far-red reversal of the red light effect (Fig. 5). There was an initial rapid decline in the ability of far-red light to reverse the red light inhibition followed by a slower and linear decline with time. It is apparent that reversal of the pigment after 1 hr or less does not prevent some expression of the red light effect on growth. The break in the curve at 2–3 hr may be interpreted as indicating that pigment conversion has been translated into metabolically and physiologically active intermediates by this time, while the slower decline in reversibility after 3 hr may indicate that continued presence of the active form of the pigment is necessary for maintenance of steady state levels of some causal agent.

[7] F. E. MUMFORD, D. H. SMITH and P. G. HEYTLER, *Biochem. J.* **91**, 517 (1964).

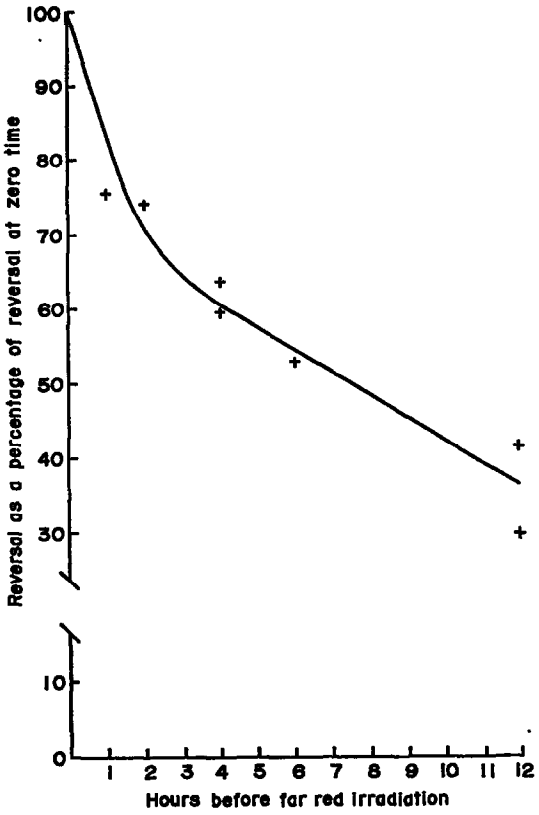

FIG. 5. KINETICS OF REVERSIBILITY OF THE RED LIGHT INHIBITION BY FAR-RED LIGHT IN INTACT 10 MM SUBAPICAL INTERNODE SECTIONS OF ETIOLATED PROGRESS SEEDLINGS.

DISCUSSION

It is evident that changes in kaempferol complexes occur shortly after light treatment in pea internode tissues. This raises two major questions: (i) what is the primary point of action of red light in intermediary metabolism, i.e. after photon absorption and pigment activation; (ii) are the changes in kaempferol complexes in any way related to the growth response and, if not, what is the biochemical basis of the physiological response?

With regard to the first question it has been pointed out by others[8,9] that the synthesis of a number of compounds is affected by red light irradiation including carotenoids, chlorophyll, and probably fatty acids in addition to flavonoids. To reduce these apparently diverse effects of light to some semblance of uniformity a common point of action in acetate metabolism has been proposed. However, it is difficult to envisage how specific stimulation of activity of enzymes concerned in acetate incorporation could account for these effects since the compounds fall into two groups according to whether synthesis occurs via malonyl-CoA or the mevalonate pathway. The problem of this apparent duality may be avoided by assuming stimulation of acetate activation or even an earlier step. Though such an assumption may appear to sacrifice a degree of the apparent specificity of the light response as judged by known

[8] L. G. PALEG, *Ann. Rev. Plant Physiol.* 16, 291 (1965).
[9] D. VINCE, *Biol. Rev.* 39, 506 (1964).

metabolic effects, this need not be so if, for example, specific isoenzymes mediate acetate activation for the different pathways of acetate incorporation. A stimulation at the level of acetate incorporation could imply an action in regulation of input to the two pathways, though the means by which this might be achieved are difficult to envisage.

The effects on kaempferol complexes obtained here show that identical irradiation régimes in different organs of the same plant lead to different biochemical consequences. Thus in the apical bud of etiolated peas transformation of phytochrome from P_r to P_{fr} leads to the *de novo* appearance of a flavonoid (quercetin) with an extra hydroxyl group.[4] This suggests that phytochrome activation might lead to changes in a hydroxylation system and results with sorghum[10] and gherkin[11] support this. It is not intended to imply that this is a direct effect but may well result from phytochrome-stimulation of some earlier step in the biosynthetic pathway. By contrast, in the stem tissue of the etiolated pea, phytochrome transformation fails to lead to quercetin production, and causes instead an enhanced synthesis of an already existing substance, kaempferol. The data do not support the proposed transformation of KG to KGC as suggested to occur in the apical buds of etiolated peas on irradiation.[7]

Previous workers have shown that in pea *apices* the concentration of KGC is not influenced by red light though at the same time QGC concentration increases.[4] The present data show that the concentration of kaempferol complexes in internode tissues does change with red light treatment. However, KGC levels are much higher in apices than in the internode tissues examined here: thus it is possible that QGC may be synthesised from KGC when the latter reaches a given concentration. This might account for their conclusion that the synthesis of KGC was not affected by light. In support of this there is evidence that quercetin is synthesised by hydroxylation of kaempferol[12].

It has been suggested[13] that since actinomycin-D inhibits phytochrome-mediated responses the activated pigment acts at the DNA level. However in the absence of conclusive evidence it is not necessary to postulate action at this level; biochemical amplification of the light signal very probably involves the synthesis of enzyme protein but this need not depend upon the direct intervention of phytochrome. If the activated pigment stimulates a particular reaction in intermediary metabolism, perhaps a rate limiting one, it is likely that this would cause an increased synthesis of enzymes mediating subsequent steps in the pathway. Inhibition of such synthesis by actinomycin-D would thus prevent expression of the red light effect.

The question of the possible causal effects of flavonoids in growth responses of the apical bud to light have also been examined.[4] Since the increases in QGC paralleled or preceded the effect on promotion of bud growth a causal relationship may be considered; it is noteworthy also that QGC acts as an inhibitor of peroxidative indolyl-3-acetic acid (IAA) destruction *in vitro*. It was interesting to find that no detectable quercetin compounds were present in internode tissues though light induced an increase in kaempferol complexes which are known to act as cofactors in *in vitro* peroxidative destruction of IAA. Thus an increase in kaempferol level might indirectly inhibit growth by promoting IAA destruction in auxin-dependent stem tissue. However the kinetic studies of growth inhibition and relative increase in KG concentration showed that changes in KG commenced at 3 hr, and even at this stage the increase was less than 10 per cent. On the other hand growth inhibition was detected at 2 hr and was very marked between hour two and hour three. It is doubtful, therefore, that

[10] H. A. STAFFORD, *Plant Physiol.* **40**, 130 (1965).
[11] G. ENGELSMA, *Nature* **208**, 1117 (1965).
[12] W. E. HILLIS and K. ISOI, *Phytochem.* **4**, 905 (1965).
[13] H. MOHR and H. LANGE, *Naturwiss.* **10**, 261 (1965).

the altered growth rate of the stem tissue can be ascribed to changes in KG, unless our analytical methods are inadequate for establishing small but meaningful differences. On the basis of the growth kinetics it might be expected that increased activity of a causal mechanism should occur between 1 and 2 hr after irradiation and that either (i) the level of the causal agent should reach a peak between 2 and 3 hr, followed by a decline to a higher than original steady state level. This would be accompanied by a corresponding rapid decline followed by a partial recovery to a relatively steady level in some growth factor such as IAA; or (ii) an abrupt increase in the causal agent may cause a temporary severe limitation of some growth-promoting factor between 2 and 3 hr followed by a recovery to a new steady growth factor level after hour three.

On the basis of the results presented it is suggested that the kaempferol complexes have no primary effect on growth. But since the responses occur very shortly after the first effects on growth some earlier intermediate in the flavonoid biosynthetic pathway may be causally related to the growth response.

EXPERIMENTAL

Seeds of *Pisum sativum* cv. Progress No. 9, were soaked in tap water with fungicide (Phygon, 1 tsp per lb of seed) for about 5 hr and sown in moist vermiculite in (10 × 10 × 10) cm^3 polyethylene containers. These were placed in flats in a darkroom at 27° ± 1° and used on the seventh day when third internodes were present. Plants to be used were selected for third internodes 25–35 mm long.

Sub-apical internode segments 10 mm long and 1·0 mm below the lower side of the apical hook were marked off with a mixture of powdered charcoal and lanolin, applied by means of two fine needles set 10 mm apart in a cork block and coated with the mixture. A third needle projecting to a lesser extent at a distance of 1·0 mm from the two marker needles enabled precise marking of the segments 1 mm below the lower side of the hook. Measurements were made at zero time and at the required time intervals by means of a rule in 0·5 mm scale divisions with which it was possible to measure reproducibly to the nearest 0·1 mm with an estimated error of ±0·075 mm.

The light sources and their spectral distribution have already been described.[2] Plants were irradiated for 10 min with red light at an intensity of 330 ergs cm^{-2} sec^{-1}. This was the optimum energy for inhibition as determined by dose response experiments. In reversal experiments, plants were irradiated with far-red light immediately after red light treatment unless otherwise stated. Exposure times were 12–13 min at an intensity of *ca.* 1000 ergs cm^{-2} sec^{-1}, thus giving about four times as much energy as used for the red light treatment.

Flavonoids were extracted from fresh or frozen tissue by homogenizing in 1-butanol followed by heating on a boiling water bath for 10 min. After centrifuging and washing the debris the combined supernatants were extracted 3 × with ½ vol. of 0·1 N NH_4OH. The aqueous solution was acidified and extracted with ether 3 ×, the ether extract being discarded. Flavonoids were extracted from the aqueous solution with butanol (3 × ½ vol.). The butanolic extract was evaporated to dryness on a Buchi evaporator and taken up in 0·2–0·3 ml of water and aliquots applied to 7-mm discs of chromatography paper which were inserted into slits at the origin of large sheets of chromatography paper (Whatman No. 1, 18 in. × 18 in.) prewashed in both solvents. Development was carried out in the first direction with butanol: acetic acid: water, (12:3:5 v/v) for about 10 hr and in the second direction with 2% (v/v) acetic acid for about 2 hr. Kaempferol complexes were located under u.v. light as dark spots. These were cut out and eluted in 2 or 3 ml of water depending on spot size, and the spectra of the eluates recorded with a Perkin Elmer model 350 recording spectrophotometer. Concentrations were calculated from known extinction coefficients of 3·0 × 10^4 at 316 nm (λ_{max}) for KGC and 2·9 × 10^4 at 266 nm (λ_{max}) for KG.

The u.v. spectrum of suspected KGC from internode tissues of dark-grown and red-light-treated Progress seedlings was identical with the u.v. spectrum of purified KGC from light-grown Alaska seedlings. Purified KGC was hydrolysed in 1 N KOH for about ¾ hr, the *p*-coumaric acid extracted with ether after acidification, and the KG then extracted with butanol. The u.v. spectrum of the KG thus obtained was identical with the spectrum of the suspected KG in extracts of etiolated tissues: the two also showed identical chromatographic behaviour on paper on development with butanol: acetic acid: water (10:2:3 v/v), propanol: water (10:3 v/v), butanol saturated with water, and water alone.