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Investigations on the Role of Ethylene in Phytochrome-mediated Photomorphogenesis

I. Anthocyanin Synthesis

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Abstract. The etiolating, intact mustard (*Sinapis alba* L.) seedling exhibits a distinct temporal pattern of ethylene production. Light, operating through phytochrome, increases the rate of ethylene production without changing the pattern. Ethylene production of the isolated plant parts (segments), added together, exceed the production of the intact system even if the wound effect is taken into account. There is no significant light effect on ethylene production of the segments. Phytochrome-mediated anthocyanin synthesis in the cotyledons is inhibited by ethylene. The responsiveness towards ethylene of the anthocyanin producing metabolic chain is decreased by phytochrome. As anthocyanin synthesis is only partly inhibited under saturating ethylene concentrations in the atmosphere around the seedlings (100 $\mu l\ l^{-1}$), a two-factor analysis becomes feasible. This analysis leads to the result that phytochrome and ethylene show multiplicative behavior, meaning that phytochrome and ethylene act on the same metabolic sequence (leading to anthocyanin) but independently of each other, and at different sites. Therefore, the hypothesis that ethylene mediates the action of phytochrome in anthocyanin synthesis and photomorphogenesis in general appears to be inapplicable.

Key words: Anthocyanin synthesis – Ethylene – Phytochrome – *Sinapis*.

Introduction

In the seedling of white seeded mustard (*Sinapis alba* L.), anthocyanin synthesis is controlled by light (Mohr, 1957). It was shown previously (Lange et al., 1971; Drumm and Mohr, 1974; Drumm et al., 1975) that light-mediated anthocyanin synthesis in this system is very probably exclusively due to the operation of phytochrome, under induction conditions (light pulses, law of reciprocity valid) as well as under continuous light ("high irradiance response"). In the present investigation, we use phytochrome-mediated anthocyanin synthesis in the mustard seedling, to study the kind of interaction, if any, between phytochrome and the gaseous plant hormone, ethylene.

It is known (e.g. Abeles, 1973; Craker et al., 1971; Craker and Wetherbee, 1973a, b) that ethylene (under suitable boundary conditions) *inhibits* light-mediated anthocyanin synthesis in seedlings. Kang and Burg (1973) in experiments with red cabbage seedlings derived the hypothesis that the actions of light (occurring via phytochrome) and ethylene on anthocyanin synthesis are aspects of the same mechanism insofar as ethylene acts as a mediator of phytochrome action with regard to anthocyanin synthesis. Kang and Burg's argument was mainly based on the following observations obtained with 1 cm apical segments from the red cabbage seedling: 1. Red light (operating through phytochrome) stimulates anthocyanin synthesis. 2. In the presence of endogenously produced or exogenously supplied ethylene, anthocyanin synthesis is inhibited. 3. Red light reduces endogenous ethylene production. 4. There seems to be a correlation between red light mediated reduction of ethylene synthesis and concomitant stimulation of anthocyanin synthesis. Kang and Burg concluded that anthocyanin synthesis is due to a lowered ethylene content in light-treated tissue. In a review, Burg (1973) stated that red light-stimulated anthocyanin synthesis is "mediated by repressed ethylene production". The same kind of argument was previously used to explain other phytochrome-mediated photomorphogenic responses such as opening of the hypocotylar hook (Goeschl et al., 1967; Kang et al., 1967) or stimulation of ca-

Abbreviations: P_{fr} = far-red absorbing form of phytochrome; P_r = red absorbing form of phytochrome; P_{tot} = total phytochrome, i.e. $[P_r]+[P_{fr}]$

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rotenoid synthesis in peas (Kang and Burg, 1972a, b).

We have reinvestigated the relationship between phytochrome and ethylene in anthocyanin synthesis since the confirmation of a mediator role of an established plant hormone (Abeles, 1973) in the causal sequence between phytochrome (P_{fr}) and a terminal photoresponse would be an important step in linking photomorphogenesis and hormone physiology together, a task which has yet to be mastered (see Mohr, 1972; Wareing and Thompson, 1976). The results of our studies, however, are not compatible with the notion that ethylene acts as a mediator in photomorphogenesis, including anthocyanin synthesis.

Materials and Methods

Seed Material

The seeds of *Sinapis alba* L. were purchased in 1971 from Asgrow Company (Freiburg-Ebnet, West Germany). The seeds were selected and grown at $25.0 \pm 0.3°$ C according to the previously described procedure (Mohr, 1966). The selected seed material (15–20 per cent of the original sample) is normally distributed with regard to seed weight. Sowing of the seed and seedling growth followed the standardized techniques for photomorphogenic research with mustard seedlings described previously (Mohr, 1966). Sowing was carried out in dim green safelight (Mohr and Appuhn, 1962).

Sealed Containers

If not indicated otherwise, 25 seedlings growing on germination paper were placed in sealed gas-tight containers (730 ml volume) (Payer et al., 1969) at 36 h after sowing. The handling of the seedlings was performed in dim green safelight. Injection of ethylene at appropriate times was done with a gas-tight syringe (100 µl) through a particularly elastic rubber stopper (rubber serum cap). Withdrawal of gas samples was performed by means of a specifically adapted valve (for details see Bühler, 1977). In experiments with segments, 10 segments were kept in 6 ml glass vials. Withdrawal of 500 µl gas samples was performed with a gas-tight syringe (500 µl).

Measurements of Ethylene

The amount of ethylene was determined by gas chromatography (Varian 1700). Ethylene levels down to 3 nl l^{-1} can be measured in a reproducible manner (for details see Bühler, 1977). All ethylene measurements were corrected for C_2H_4-contamination in the air at the moment the containers were sealed.

Measurement of Anthocyanin

Anthocyanin was extracted from 20 pairs of cotyledons (including petioles) in 10 ml extraction medium and measured (1 cm pathlength) as described previously (Lange et al., 1971). Correction for scattering of the sample was applied as described previously (Lange et al., 1971). The small absorption measured at 535 nm in extracts of dark grown seedlings is in part due to non-anthocyanin, unknown substances ($A = 0.025$, virtually constant between 24 and 60 h after sowing) and in part due to the formation of very small amounts of "dark anthocyanin" induced by the safelight during the transfer of the seedlings from the germination boxes to the sealed containers at 36 h after sowing. This "dark anthocyanin" synthesis is totally suppressed by the application of 10 µl l^{-1} ethylene while the non-anthocyanin background absorption is unaffected by ethylene. The data given in the figures and tables are not corrected either for "background absorption" or for "dark anthocyanin".

Light sources

A standard far-red source (emission maximum at 740 nm, bandwidth 123 nm, energy fluence rate 3.5 Wm^{-2}) and a standard red source (emission maximum at 658 nm, bandwidth 15 nm, energy fluence rate 0.67 Wm^{-2}) were used for light treatments (see Mohr, 1966).

Statistics

The values given in the figures and tables are arithmetic means from 8–16 (4–8 independent) parallel experiments. The estimates of the standard errors of the means lie (as a rule) between 3 and 5 per cent.

Results and Discussion

1. Inhibition of Anthocyanin Synthesis by Ethylene

Figure 1 shows that ethylene in the gas phase surrounding the mustard seedling strongly inhibits anthocyanin synthesis induced by a 5 min red light pulse. The concentration-effect-curve is similar to the ones reported previously for other plant material (see Abeles, 1973) including red cabbage seedlings (Kang and Burg, 1973). While our system is quite sensitive towards ethylene, even a saturating concentration (100 µl l^{-1}) does not completely prevent light-mediated anthocyanin synthesis.

When the mustard seedlings were pretreated with ethylene between sowing of the seed and 27 h after sowing (the point at which competence is reached with regard to P_{fr} and anthocyanin synthesis if development takes place at 25° C, see Steinitz et al., 1976) but kept in an ethylene-free atmosphere during the red light pulse and during the development of the response, no effect of the pretreatment was found. Further experiments confirmed the fact that in the mustard seedling ethylene affects anthocyanin synthesis only if the gas is present during the period of actual pigment synthesis. Under similar conditions Craker and Wetherbee (1973a) noticed a stimulation of anthocyanin synthesis in *Sorghum* and red cabbage seedlings by a pretreatment. However, this report must be considered a misinterpretation of data due

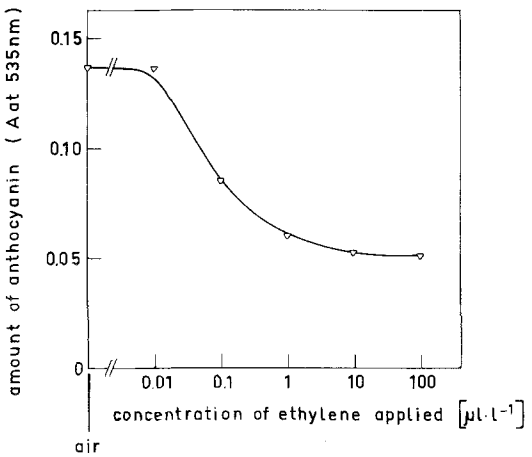

Fig. 1. Concentration-effect-curve for inhibition of anthocyanin synthesis in the intact mustard seedling by applied ethylene after a 5 min red light pulse. Seedlings were placed in a sealed container at 36 h after sowing, treated with a 5 min red light pulse and kept in darkness for 12 h (extraction of anthocyanin at 48 h after sowing). At a concentration of 10 or 100 µl l^{-1} ethylene, formation of "dark anthocyanin" (see Materials and Methods) is totally suppressed

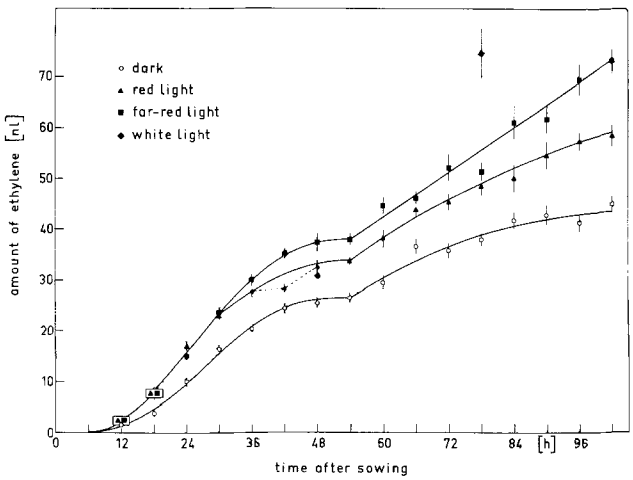

Fig. 2. Kinetics of ethylene accumulation by intact mustard seedlings in gas-tight, large volume containers in the dark and under different light treatments. At the times indicated, the amount of ethylene, accumulated by 25 seedlings, was measured. Control measurements in white light (fluorescent; 7.000 lx) were only performed at 48 and 72 h after sowing

to an effect of the pretreatment on the system of reference (10 mm-segments!). Since the ethylene pretreatment reduces lengthening of the axis, a 10 mm-segment from a pretreated plant will contain more cells than a 10 mm-segment from the control plant (for details see Bühler, 1977).

2. *Morphological Responses of the Mustard Seedling in the Presence of Ethylene*

In the presence of ethylene, the mustard seedlings undergo the usual "triple response" (see Abeles, 1973; Burg, 1973) consisting of a reduction in the rate of hypocotyl elongation, thickening of the subapical part of the hypocotyl, and horizontal nutation of the hypocotyl. In addition, ethylene leads to a coiling of the root tip, total closure of the hypocotylar hook, and inhibition of hair formation along the hypocotyl. These conspicuous morphological effects of ethylene on the axis system have prompted us to measure anthocyanin synthesis only in the cotyledons. In a mustard seedling, approximately 75 per cent of total anthocyanin are formed in the cotyledons. The cotyledons are well suited as a system of reference since there is no change of cell number (Lovell and Moore, 1970) and of DNA content (Mösinger, personal communication) over the period of our experiments (see also Mohr, 1972). We have not found any indications that ethylene affects appearance or morphology of the cotyledons.

3. *Does Phytochrome Affect Endogenous Ethylene Production?*

Figure 2 shows that light (probably operating exclusively through phytochrome) *increases* the rate of ethylene formation in the intact mustard seedling. The kinetics exhibit two distinct phases. It seems that the *temporal pattern* of ethylene production in the intact seedling is not influenced by light; it is only the actual rate which is increased. Light-mediated increase of ethylene production by intact seedlings was reported previously, e.g. by Craker et al., 1973.

Table 1 verifies the operational criteria for the involvement of phytochrome in the light-mediated response. The amount of ethylene, accumulated as a response to 4 red light pulses, is approximately the same which is accumulated during the same time as a response to continuous red light.

Table 1. Test for operational criteria (light pulse treatments) for the involvement of phytochrome in light-stimulated endogenous ethylene production of intact mustard seedlings. Pulses were applied at 12, 24, 36, and 48 h after sowing. The accumulated amount of ethylene was measured at 60 h after sowing

Treatment	Amount of ethylene (nl)
4 × 5 min red light	36.7 ± 0.9
4 × (5 min red + 5 min far-red light)	32.1 ± 1.0
4 × 5 min far-red light	31.4 ± 0.8
dark	30.2 ± 0.5

4. *Autoinhibition of Endogenous Ethylene Production*

Since in our experiments the rate of ethylene synthesis was estimated from the cumulative amounts of ethylene evolved by the seedlings in a sealed container, the possible role of an autocatalytic or an autoinhibitory effect had to be investigated. This problem was approached by absorbing the ethylene within the sealed container in a $Hg(ClO_4)_2$-solution (see Young et al., 1952; Abeles, 1973). The ethylene mercury complex formed is decomposed by the addition of a corresponding amount of a 4 mol l^{-1} NaCl-solution. At 20° C decomposition occurs at such a rate that after 1.5 h 80% of the total trapped ethylene are liberated from the complex. The values given in Table 2 are based on decomposition kinetics, corrected for the ethylene contamination of the laboratory air and for the ethylene content of the mercuric perchlorate solution before the onset of the experiment (for technical details see Bühler, 1977). Table 2 indicates that there is in fact a considerable autoinhibition of ethylene production in a closed system (sealed container). The *relative* inhibition does not seem to depend on light. Therefore, we conclude that the time courses shown in Figure 2 are not affected *specifically* even though quantitatively by the observed autoinhibition of ethylene synthesis which indicates negative feedback control.

The problem posed by a negative feedback control could be circumvented by working in an open system, i.e. passing an air stream over the seedlings and bubbling the air through a mercuric perchlorate solution. However, DeGreef et al. (1976) have described the critical shortcomings of this method, in particular that the trapping efficiency by this method is very dependent on the flow rate. Concerning the problem to be solved in the present investigation, the existence of a negative feedback control is largely irrelevant. Therefore, we have decided to continue to work with the closed system.

In the ethylene literature, an autocatalytic effect of C_2H_4 dominates (see Mapson 1969; Mayak and Dilley, 1976). However, autoinhibition of ethylene formation was shown recently by Zeroni et al. (1976) in immature stages of the fruits of sycomore fig.

5. *Does Ethylene Affect the Phytochrome System as Such?*

The results of detailed studies (see Bühler, 1977) can be summarized as follows: neither the formation of P_r nor the destruction of P_{fr} in the mustard seedling cotyledons are significantly affected by the presence or absence of ethylene. Formation of P_r (operationally, increase of P_{tot} in the absence of P_{fr}) and destruction of P_{fr} (operationally, decrease of P_{tot} with phytochrome in the P_{fr} form) were measured over a period of 12 h or 2 h, respectively, with a dual wavelength photometer as described previously (Schäfer et al., 1972).

6. *Further Characteristics Regarding the Inhibition of Light-Mediated Anthocyanin Synthesis by Ethylene*

When the onset of light is at 36 h after sowing (=time of transfer of the seedlings to gas-tight containers= time of application of exogenous ethylene) the time courses of anthocyanin accumulation with and without ethylene are similar, i.e. transformable into each other by a constant factor, as a rule up to 54 h after sowing. Figure 3 shows a representative pair of time courses of anthocyanin accumulation under continuous red light.

End point determinations of anthocyanin levels were performed at 48 h, i.e. only 12 h after the onset of light. Up to 48 h there are no indications that anthocyanin turnover comes into play. Anthocyanin turnover and the *inhibitory* effect of ethylene on this process need to be considered only beyond 60 h after sowing (see Bühler, 1977).

Figure 4 shows the concentration effect curves for the inhibitory effect of externally applied ethylene on anthocyanin synthesis under different light treatments. It seems that the responsiveness of the anthocyanin producing system towards ethylene decreases with increasing light effect. Formation of "dark anthocyanin" (in fact induced by safelight at 36 h, see

Table 2. The inhibitory effect of accumulated, endogenous ethylene on ethylene accumulation (presumably synthesis). Ethylene was absorbed by a mercuric perchlorate solution in the sealed containers. The amount of ethylene was determined at 48 h after sowing. Onset of light at the time of sowing

Treatment	Amount of ethylene (nl)		% inhibition[a] during accumulation
	accumulation	absorption	
dark	24	43	44
red light	30	47	36
far-red light	32	57	44

[a] Based on absorbed ethylene = 100%

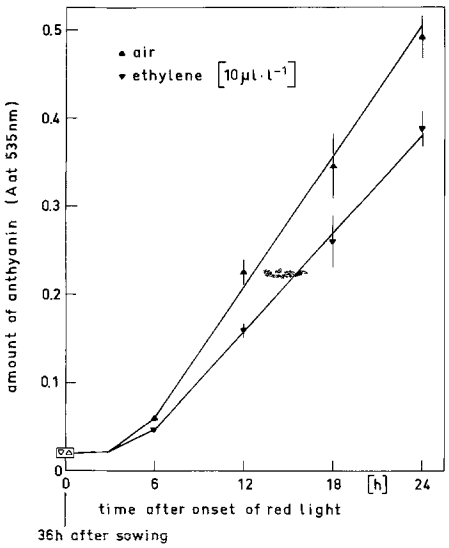

Fig. 3. Kinetics of anthocyanin accumulation in continuous red light. ▲, seedlings without application of ethylene; ▼, seedlings kept in an atmosphere with 10 µl l^{-1} C_2H_4. The lag-phase of anthocyanin accumulation after the onset of light is always close to 3 h (onset of light at 36 h after sowing)

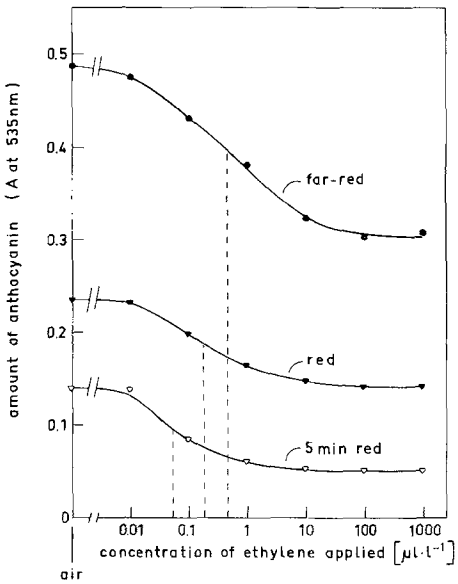

Fig. 4. Concentration-effect-curve for inhibition of anthocyanin synthesis in the intact mustard seedling by externally applied ethylene in continuous far-red or red light, or in the dark following a red light pulse applied at 36 h after sowing. Onset of light at 36 h after sowing; extraction of anthocyanin at 48 h after sowing. Vertical dashed lines, concentrations of ethylene required for half-maximum inhibition

Material and Methods) is totally suppressed by 10 µl l^{-1} ethylene. Thus, with regard to "dark anthocyanin" responsiveness towards ethylene is the highest. These findings elaborate the previous observations (Burg and Burg, 1968; Goeschl and Pratt, 1968) that dark-grown plants are more responsive towards ethylene than light-grown plants of the same population.

To account for any light-dependent differences in responsiveness towards ethylene, a concentration of 100 µl l^{-1} was chosen in the experiments related to two factor analysis (see 9). This ethylene concentration in the ambient atmosphere eliminates any differences between the seedlings due to the effect of light on ethylene production (Fig. 2) and/or responsiveness towards ethylene (Fig. 4).

7. *Does the CO_2 Partial Pressure Affect the Ethylene Effect on Anthocyanin Accumulation?*

There are reports in which CO_2 is attributed the role of a competitive inhibitor in ethylene-mediated responses (see Abeles, 1973). Since phytochrome affects the rate of CO_2-production in the mustard seedling (Friederich and Mohr, 1975), one might suggest that CO_2, accumulated in the closed system (sealed container), is the reason for the decreasing responsiveness towards ethylene with increasing intensity of phytochrome action. However, Table 3 shows that this suggestion is not supported by experimental findings. Anthocyanin was measured under conditions where endogenous CO_2 accumulates in the closed system as well as in a virtually CO_2-free system. (CO_2 absorption by a 10 per cent KOH-solution in the sealed container; control with an iso-osmotic KCl-solution). It is obvious from Table 3 that CO_2 influences neither the effect of endogenously accumulated ethylene nor the effect of exogenously supplied ethylene (10 µl l^{-1}). This result excludes stimulation of endogenous CO_2-production by light as an explanation for the light-mediated decrease of responsiveness towards ethylene. Even with 5 and 10 per cent CO_2 in the sealed container we could not observe any significant inhibition of the ethylene effect by CO_2, neither in anthocyanin synthesis nor with regard to morphological responses such as hook closure or hypocotyl elongation. Rather, at 10% CO_2 both gases tend to act in the same direction. This effect (imitation of ethylene effects by CO_2 at high partial pressure) was observed previously by Craker and Wetherbee (1973b). A *stimulatory* effect of CO_2 on the ethylene effect was observed by Ku et al. (1970) and Negm et al. (1973). In view of these reports, the *generalization* (Burg, 1973) that ethylene action is competitively inhibited by CO_2 certainly needs revision.

Table 3. A test for the effect of endogenously accumulated CO_2 on the effect of endogenously accumulated and exogenously applied ethylene (10 µl l^{-1}) on anthocyanin accumulation of the intact mustard seedling. The iso-osmotic KCl-solution served as a control for a potential osmotic effect of the 10% KOH-solution in the sealed container. Extraction of anthocyanin at 60 h after sowing

Experimental conditions	Amount of anthocyanin (A at 535 nm)		
	−KOH/−KCl	+KOH	+KCl
Safe light only (air)	0.060 ± 0.003	0.061 ± 0.003	0.059 ± 0.004
Far-red light (air)	1.07 ± 0.03	1.12 ± 0.04	1.10 ± 0.05
Far-red light (10 µl l^{-1} C_2H_4)	0.98 ± 0.04	1.06 ± 0.04	1.06 ± 0.02

8. *Investigations With Segments*

So far we have dealt with ethylene production of the whole, intact mustard seedling while anthocyanin synthesis has been measured in the cotyledons. We now pose the question to what extent the 3 main parts of the seedling – cotyledons, hypocotyl and taproot – contribute to ethylene production of the intact seedling. A further question is whether all three organs respond to light in qualitatively the same way (i.e. with an *increase* of the rate). Moreover, anthocyanin accumulation of the intact seedling was compared with anthocyanin production in the isolated parts.

a) Ethylene Production by Segments. Figure 5 shows the kinetics of ethylene accumulation in the closed system (6 ml vials) with segments isolated at 66 h after sowing, i.e. during the 2 nd phase of ethylene production by the intact seedling (see Fig. 2). The same

type of kinetics was obtained when the segments were isolated during the 1st phase of ethylene production by the intact seedling (isolation of segments at 42 h after sowing). Surprisingly, neither red light pulses nor continuous far-red light cause any *appreciable* change in the ethylene output of the segments. There was only a weak tendency (statistically insignificant) for stimulation in the cotyledons and for descrease in the hypocotyl (see Bühler, 1977).

An interpretation of the segment data is difficult, if not impossible, since the crucial question of how much of the ethylene produced by the segments is wound ethylene (see Goeschl et al., 1966; Abeles, 1973) cannot be answered conclusively. To exclude the wound effect as far as possible, the segments were kept for 4 h on wet germination paper after excision. After this period of adaptation, ethylene output (accumulation) was measured over another 6 h, i.e. between 4 and 10 h after segment excision (Table 4, 5). It is clear from the data that cotyledons produce much less ethylene than roots or hypocotyls. Tendencies seem to exist for light stimulation of ethylene production in cotyledons and for an inhibition of ethylene production in the hypocotyl.

The significance of the data obtained with segments becomes doubtful when we compare the segment data with the data obtained with the intact seedling (see Fig. 2). The comparison shows that the combined segments between 46 and 52 h after sowing yield considerably more ethylene than the intact seedling. Between 70 and 76 h after sowing the added figures are still approximately 50 per cent above the figure obtained with the intact system. Although autoinhibition makes a quantitative evaluation of the data difficult, the results illustrate the well-known limitations of "segment physiology", in particular it signals extreme caution if segment data are considered to represent in quantitative terms the behavior of the particular organ within the whole, intact organism.

However, an unambiguous conclusion can be drawn from our data: anthocyanin synthesis in the cotyledons cannot be attributed to a light-mediated *inhibition* of ethylene production. Burg's (1973) lapidary statement that light-stimulated anthocyanin synthesis in seedlings is "mediated by repressed ethylene production" is incorrect.

b) Anthocyanin Production by Segments. There seems to be a tendency that light decreases the output of ethylene in the hypocotyl. One would expect, if ethylene in fact plays a mediator role in anthocyanin synthesis in this organ, that anthocyanin accumulation in isolated hypocotyls is fostered by the light treatment compared with isolated cotyledons. Table 6 shows that this is not the case. While anthocyanin synthesis in the isolated hypocotyl is strongly decreased (compared with anthocyanin synthesis in the hypocotyl

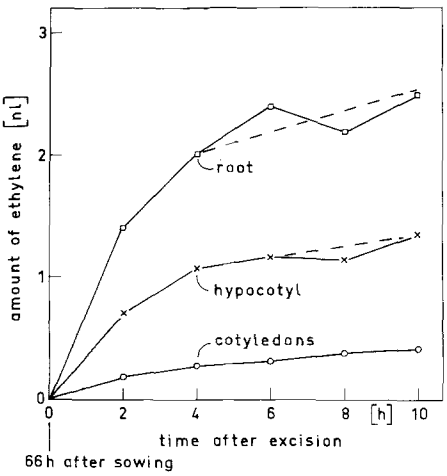

Fig. 5. Kinetics of ethylene accumulation by isolated taproots, hypocotyls, and cotyledons in darkness between 66 and 76 h after sowing. Excision of the parts was performed immediately before they were placed in gastight small containers (10 segments in 6 ml volume). The dashed lines indicate the probable interpolations

Table 4. Amount of ethylene accumulated in the dark or under continuous far-red light by 10 segments each between 46 and 52 h after sowing in sealed containers. Time of excision: 42 h after sowing. The sum of the partial amounts is indicated in the last column

Treatment	Amount of ethylene (nl)			
	cotyledons	hypocotyl	taproot	
Dark	0.45	0.80	0.89	2.14
Far-red light	0.49	0.76	0.97	2.22

Table 5. Amount of ethylene accumulated in the dark or under continuous far-red light by 10 segments each between 70 and 76 h after sowing in sealed containers. Time of excision: 66 h after sowing. The sum of the partial amounts is indicated in the last column

Treatment	Amount of ethylene (nl)			
	cotyledons	hypocotyl	taproot	
Dark	0.37	0.66	1.13	2.16
Far-red light	0.40	0.61	1.09	2.10

Table 6. Amount of anthocyanin accumulated in continuous far-red light by the intact mustard seedling or by 10 segments each between 42 and 52 h after sowing. Time of excision: 42 h after sowing. The taproot does not form any anthocyanin either in the intact seedling or as an isolated organ

Segment type and treatment	Amount of anthocyanin (A at 535 nm)	
	segments	intact
Cotyledons, far-red light	0.327	0.308
Hypocotyl, far-red light	0.012	0.091

of the intact seedling), anthocyanin synthesis is even increased in the isolated cotyledons compared with the cotyledons of the intact seedling. These data are of limited value either (in particular the hypocotyl data), since the translocation of organic matter, including substrate for anthocyanin synthesis, from the cotyledons to the hypocotyl is abolished by segmentation. However, the conclusion can be drawn that the segment data, too, are incompatible with the hypothesis that ethylene acts as a mediator in phytochrome-induced anthocyanin synthesis.

9. *Simultaneous Action of Phytochrome (P_{fr}) and Ethylene on Anthocyanin Synthesis*

In this section, we will attempt to solve the question proper of the present paper. The previous sections have served the function of supplying the knowledge required for a correct "two factor analysis". The major facts obatined so far can be summarized as follows: a) Anthocyanin synthesis in the mustard seedling is mediated by light via phytochrome. There is very probably no anthocyanin synthesis at all without P_{fr}. b) While anthocyanin synthesis is always inhibited by ethylene, inhibition is not complete even at saturating levels of ethylene in the atmosphere around the seedlings. c) While the sensitivity of the mustard seedling towards ethylene is decreased by phytochrome, a level of 100 $\mu l\, l^{-1}$ ethylene in the atmosphere around the seedlings suffices under all light treatments in saturating the inhibitory effect of ethylene on anthocyanin synthesis. d) The mustard seedling produces ethylene throughout its development. In the intact seedling, ethylene production is always *stimulated* by light. e) The light effect on ethylene production is mediated by phytochrome. f) Properties of the phytochrome system such as rate of P_r synthesis and rate of P_{fr} destruction are unaffected by ethylene.

The "two factor analysis" (simultaneous action of P_{fr} and C_2H_4, with regard to anthocyanin synthesis) is based on a theoretical model described previously (see Mohr, 1972, 1977). The essentials are as follows: If two factors act *independently* on the same causal sequence, "multiplicative behavior" of the two factors will be observed. As a formula: $A_{F_1F_2} = a\, A_{F_1}$. Expressed in words: the response (rate) $A_{F_1F_2}$, obtained with 2 factors, applied simultaneously, is a defined fraction of the response (rate) obtained with one factor irrespective of the magnitude of the response (rate) A_{F_1}. If a is >1, the two factors act in the same direction; if $a<1$, the two factors act against each other. An example for "multiplicative behavior" is illustrated in our Figure 6. The theoretical alternative is that the two factors act on different causal sequences that lead entirely independently to the response A. An example would be that the same molecule is produced independently in two compartments of the cell and under the regulatory control of independent factors. Under these circumstances, "numerically additive behavior" fo the two factors will be observed. As a formula: $A_{F_1F_2} = A_{F_1} \pm A_{F_2}$. Expressed in words: a response (rate) $A_{F_1F_2}$ which is caused by the two factors F_1 and F_2 (if applied simultaneously), is identical with the sum of the responses caused by the two factors if applied separately. The essential point is that multiplicative as well as numerically additive behavior of the two factors indicates "no interaction". Any other experimental outcome means "interaction", as a rule peculiar and system specific.

Table 7. Inhibition of anthocyanin accumulation by a constant ethylene concentration in the atmosphere around the seedlings (100 $\mu l\, l^{-1}$) at different energy fluence rates in continuous red and far-red light. Onset of the experiment: 36 h after sowing. Extraction of anthocyanin: 48 h after sowing. For a representative example of the kinetics of anthocyanin accumulation between 36 and 48 h after sowing see Figure 3. 1/1 far-red light: standard far-red light, 3.5 Wm^{-2}; 1/1 red light: standard red light, 0.67 Wm^{-2}

Relative fluence rate	Anthocyanin (A at 535 nm)		$\frac{A_{C_2H_4}{}^a}{A_{air}}$
	air	C_2H_4	
1/1 far-red light	0.466	0.282	0.61
1/10 far-red light	0.356	0.225	0.63
1/100 far-red light	0.231	0.136	0.59
10/1 red light	0.306	0.197	0.64
1/1 red light	0.241	0.145	0.60
1/10 red light	0.198	0.115	0.58

[a] In the case of multiplicative calculation, the expectation is: $A_{C_2H_4} = a \cdot A_{air}$
The experimental result is: $A_{C_2H_4} = 0.61 \cdot A_{air}$

In the following experiments, we have used a constant ethylene concentration (100 $\mu l\, l^{-1}$) in the atmosphere around the seedlings. By the application of this *saturating* concentration (see Fig. 4) we exclude all differences between the mustard seedlings due to ethylene sensitivity or endogenous ethylene production. The fluence rate of the light (and thus the *intensity* of phytochrome action, see Mohr, 1972) was varied. The results are shown in Table 7. As well in continuous far-red light as in continuous red light, the two factors (light and ethylene) show multiplicative behavior. The fact that the constant factor *a* is the same (0.61) in both kinds of light confirms our assumption (justified for other reasons; see Mohr, 1972) that continuous red and far-red light operate through the same "mechanism" ("high irradiance reaction" of phytochrome).

Multiplicate behavior means that P_{fr} and ethylene act independently of each other at different sites on the same metabolic channel leading to anthocyanin. While the factor P_{fr} *stimulates* anthocyanin synthesis,

the factor ethylene *inhibits* the flow in the pertinent metabolic channel ($a < 1$).

To our knowledge there is only a single paper in print (Janes et al., 1976) in which a *conclusive* two factor analysis about the interaction, or kind of simultaneous action, between light and ethylene was undertaken. In this paper, hook closure in intact lettuce seedlings was investigated with the result that both factors (red light and ethylene) act independently of each other on this system. In this particular case, the numerically additive model of non-interaction is adequate in describing the data. The authors conclude that the effect of red light on hook closure is not mediated through ethylene.

Conclusion

Figure 6 describes the action of light (phytochrome, P_{fr}) and ethylene (C_2H_4) on anthocyanin synthesis in the mustard seedling. We have shown that two kinds of interaction between the two factors exist: (1) P_{fr} stimulates the rate of endogenous ethylene production (see Fig. 2). (2) P_{fr} decreases the sensitivity of the seedling towards ethylene with regard to the anthocyanin response (see Fig. 4). Concerning the stimulatory action of P_{fr} (3) and the inhibitory action of ethylene (4) on anthocyanin synthesis we have shown that P_{fr} and C_2H_4 operate totally independent of each other at different sites along the same biogenetic sequence, leading from the basic metabolism to anthocyanin (aglycon cyanidin). This conclusion is drawn from the observation that both factors act multiplicatively under conditions where the interactions (1) and (2) are no longer significant since the system is saturated with C_2H_4 (100 $\mu l\, l^{-1}$ in the atmosphere around the seedlings). Since even at saturating concentrations, ethylene only *partly* closes the metabolic channel leading to anthocyanin, a precise two factor analysis became possible in deciding the question of whether multiplicative or numerically additive behavior of the two factors is involved.

The question posed in the Introduction can now be answered clearly: Ethylene does *not* play the role of a mediator in phytochrome-induced anthocyanin synthesis (at least in the mustard seedling). The hypothesis advanced by Kang and Burg (1973) emphasizing that ethylene mediates the action of phytochrome in anthocyanin synthesis (and in other photoresponses as well; Burg, 1973) is not substantiated.

A further question is what is the function of ethylene during seedling photomorphogenesis? — The mustard seedling produces considerable amounts of ethylene, and this production is stimulated considerably by light via phytochrome. Ethylene accumulation

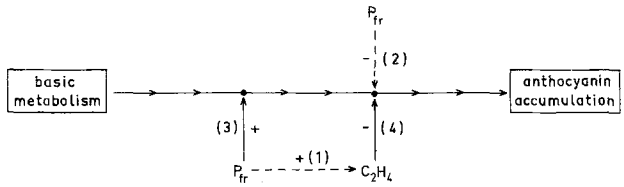

Fig. 6. Model to describe the action of light (phytochrome, P_{fr}) and ethylene (C_2H_4) on anthocyanin synthesis in the cotyledons of the intact mustard seedling. The dashed arrows (1, 2) indicate interactions; the solid arrows (3, 4) indicate totally independent actions of P_{fr} and C_2H_4. +, stimulatory actions; −, inhibitory actions

leads to an inhibition of ethylene production (autoinhibition rather than autocatalytic response as reported for fruits just prior to fruit ripening, e.g. Mapson, 1969, or flowers, e.g. Mayak and Dilley, 1976). Morphologically, the mustard seedling responds to elevated levels of C_2H_4 in the usual manner ("triple response"; see Abeles, 1973) but these responses can hardly be considered as physiologically meaningful under normal conditions of germination and seedling's growth, except perhaps closure of the apical hook as long as the seedling is still to penetrate the surface of the soil.

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