Phytochemistry, 1970, Vol. 9, pp. 763 to 773. Pergamon Press. Printed in England.

PHOTOCONTROL OF C-GLYCOSYLFLAVONES IN BARLEY SEEDLINGS*

JERRY W. MCCLURE and K. G. WILSON

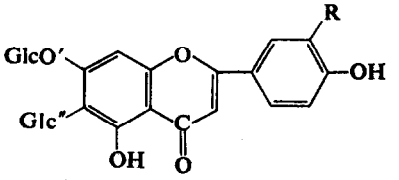
Department of Botany, Miami University, Oxford, Ohio 45056, U.S.A.

(*Received* 29 *August* 1969)

Abstract—The sole flavonoid of etiolated barley plumules is saponarin, a C-glycosylflavone restricted to the primary leaves. Increased saponarin synthesis is under a typical low-energy phytochrome control in both etiolated plumules and in those grown in white light. Prolonged illumination with white light, or high levels of blue light followed by red light, greatly increases saponarin and initiates the synthesis of lutonarin; this response is characteristic of high-energy photoresponses (HER). Kinetic studies show a typical lag of about 4 hr for phytochrome control of saponarin in etiolated plumules. Surprisingly, during this lag-phase and within 2 min following a red-light (132 Kerg cm^{-2}) treatment, there is a 25 per cent decrease in saponarin recovered from whole plumules. Within 1 hr the light-treated plants yield only about half as much saponarin as do the dark controls. After 1 hr apparent saponarin content increases and by the fourth hour the flavonoid content of the light-treated and dark-control plants is similar. The fate of this "lost" saponarin is not known; it may be in part translocated into other parts of the seedling as the apical 1 cm of the plumule shows a more rapid recovery under these conditions, a part may be complexed into forms not recoverable by our techniques. Mediatory aspects of changes in saponarin localization within the tissues are considered.

INTRODUCTION

THE C-GLYCOSYLFLAVONES saponarin (R = H), lutonarin (R = OH), and lutonarin 3′-methyl ether (R = OCH_3) are the major flavonoids of barley leaves.[1-3]



(I)

Many lines of evidence[4,5] support the contention that *O*-glycosylation is a late or terminal biosynthetic step but that C-glycosylation takes place at an earlier stage. For example, Wallace *et al.*[6] cultured various Lemnaceae in media containing ^{14}C-flavones and followed their incorporation into various flavone *O*- and C-glycosides. The ^{14}C-flavone aglycones were *in vivo* *O*-glucosylated, *O*-methylated and 3′-hydroxylated, but not C-glucosylated. When

* Part I in a projected series "Phenolic Biosynthesis in Barley Seedlings".

[1] M. K. SEIKEL and T. A. GEISSMAN, *Arch. Biochem. Biophys.* **71**, 17 (1957).

[2] M. K. SEIKEL and A. J. BUSHNELL, *J. Organic Chem.* **24**, 1995 (1959).

[3] M. K. SEIKEL, A. J. BUSHNELL and R. BIRZGALIS, *Arch. Biochem. Biophys.* **99**, 451 (1962).

[4] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, Academic Press, New York (1967).

[5] H. GRISEBACH, in *Recent Advances in Phytochemistry* (edited by T. J. MABRY, R. E. ALSTON and V. C. RUNECKLES), p. 379, Appleton-Century-Crofts, New York (1968).

[6] J. W. WALLACE, T. J. MABRY and R. E. ALSTON, *Phytochem.* **8**, 95 (1969).

labeled C-glucosylflavones were added, they too were *O*-glycosylated but they were not further C-glycosylated into the di-C-glycosylflavones, nor is it likely that they were 3'-hydroxylated or interconverted into their isomeric forms.[6] Thus the biosynthesis of C-glycosylflavones may parallel that of the *O*-glycosides with the pathways diverging at some early point.

It is well known that the production of flavonoids is under close genetic and environmental regulation.[4,5,7] And just as the genetic controls work in a sequential fashion, it is now apparent that the environmental controls do so as well. The most important regulatory influence of the environment seems to be light[4,7] and specific photocontrolled steps such as the availability of flavonoid precursors,[8] control of B-ring substitution patterns[9] and establishment of the anthocyanin oxidation level,[10] can often be separated from general effects on growth and phenolic biosynthesis.

Photocontrol of several classes of *O*-glycosides is well established yet light is known to influence the pattern of C-glycosylflavones only in several species of Lemnaceae.[10,11] While *Spirodela* and other members of the Lemnaceae are excellent experimental plants for incorporation studies,[6] it is necessary to culture them for several days or weeks in order to obtain sufficient plant material for analysis. By this time the cultures contain plants of mixed ages and physiological states and this complicates the interpretation of the results.[10] It is also difficult to study the effects of light on etiolated Lemnaceae as they grow in the dark only on media containing growth factors such as kinetin and the growth factors themselves may alter flavonoid biosynthesis.[12] Perhaps it is for these reasons that it has not been possible to demonstrate clear-cut photocontrols of C-glycosylflavones in *Spirodela*,[10,11] or to clearly separate the effects of light on flavonoid synthesis from its effects on growth.[12]

If the *O*- and C-glycosides are synthesized by parallel biosynthetic pathways,[6] this raises the question of similarities or differences in the manner in which the plants control the production of these two classes of glycosides. Seikel and Geissman[1] have shown that saponarin production is increased by culturing Atlas 46 barley seedlings in the light. The present study shows that the production of increased amounts of saponarin in young barley is controlled by the low-energy phytochrome system while traces of lutonarin are found only in those plants given high levels of white light, or blue followed by red light.

RESULTS

Special Problems in Estimating the Production of Barley C-Glycosylflavones

The flavonoids were extracted from the tissues in acidified methanol, separated by two-dimensional chromatography, eluted and measured spectrophotometrically. When varying amounts of saponarin were added to the plant material before extraction, or chromatographed alone, about 44·5 per cent was recovered. When applied directly to paper elipses (see Experimental) and these air-dried and then eluted, about 82·7 per cent was recovered. Thus a considerable loss seems to take place before elution although there are no visible indications of decomposition on the chromatograms and these compounds are well resolved.

[7] S. B. HENDRICKS and H. A. BORTHWICK, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 405, Academic Press, New York (1965).
[8] H. SCHERF and M. H. ZENK, *Z. Pflanzenphysiol.* **57**, 401 (1967).
[9] W. BOTTOMLEY, H. SMITH and A. W. GALSTON, *Nature* **207**, 1311 (1965).
[10] J. W. MCCLURE, *Plant Physiol.* **43**, 193 (1968).
[11] M. FURUYA and K. V. THIMANN, *Arch. Biochem. Biophys.* **108**, 109 (1964).
[12] A. M. NORMAN, M.A. Thesis, Miami University (1968).

These flavonoids are extremely difficult, almost impossible, to crystallize[1-3] and they are unstable in air at room temperature in anything but acidic environments, decomposing into uncharacterized bright-yellow compounds.[3] For these reasons it has not been possible to determine a reliable molecular extinction coefficient for saponarin (the only flavonoid consistently found in sufficient quantity for estimation) and we have reported its concentration as absorbance of the chromatographic eluates at 333 nm (λ_{max} saponarin) per gram of tissue. Other C-glycosylflavonoids have been crystallized, however and the value ϵ $1{\cdot}750 \times 10^4$ was determined for vitexin and isovitexin.[6,10] Saponarin is a 7-*O*-glucoside of isovitexin[1] and the 7-substitution should have little effect on the absorptivity of the long-wave band.[13] Considering all of the foregoing it is likely that an absorbance of 1·0 from the chromatographic eluates is equal to about 0·641 μM of saponarin per g of fresh tissue.

While these are low recovery values, most experiments were done in at least triplicate and the statistical analyses indicatet hat absorptivity at 333 nm per g of fresh tissue is a reliable indication of the saponarin content of the plants. For example, an analysis of variance of all replicated experiments in this paper shows an error mean square of 0·0201 where $n = 206$.

Flavonoids of Etiolated Barley Seedlings

When grown for 4 days in the dark at 23° the total plumule of Atlas 46 barley contains a small amount of saponarin (Table 1). We have found no other flavonoids in dark-grown seedlings. Furthermore, this flavonoid is not detectable in the roots, seed coat and endosperm, or in the coleoptile. Saponarin is apparently restricted to the primary leaves of young barley seedlings and the highest concentration is found in the apical centimeter (Table 1).

TABLE 1. THE DISTRIBUTION OF SAPONARIN IN 4-day-OLD ETIOLATED ATLAS 46 BARLEY SEEDLINGS

Tissue	Saponarin content
Primary leaves	0·86
Coleoptile	N.D.
Roots	N.D.
Endosperm and seed coats	N.D.
Whole plumule	0·36
(Plumule segments)	
apex–1 cm from apex	0·78
1–2 cm from apex	0·26
2–3 cm from apex	0·27
3–4 cm from apex	0·09

At this stage the plumules (primary leaves enclosed within the coleoptyle) are about 4 cm long. The plants were separated and the tissues homogenized under a dim green safelight. N.D. = not detected. Saponarin expressed as absorbance at 333 nm per g fresh tissue. $S^2_{\bar{x}} = 0{\cdot}0034$ ($n = 3$).

Response of Barley Plumules to Low-Energy Red and Far-Red Light

Four-day-old, dark-grown seedlings were exposed to red light for 30 sec to 16 min and returned to the dark for 24 hr. Other plants were given 4 min of red light and then far-red light

[13] B. SKARZNSKI, *Biochem. Z.* **301**, 150 (1939).

for 2 to 16 min. Still others were given only the far-red light. Using similar filters and light sources, Hillman[14] determined that red light establishes about 90 per cent active phytochrome (P*fr*) and far-red light, when following red light or given alone, results in about 5–10 per cent P*fr*. From Fig. 1 it is apparent that increased saponarin synthesis is a typical low-energy phytochrome response.[15]

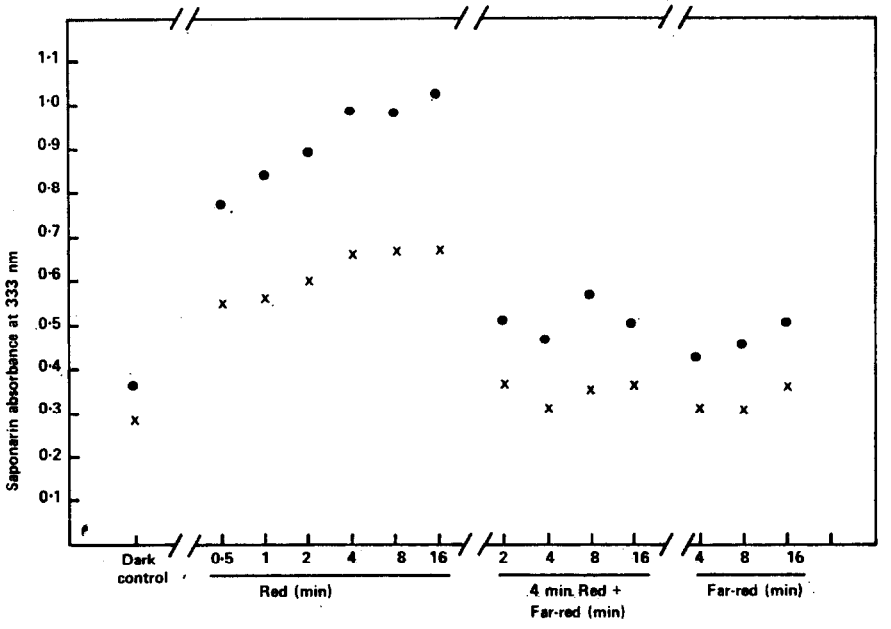


FIG. 1. SAPONARIN PRODUCTION IN ATLAS 46 BARLEY IN RESPONSE TO VARYING AMOUNTS OF RED, RED + FAR-RED, AND FAR-RED LIGHT.

●, per g fr. wt.; ×, per 10 plumule sample. Etiolated 3-day-old plants were given light as indicated and returned to the dark for 24 hr. Saponarin was separated by two-dimensional paper chromatography of methanolic homogenates and eluted in acidified 80% methanol. $S_{\bar{x}}^2 = 0{\cdot}0072$ ($n = 3$).

TABLE 2. EFFECTS OF RED AND FAR-RED LIGHT ON SAPONARIN PRODUCTION IN ATLAS 46 BARLEY DURING EARLY STAGES OF GROWTH

Treatment	Saponarin content			
	3 days	4 days	5 days	6 days
Darkness (controls)	0·21	0·29	0·26	0·41
Red	0·63	0·94	0·97	1·14
Red + far-red	0·33	0·50	0·51	0·74

Red for 4 min, far-red for 2 min. The seedlings were returned to the dark for 24 hr before harvest. Days refer to time since planting. Saponarin as absorbance at 333 nm per g fresh tissue (whole plumules). $S_{\bar{x}}^2 = 0{\cdot}0024$ ($n = 3$).

[14] W. S. HILLMAN, *Plant Cell Physiol.* 6, 499 (1965).

[15] For recent reviews on the biochemistry and physiology of phytochrome see: (*a*) M. FURUYA in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 1, p. 347, Interscience, New York (1968); and (*b*) W. S. HILLMAN, *Annual Review of Plant Physiol.* 18, 301 (1967).

To confirm the efficacy of using 4-day-old seedlings in subsequent investigations, the red-promotion far-red-reversal characteristics for saponarin synthesis were examined in seedlings from 3 through 6 days of age. Table 2 shows that the phytochrome control of saponarin operates throughout this period and synthesis is quite rapid between the third and fourth day. The low values for the 5-day-old plants are attributable to the rapid rate of elongation between the fourth (plumules *ca.* 4 cm long) and fifth (*ca.* 7 cm long) days after planting.

Saponarin Production as a Varietal Response

Atlas 46 barley was originally chosen on the basis of previous work on its flavonoids by Seikel and her associates.[1-3] However, phytochrome responses in seed germination, etc., are often varietal characters. For this reason, six other varieties of barley were investigated. The red-light promotion of saponarin was not limited to Atlas 46 barley (Table 3).

TABLE 3. SAPONARIN FORMATION IN 4-DAY-OLD SEEDLINGS OF SEVERAL BARLEY VARIETIES IN DARKNESS AND IN RESPONSE TO RED LIGHT

Barley variety	Saponarin content	
	Darkness	Red light
Penrad	0·52	1·21
Harrison	0·25	0·99
Dayton	0·45	1·07
Atlas 46	0·37	1·10
Trophy	0·23	1·04
Dickson	0·23	0·95
Larker	0·28	0·98

Plants were given 4 min of red light 3 days after planting and harvested after an additional 24 hr in the dark. Penrad, Harrison, Dayton and Atlas 46 are winter barleys; Trophy, Dickson and Larker are spring barleys. Saponarin is expressed as absorbance at 333 nm per g fresh tissue (whole plumules). $S_x^2 = 0{\cdot}0164$ ($n = 3$).

C-Glycosylflavone Production Controlled by Phytochrome After Culture in White Light

A single saturating dose of red light given on the third day, 24 hr before harvest, will not elicit maximum saponarin synthesis in 4-day-old barley seedlings (Table 4). Also, lutonarin was detected only when the plants were given 11,000 lx of fluorescent light for 4 days.

If grown under 3300 lx of fluorescent light (*ca* 70 per cent P*fr*)[16] and placed in the dark for the last 24 hr before harvest, the amount of saponarin is reduced when compared with plants maintained in this light throughout the 4-day period. However, if the plants are given 4 min of red light immediately before they are placed in the dark, saponarin production is increased. This increase is reversible by far-red light. All of these responses following 3 days of white light are consistent with a phytochrome control based on the establishment of various percentages of P*fr* on entering the final 24-hr dark period. Furthermore, the marked difference in the amount of flavonoid in the 11,000 and the 3300 lx fluorescent light treatments suggest mediation of other light reactions, possibly through the high-energy response (HER).

[16] W. S. HILLMAN, personal communication.

TABLE 4. C-GLYCOSYLFLAVONE PRODUCTION IN 4-DAY-OLD ATLAS 46 BARLEY AFTER WHITE, OR WHITE FOLLOWED BY OTHER, LIGHT TREATMENTS

Light regime	C-Glycosylflavone content	
	Saponarin	Lutonarin
Darkness (controls)	0·40	N.D.
White, 9900 lx 4 days continuous	4·61	trace (est. 0·08)
White, 3300 lx 4 days continuous	3·73	N.D.
White, 3300 lx 3 days plus:		
24 hr dark	3·18	N.D.
4 min R, 24 hr dark	3·36	N.D.
4 min R + 2 min FR, 24 hr dark	2·84	N.D.
2 min FR, 24 hr dark	2·74	N.D.

The seedlings were grown under cool-white fluorescent lights in culture chambers in the dark room and harvested 4 days after planting. Red (R) and far-red (FR) lights were from the usual sources. C-Glycosylflavones expressed as absorbance at 333 nm (saponarin) or 349 nm (lutonarin) per g fresh plumule. $S_{\bar{x}}^2 = 0{\cdot}097$ ($n = 3$).

Blue Light and C-Glycosylflavone Synthesis

As phytochrome has an appreciable absorption in the blue portion of the spectrum,[15] treatment of the plants with a few minutes of blue light should establish a P*fr* level intermediate between that achieved with either red or far-red light.[14] However, long-term illumination with blue light often leads to HER photoreactions which usually require a subsequent treatment with red light for their effects to be fully expressed.[7] These controls were investigated for flavonoid synthesis in barley seedlings.

When the plants were given 2 min of blue light 24 hr before harvest, saponarin production was increased to a level intermediate between that found in the dark-grown control plants and those given 2 min of red light (Table 5). This is consistent with blue light establishing a 50 per cent P*fr* status.[14] Longer term irradiation with blue light, this time 20 min, increased the yield of saponarin slightly. However, when the 20 min of blue light was followed by 4 hr of darkness and then 2 min of red light, saponarin increased markedly and a trace of lutonarin was found.

TABLE 5. EFFECT OF BLUE LIGHT ON C-GLYCOSYLFLAVONE PRODUCTION IN 4-DAY-OLD ATLAS 46 BARLEY PLUMULES

Treatment	C-Glycosylflavone production	
	Saponarin	Lutonarin
Darkness (controls)	0·36	N.D.
Blue, 2 min	0·74	N.D.
Blue, 20 min	0·81	N.D.
Blue, 20 min and, after 4 hr dark, 4 min red	1·84	trace (est. 0·05)
Blue, 20 min and, after 4 hr dark, 4 min red + 2 min far-red	1·02	N.D.

The plants were returned to the dark for 24 hr before harvest. C-Glycosylflavones expressed as absorbance at 333 nm (saponarin) or 349 nm (lutonarin) per g fresh plumule. $S_{\bar{x}}^2 = 0{\cdot}0143$ ($n = 3$).

This red-promotion was reversible by far-red light. These results, along with the white-light experiments, support the involvement of the HER in increased saponarin synthesis.

It was considered that some lutonarin, below the level required for visual detection on the chromatograms, might be produced under all conditions. However, saponarin and lutonarin are well separated on the chromatograms in these solvents[2] and when the amount of tissue (10 plumules) normally processed for each determination was doubled and the chromatograms sprayed with saturated aqu. Na_2CO_3, lutonarin was found only under the aforementioned conditions.

Kinetic Studies of Saponarin Synthesis Following Red-Light Treatment

A large number of samples were given red light and harvested at various time intervals thereafter. Increased saponarin synthesis is clearly apparent after 4 hr and the trend persists for at least 32 hr (Fig. 2B). Surprisingly, for the first few hours the red-light treatment results in an apparent loss of saponarin. In fact, only after the fourth hour following the light treatments do the treated plants yield as much saponarin per g of tissue as do the dark controls (Fig. 2A).

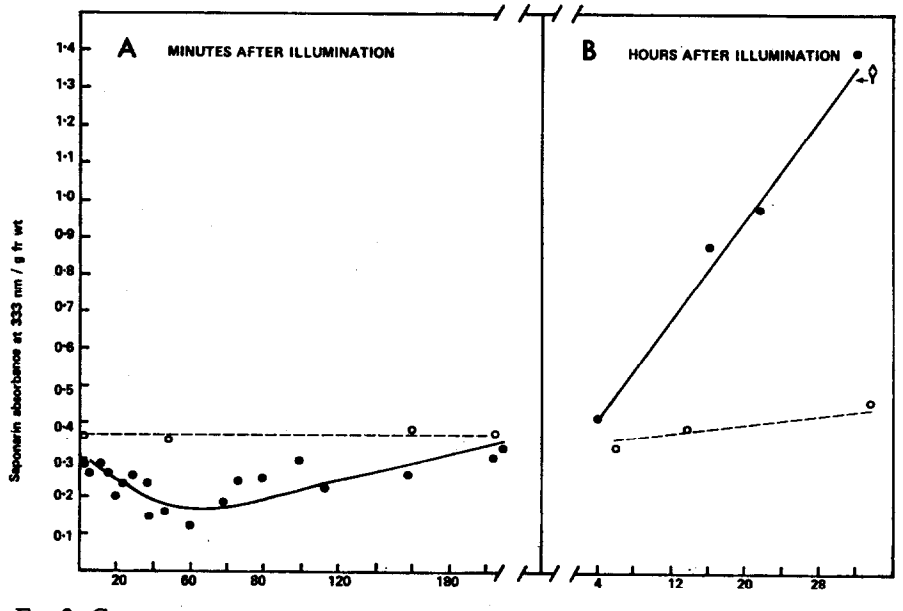


FIG. 2. CHANGES IN SAPONARIN CONTENT OF WHOLE BARLEY PLUMULES FOLLOWING RED LIGHT.

●——●, red light; o- - -o, dark controls. Intact 4-day-old seedlings of Atlas 46 barley were given 4 min of red light and returned to the dark. Minutes ($n = 1$) and hours ($n = 3$, $S_{\bar{x}}^2 = 0{\cdot}0014$) refer to the time of harvest after the light treatments.

Apparent Saponarin Loss in Apical Segments Following Red or Red + Far-Red Light

As the apical centimeter of the plumule contains much of the saponarin of the total seedling (Table 1), we examined the flavonoid content of 1 cm apical segments taken at various times after the intact plants were given 4 min of red, or 4 min of red + 2 min of far-red, light. The results are shown in Fig. 3. As before, the red-light treatment caused an almost immediate reduction in saponarin content yet the values returned more quickly to the level maintained

in the dark controls. In contrast, an unusually large and quite variable increase in saponarin was caused by following this red light by far-red light (Fig. 3).

The rapidity and magnitude of these changes suggest that a part of the saponarin is translocated, or complexed into a form no longer recoverable by our techniques, or both. It will be interesting to determine the wavelength specificity of these short-term responses in various regions of the plumule, especially as the far-red light causes an increase while the red light causes a decrease in saponarin recovered.

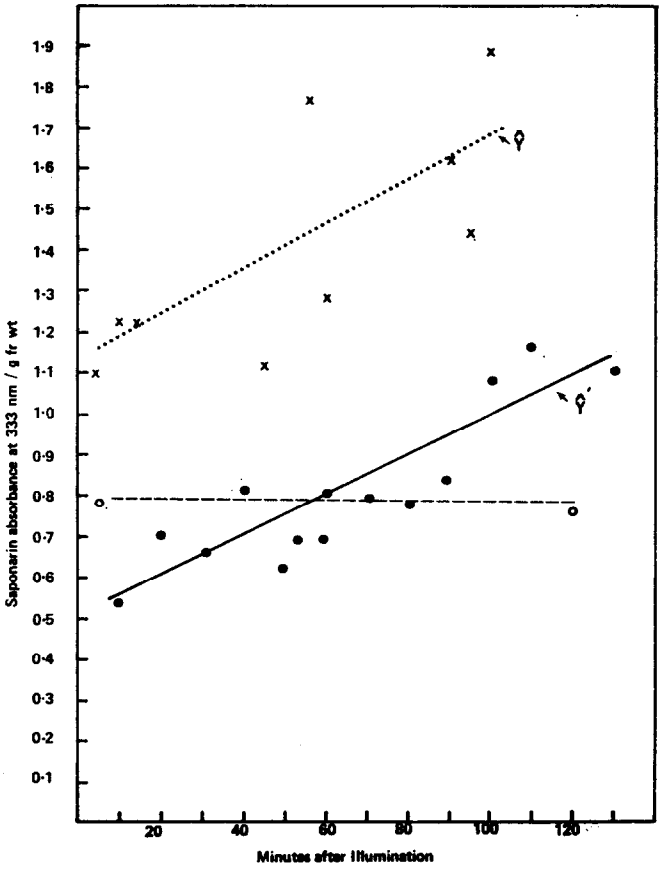


FIG. 3. CHANGES IN SAPONARIN CONTENT OF APICAL 1 cm SEGMENTS OF ATLAS 46 BARLEY.

●——●, red light; ×·····×, red + far-red light; o– – –o, dark controls; $\hat{Y}$, regression line. Intact 4-day-old etiolated plants were given 4 min of red, or 4 min red + 2 min far-red, light and returned to the dark. Samples (twenty apices) were excised and homogenized at the indicated times. $n = 1$.

DISCUSSION

Control of phenolic biosynthesis through the low-energy phytochrome and the high-energy photosystem has long been examined and it is now well established that they are common phenomena in higher plants.[7,15] Phytochrome has been isolated and characterized from several plants and much is known about its biochemistry and physiology.[15] While the HER photoreceptor has not been conclusively identified there is some evidence that it may be

phytochrome itself.[17–19] Still, little is known about the mechanisms by which these pigments mediate the conversion of light energy into events ultimately measurable as changes in plant constituents. This is complicated by the lag periods usually intervening between irradiation and detectable phytochemical responses which generally rule out primary responses.

To reconcile various changes attributable to phytochrome and the HER, two major types of mechanisms seem to be working; indirect changes which alter nucleic acid metabolism and more direct changes controlling membrane permeability.[14] We suggest that both of these mechanisms are involved in determining the pattern of saponarin synthesis and distribution in etiolated barley seedlings.

Photocontrol of Increased C-Glycosylflavone Synthesis

In many plants it has been established that phytochrome controls the *de novo* synthesis of phenylalanine ammonia–lyase,[8] the production and hydroxylation of various polyphenols,[9] and light is usually required for the production of any anthocyanin.[4,7] Most of these investigations can probably be explained through the activation of appropriate genes by P*fr*; the fact that there is usually a general increase in protein and enzyme synthesis in the presence of P*fr* also suggests the involvement of RNA metabolism in phytochrome induced photoresponses.[15]

It has been suggested that these light reactions, especially the HER, controlled the production of enzymes involved in acetate or malonate production for flavonoid A-rings.[7] Recently, Scherf and Zenk[17] have shown that the coupled HER, low-energy red light controls for rutin, leucocyanidin, chlorogenic acid and several cyanidin glycosides in *Fagopyrum* (buckwheat) are not likely to be closely related to increased A-ring precursor. Their *in vivo* and *in vitro* experiments show that light has little effect on "acetate" metabolism under conditions in which polyphenol metabolism is markedly altered. In contrast, the activity of phenylalanine ammonia–lyase in *Fagopyrum* seedlings is increased about 6-fold by illumination. Our preliminary (unpublished) experiments show that phenylalanine ammonia–lyase activity is also increased by light in barley seedlings. From other investigations[7,8,15,17] it is anticipated that any compound biosynthetically derived from cinnamic acid should be increased within a few hours after etiolated plants are treated with red light. Our results with saponarin synthesis in barley fit this pattern (Fig. 2A).

The detection of lutonarin only in plants given prolonged treatment with white light (Table 4), or blue light followed by low energies of red light (Table 5), are more typical of the HER responses.[7] Based on Stafford's work on *Sorghum* phenolics,[20] it is likely that this HER operates in part to organize the system, perhaps within the plastids of the primary leaves, incorporating oxygen into the flavone B-ring at the 3′ position. It is still not known when this hydroxylation takes place. While Grisebach and his associates[5] have shown by elegant dual labelling experiments that 3′-hydroxylation almost surely takes place at the level of the dihydroflavonol precursor of the flavonol quercetin and the anthocyanin cyanidin, the flavones are probably formed by dehydrogenation of flavanones.[5] Genetic studies in *Mimulus*[21] suggest that different mechanisms control 3′-hydroxylation of flavones and anthocyanins, and ^{14}C-apigenin is a good precursor of ^{14}C-luteolin in several of the Lemnaceae.[6]

[17] H. Scherf and M. H. Zenk, *Z. Pflanzenphysiol.* **56**, 203 (1967).
[18] K. M. Hartman, *Z. Naturforsch.* **22**, 1172 (1967).
[19] E. Wagner and H. Mohr, *Photochem. Photobiol.* **5**, 397 (1966).
[20] H. A. Stafford, *Phytochem.* **8**, 743 (1969).
[21] K. G. Wilson, R. K. Vickery, Jr. and H. G. Pollock, *Genetics* **61**, S64 (1969).

Photocontrol of the C-glycosylflavones vitexin and orientin, as well as glycosides of the flavonols kaempferol and quercetin and the anthocyanin cyanidin 3-glucoside have been shown in *Spirodela intermedia*.[10] Without regard to flavonoid class, the 4'-hydroxylated flavonoids are formed in comparable yield under all conditions of culture, while light is required for 3'-substitution of non-cyanic flavonoids. It is fairly certain that none of these compounds is interconverted *in vivo*.[5,6] This closely paralleled regulation of flavonoid B-ring substitution patterns under many conditions of culture suggests that the biosynthesis of the C-glycosylflavones and other classes of non-cyanic flavonoids are under similar controls and limited by common precursors. It is also probable that the specific high-energy light requirement for anthocyanin synthesis operates in many plants to determine the oxidation of a dihydroflavonol into the corresponding anthocyanin since quercetin is produced in good yield under photoconditions in which cyanidin is not detected.[10] Thus a direct comparison of work on the photocontrol of anthocyanins and non-cyanic flavonoids may relate only in their earlier biosynthetic stages before the pathways diverge.

Photocontrols Determining C-Glycosylflavone Distribution within Barley Plumules

In addition to light resulting in a strict biochemical transformation of flavonoid substrates, it is probable that some of the intermediates are transported and that this too is under photocontrol. For example, Grill and Vince[22] show that anthocyanin synthesis in *Sinapis* seedlings begins in the cotyledons and that some substance is moved from there into the hypocotyl where a second photoreaction is required for pigment production. This translocated substance has not been identified, although they speculate that it is a fairly late precursor as when they fed the hypocotyls several early precursors of anthocyanins, no pigment was formed in response to this last light reaction. Konishi and Galston[23] also found that phytochrome had no apparent effect on the transport of phenylalanine, tyrosine or cinnamic acid in etiolated *Pisum* epicotyls. The observed changes in saponarin content and its distribution within the barley plumules may be a related translocation phenomenon, especially the rapid and marked saponarin increase in the apical centimeter of the plumule following red + far-red light treatments.

Other investigators, notably those at Beltsville and Brookhaven,[15] have worked with rapid photomorphogenetic responses and conclude that some phytochrome responses are best explained by assuming that the molecule is a part of, or closely associated with, a membrane system and that light thus alters membrane permeability. In fact, Tanada[24] reports a phytochrome mediated control of the adhesion of excised barley roots to glass surfaces within 15 sec from the beginning of illumination, interpreting this as an initial expression of phytochrome action. Thus another possible explanation for the apparent rapid loss of saponarin from whole plumules when etiolated plants are given red light, is that light changes the permeability of the vacuolar membrane. This would allow the flavonoids to move out into the cytoplasm and combine—possibly with cytoplasmic proteins—into forms not methanol soluble and thus not recoverable by our techniques. Such protein-polyphenol complexes are readily formed *in vitro*.[25] The biological consequences of such a change in the localization of flavonoid as they pass from cell to cell could be very important. A number of flavonoids are

[22] R. GRILL and D. VINCE, *Planta* **70**, 1 (1966).
[23] M. KONISHI and A. W. GALSTON, *Phytochem.* **3**, 559 (1964).
[24] T. TANADA, *Proc. Nat. Acad. Sci. U.S.* **59**, 276 (1968).
[25] A. M. FIRENZUOLI, P. VANNI and E. MASTRONUZZI, *Phytochem.* **8**, 61 (1969).

inhibitory to protein synthesis in excised plant tissues,[26] they have been implicated in rapidly induced growth movements in tendrils,[27] and a host of other metabolic activities have been demonstrated for various polyphenols.[28]

In the apices, the rapid increase of saponarin following red + far-red light may also reflect translocation; its kinetics seem to preclude both *de novo* enzyme synthesis and enhanced flavonoid production. Work has commenced to determine the wavelength dependency and distribution patterns of these rapid changes in saponarin content of barley plumules.

EXPERIMENTAL

Plant Material and Light Treatments

Atlas 46 barley (*Hordeum vulgare* L.) was obtained from C. W. Schaller, University of California, Davis, and from G. Wiebe, United States Department of Agriculture, Beltsville, Maryland. Other varieties were provided by D. A. Ray, Ohio State University, Columbus. Seeds were soaked for 8 hr in the dark at 23° in distilled water. From thirty to forty seeds were scattered on the surface of water-saturated vermiculite in 250-ml glass beakers under a dim-green safelight. The beakers were covered with perforated aluminum foil and placed in light-tight culture chambers maintained at 23° in the dark room. The foil was removed 24 hr before harvest. By the fourth day the plumules were about 4 cm long. The light sources used in the various treatments were the same as those described by McClure.[10] Energy levels (1·1, 1·2 and 34 Kerg cm^{-2} sec^{-1} for red, blue and far-red, respectively) were measured with YSI-Kettering Model 65 Radiometer. All manipulations and harvesting steps through homogenization were carried out in the dark room under the safelight.

Extraction and Determination of C-Glycosylflavones

The plumules (usually 10, *ca.* 700 mg fresh weight, but varying somewhat with the experimental modifications) were thoroughly ground in 5 ml of methanol containing 0·5% HCl (v/v) in a Ten Broeck homogenizer. The homogenate and washings were pooled and adjusted to 30 ml by the addition of methanol. After centrifuging and washing the pellet (2 × 30 ml) in methanol, the combined supernatants were concentrated *in vacuo* for chromatography. The total extract and washings were applied to a disk 2·5 cm in diameter of Whatman No. 3MM filter paper suspended on stainless-steel pins. The air-dry disks were inserted through four parallel slits 5 mm apart on the starting line of a sheet of Whatman No. 3MM paper. Two-dimensional chromatographic separation was carried out by descent using first TBA (*t*-BuOH–HOAc–H_2O, 3:1:1 v/v) in the short (46 cm) direction and then in 5% HOAc. C-glycosylflavones were detected in u.v. light and, using a template, the spots were cut from the paper as ellipses of 45·5 cm^2. With but a few exceptions (as noted) only saponarin was found. Saponarin was eluted from the ellipses in tightly sealed Erlenmeyer flasks on a mechanical shaker in 5 ml of a mixture of 0·01 N HCl and methanol (2:8 v/v) for 3 hr and estimated spectrophotometrically by its absorbance at 333 nm (λ_{max}). Special problems involved in quantitating these compounds are detailed in the results section.

Acknowledgements—The work was supported by the National Science Foundation (Grant GB 4132) and by a graduate assistantship from the Miami University Research Committee for our technical assistant, Mr. Richard Carlin. The authors express their thanks to Mr. Carlin for his diligence and to the late Dr. M. K. Seikel for her valuable suggestions.

[26] E. V. PARUPS, *Can. J. Biochem.* **45**, 427 (1967).
[27] M. J. JAFFE and A. W. GALSTON, *Annual Review of Plant Physiol.* **19**, 417 (1968).
[28] M. K. ZUCKER, R. HANSON and E. SONDHEIMER, in *Phenolic Compounds and Metabolic Regulation* (edited by B. J. FINKLE and V. J. RUNECKLES), p. 68, Appleton-Century-Crofts, New York (1967).