Kinetic of Lipoquinone and Pigment Synthesis in Green *Hordeum* Seedlings during an Artificial Day-Night Rhythm with a Prolonged Dark Phase

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Chloroplast Prenyllipid Synthesis, Light-Dark Change, Thylakoid Turnover

The effect of a prolonged darkness (48 h) with a following re-illumination on the prenyllipid metabolism of chloroplasts is tested in green *Hordeum* seedlings. Continuous darkness induces thylakoid and prenyllipid breakdown and changes the remaining lipoquinone and carotenoid metabolism of chloroplasts to that of etioplasts. Re-illumination, in turn, reverses the effects of darkness and regenerates the photosynthetic apparatus within 24 hours by a continuous and specific synthesis of certain thylakoid prenyllipids.

1. The level of chlorophylls, vitamin K_1 and that of the oxidized benzoquinone forms, plastoquinone-9 and α-tocoquinone, decreases continuously in darkness. All these prenyllipids are re-synthesized upon re-illumination, whereby a faster destruction rate in the dark (α-tocoquinone + plastoquinone-9 > vitamin K_1 > chlorophylls) corresponds to a faster re-accumulation rate in the light.

2. The concentration of the reduced benzoquinones plastohydroquinone-9 and α-tocopherol, which are preferentially deposited in the osmiophilic plastoglobuli, increases until 24 hours of darkness and decreases thereafter either continuously (plastohydroquinone-9) or exhibits another strong increase (α-tocopherol). Re-illumination results only in the accumulation of the oxidized quinone forms (mainly plastoquinone-9 and little α-tocoquinone); the level of plastohydroquinone remains, however, almost constant with a concomitant and steady decrease in the concentration of α-tocopherol. Darkness changes the main metabolite flow in benzoquinone synthesis via the formation of α-tocopherol (a trimethyl-, phythyl-1,4-benzoquinone derivative) and light (re-illumination) *via* the production of plastoquinone-9 (a trimethyl, solanesyl-1,4-benzoquinone).

3. Carotenoids are enriched throughout the dark phase (mainly xanthophylls, little β-carotene) with a higher accumulation rate than after re-illumination, which yields again a higher portion of β-carotene. The correlation of prenyl chain synthesis (phytyl chain for chlorophylls and vitamin K_1, solanesyl chain for plastoquinone-9) in the light with a reduced rate of carotenoid formation is discussed with respect to prenyl biosynthesis.

4. It is concluded that breakdown and re-synthesis of thylakoids and their prenyllipds, which are described here in a prolonged dark phase with following re-illumination, also occur during natural day-night growth of plants. The turnover of the thylakoid membranes and their lipids does, however, not get visible, since the decomposition in the night is recompensated by new synthesis during day. From the destruction rate one can calculate in a first approximation the biological half life time ($\tau\ ^1/_2$) which gives values in the range of 7 to 2,5 days for all thylakoid prenyllipids.

Introduction

Morphogenesis of green plants is controlled by light, which is effective *via* the photoconvertible phytomchrome system [1]. The active phytochrome P_{fr} also regulates growth and development of photosynthetic active chloroplasts including the formation of their photochemical active thylakoids and prenyllipids [2]. Dark grown etiolated *Hordeum* seedlings contain thylakoid-free etioplasts whose metabolism is changed to that of chloroplasts within 24 to 30 hours of illumination with white light [3]. On the other hand, light cultivated green *Hordeum* plants, when brought into continuous darkness, change their carotenoid and lipoquinone metabolism to that of etiolated plants already after a dark period of about 24 hours [4]. While chlorophylls and naphthoquinone vitamin K_1 are destroyed successively in continuous darkness, there is still an increase of the plastoquinone-9 (a dimethylbenzoquinone derivative) and β-carotene level up to 24 hours of darkness. The trimethylbenzoquinone derivative α-tocopherol and the xanthophylls, the main etioplast carotenoids, in turn, are accumulated up to three days of darkness; their concentration is decreased thereafter by destruction [4].

In the present paper we examine whether the changes in the lipid metabolism of chloroplasts, *e. g.* immediate destruction of some prenyllipids and in-

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creased synthesis of others as induced by continuous darkness may be reversed by light. In addition, it is shown that this system of prolonged darkness with following re-illumination represents a model system for the examination of the turnover rate of thylakoids and their prenyllipids.

Methods

Barley seedlings (*Hordeum vulgare* L. Asse) were cultivated in a growth chamber (24 °C), 50% relative humidity) in continuous white light (1800 lx, Osram Fluora lamps 55 W) until the age of 6 days (initial value). Thereafter all plants were brought into darkness and kept there for two days and then either re-illuminated for two days or left in darkness for two more days. The lipid analysis was performed with 200 plants each (primary leaves + coleoptile). The leaf material was homogenized and extracted at 5 °C with an acetone-petrol ether mixture in an "Ultraturrax-Homogenisator". Plastid pigments and lipoquinones were separated by thin-layer chromatography. The quantitative estimation was carried out photometrically [3]. The total nitrogen content was determined according to the method of Kjeldahl. The results represent mean values of several experiments.

Results

In a previous paper we have shown that six day old green *Hordeum* seedlings exhibit under exclusion of light not only the typical morphological signs of etiolement but also a change in the synthesis of their chloroplast prenyllipids [4]. This change in prenyllipid composition is a result of the fact that the individual thylakoid lipids are synthesized in the dark not at all (chlorophylls, vitamin K_1) or with a different rate to the light (lipophilic benzoquinones, carotenoids).

These former results are confirmed in this investigation. In addition, we have now determined the dry weight of the shoots, their total nitrogen and their total lipid content in continuous darkness. These data also show that two days of darkness are sufficient to induce in the plant catabolic processes. Light reverses these as is shown by the immediate increase of dry weight, of the lipid content and the total nitrogen level (Table I). Length growth of shoots and roots, which is promoted in darkness, slows down upon re-illumination (Fig. 1). Furthermore, light cancels out the changes in prenyllipid formation induced by darkness. The synthesis rate in the light compensates and exceeds their destruction rate in darkness which results in an increase of the concentration of the major chloroplast prenyllipids (Fig. 2).

Table I. Changes in analytical data of green 6 day old *Hordeum* seedlings in prolonged darkness and upon re-illumination (values in g per 100 shoots).

	Light	Dark		Light	
	6 d	+1 d	+2 d	+1 d	+2 d
dryweight	1.51	1.81	1.55	1.64	1.91
total lipid content	0.175	0.170	0.143	0.166	0.193
total nitrogen content	0.055	0.068	0.066	0.069	0.074
water content	12.2	11.1	12.8	13.6	15.5

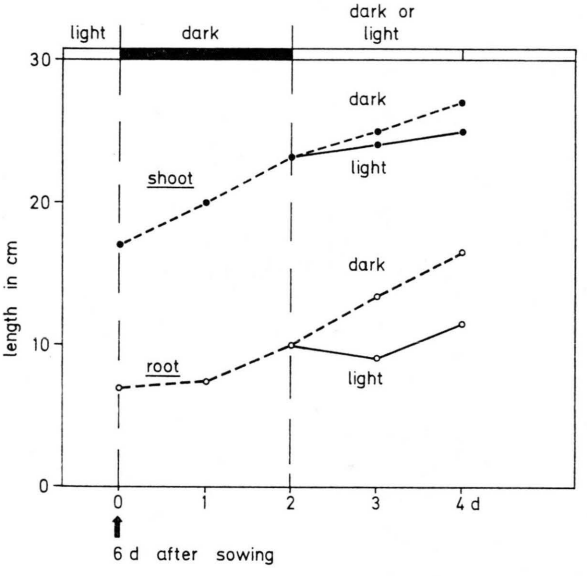

Fig. 1. Length growth of 6 day old green *Hordeum* seedlings in light-dark change.

a. *Chlorophylls*

In continuous darkness chlorophyll a and b are destroyed successively. After two days about 15% are destroyed. The chlorophyll decomposition carries on in darkness. Upon re-illumination (after two days of darkness) the chlorophyll level increases and reaches the initial value after 24 hours (Fig. 2). Destruction and new synthesis rate are about equally valid for chlorophyll a and b. Thus, the chlorophyll a/b ratio with 2.9 to 3.1 remains about the same during dark and light period.

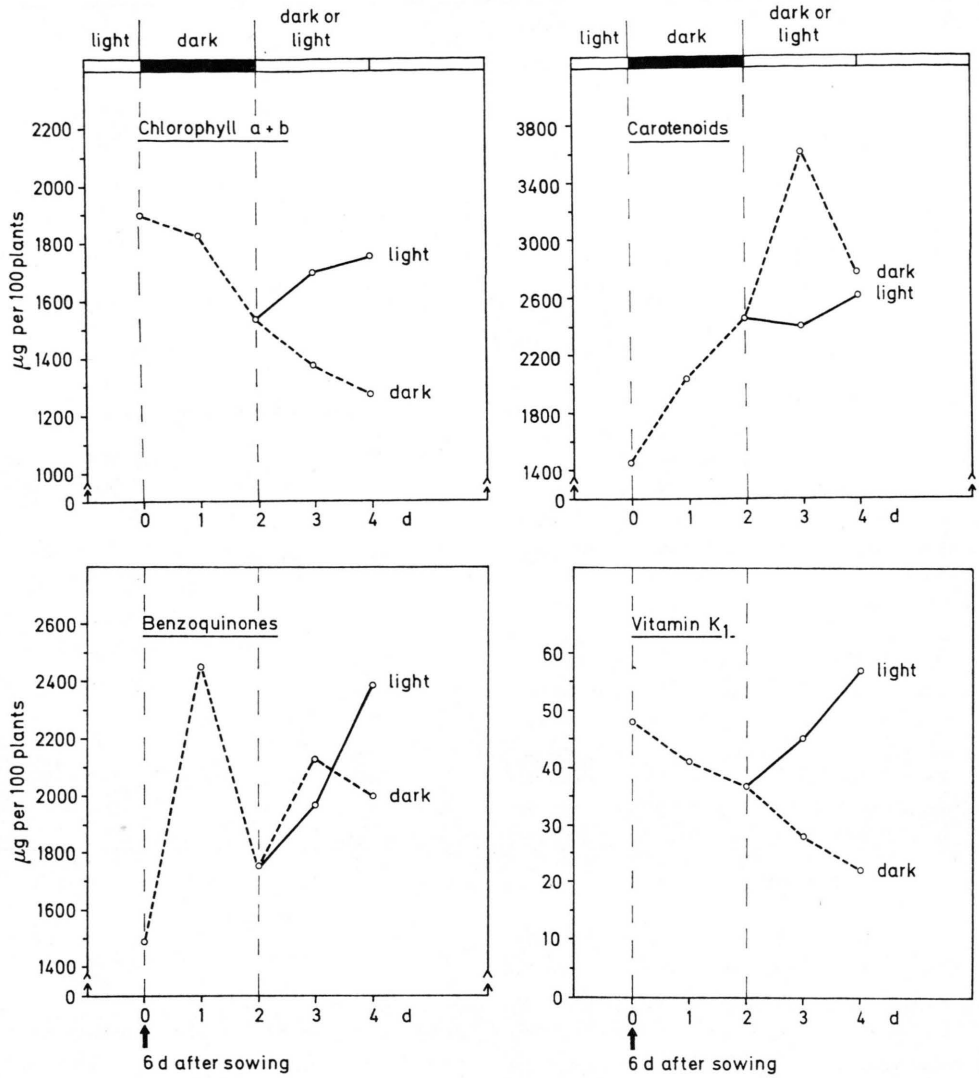

Fig. 2. Accumulation and destruction of chloroplast pigments and lipoquinones in green *Hordeum* seedlings in light or darkness.

b. *Carotenoids*

In contrast to the chlorophylls, the synthesis of total carotenoids continues in darkness and leads to a considerable increase of the xanthophyll content from the second to the third day of continuous darkness. Only thereafter can a decrease of the total carotenoid level by decomposition be visualized (Fig. 2). β-Carotene, whose synthesis is strongly promoted by light, is enriched in the dark only up to 24 hours. Its concentration then remains on the same level to that of the second day of darkness and decreases thereafter.

Re-illumination gives an immediate increase in the β-carotene level by new synthesis (Fig. 3). Lutein, the main xanthophyll component, and violaxanthin are initially enriched in darkness with the same synthesis rate as in the light. The large increase of the total carotenoid content from the second to third day of darkness which is due to a vast promotion of lutein and violaxanthin synthesis, is suppressed by re-illumination (Fig. 3). The pool-size of zeaxanthin, which is particularly enriched from the first to the second day of darkness [4], decreases in continued darkness and also upon re-illumination. In both cases it may get transferred to

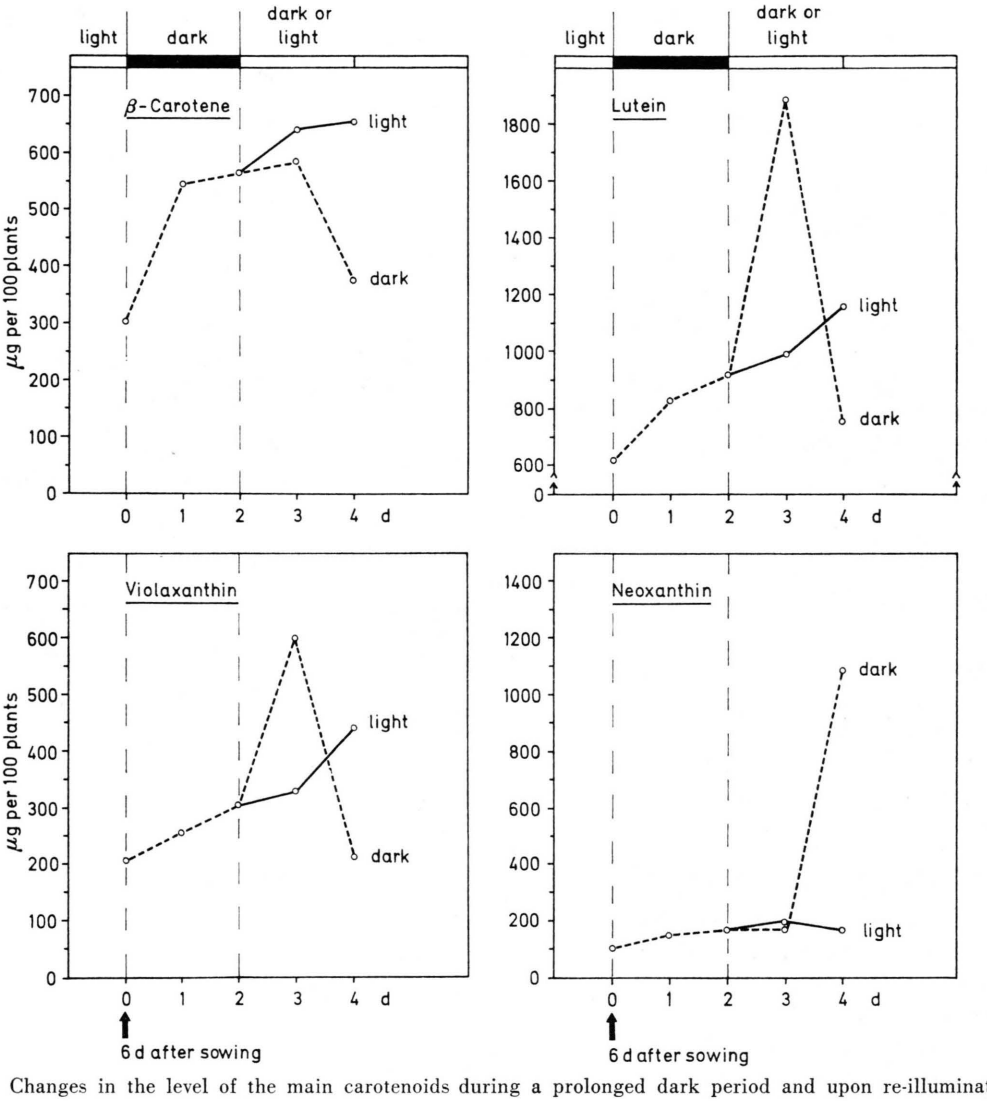

Fig. 3. Changes in the level of the main carotenoids during a prolonged dark period and upon re-illumination.

violaxanthin or to its stereo-isomere lutein. The level of the other xanthophylls, which are present in lower concentrations, are less influenced by the dark-light change.

The different synthesis rate of the single carotenoids in darkness results in a change of the percentage composition of the carotenoids. The relative portion of β-carotene, violaxanthin and lutein is diminished within two days of darkness, while the portion of the other carotenoids is increased (Table II). Re-illumination reverses this process. The results thus show that the carotenoid metabolism of chloroplasts is changed in continuous darkness successively to that of etioplasts. Upon re-illumination the carotenoid composition of functioning chloroplasts is reached within one to two days. After re-illumination the portion of zeaxanthin with 0.8% remains significantly beyond the initial value (9% in six day old plants). This decrease in the relative zeaxanthin level, however, is dependent on the development stage and is found in the light-grown green control plants, too.

c. *Plastid-quinones*

Continuous darkness leads in the first 24 hours to a strong increase of the total benzoquinone content of chloroplasts. Then its level is decreased considerably by decomposition. After the second to

Table II. Changes in the percentage composition (weight %) of carotenoids in green *Hordeum* seedlings in darkness and upon re-illumination.

	Light 6 d	Dark +1 d	Dark +2 d	Dark or Light +1 d	Dark or Light +2 d	
β-carotene	21.2	27.0	23.0	16.2	14.7	dark
				26.8	*25.1*	light
lutein	43.7	40.9	37.4	52.5	29.8	dark
				41.4	*44.6*	light
violaxanthin	14.4	12.7	12.5	16.6	8.3	dark
				13.9	*16.9*	light
neoxanthin	7.4	7.7	7.0	4.9	42.9	dark
				8.1	*6.6*	light
zeaxanthin	9.0	7.4	14.6	5.0	1.1	dark
				1.9	*0.8*	light
luteinepoxid	0.5	0.8	1.0	0.8	1.3	dark
				1.3	*1.1*	light
antheraxanthin	2.1	1.7	1.8	2.0	0.4	dark
				3.6	*2.4*	light
neoxanthin a	1.7	1.8	2.7	2.0	1.5	dark
				3.0	*2.5*	light
carotenoids	100.0	100.0	100.0	100.0	100.0	dark
				100.0	100.0	light

fourth day of darkness there is again a low net increase of the total benzoquinone concentration (Fig. 2). The individual benzoquinones, which consist of the two lipophilic redox systems plastohydroquinone-9 (+ plastoquinone-9) and α-tocopherol (+ α-tocoquinone) behave, however, in a different way.

The level of total plastoquinone-9 is increased only up to 24 hours of darkness and decreases thereafter successively (Fig. 4). The concentration of the oxidized form (plastoquinone-9) decresases immediately in darkness.

Re-illumination results in an increase of total plastoquinone-9 content which is due exclusively to the formation of the oxidized form plastoquinone-9. The level of plastohydroquinone-9, in turn, is decreased in the light in a similar way as in continued darkness (Fig. 4).

The level of the trimethyl-, phytyl-1,4-benzoquinone derivative α-tocopherol shows an increase up to 24 hours of darkness and a significant decrease thereafter. Further darkness results in a considerable re-accumulation of α-tocopherol, which paralleles in time the decomposition of chlorophylls and which is similarly found during chloroplast degeneration [6] and during development of chromoplasts from chloroplasts [7, 8]. The level of its oxidized form α-tocoquinone, however, decreases successively in continuous darkness (Fig. 4). Upon re-illumination the concentration of α-tocopherol decreases continuously in the light. The level of its oxidized form α-tocoquinone, which is present in much lower concentration, however, is raised in the light. In contrast to the benzoquinones the synthesis of the naphthoquinone vitamin K_1 is similarly regulated to that of the chlorophylls (Fig. 2). The K_1 concentration decreases continuously in darkness and is raised again upon re-illumination. These light and dark changes in benzoquinone and vitamin K_1 synthesis result in the differences in the percentage composition of the lipoquinones as is shown in Table III. The relative portion of α-tocopherol in-

Table III. Percentage composition (weight %) of chloroplast lipoquinones in 6 day old green barley seedlings in a prolonged dark phase and upon re-illumination.

	6 d	Dark +1 d	Dark +2 d	Dark or Light +1 d	Dark or Light +2 d	
plasto-quinone-9	17.5	6.9	7.4	4.0	2.0	dark
				17.9	*40.8*	light
plastohydro-quinone-9	43.1	50.1	49.9	42.6	41.4	dark
				44.8	*31.5*	light
α-tocopherol	34.3	40.7	40.4	51.9	55.4	dark
				34.8	*24.3*	light
α-tocoquinone	2.0	0.7	0.2	0.1	0.1	dark
				0.2	*1.1*	light
vitamin K_1	3.1	1.6	2.1	1.4	1.1	dark
				2.3	*2.3*	light
total lipo-quinones	100.0	100.0	100.0	100.0	100.0	dark
				100.0	100.0	light

creases with continuous darkness from 35 to 55% of total lipoquinone content while that of total plastoquinone-9 is diminished from 60 to 43% after 4 days darkness. Re-illumination reverses this process. After two days light the total plastoquinone-9 content rises up to 72%, while that of α-tocopherol decreases to 24% of total chloroplast lipoquinones. The relative lipoquinone composition in darkness thus resembles that of etioplasts and upon re-illumination again that of chloroplasts.

d. *Prenyl synthesis*

Lipoquinones and chlorophylls with their isoprenoid side chains belong, together with the carotenoids, to the group of isoprenoid plastid lipids (prenyllipids). Phytosterols also belong to this group, but they are present in chloroplasts only in traces. Thus by formation of the sum of carotenoids and prenyl side chains one obtains a rough approximation on the extent of total chloroplast prenyl syn-

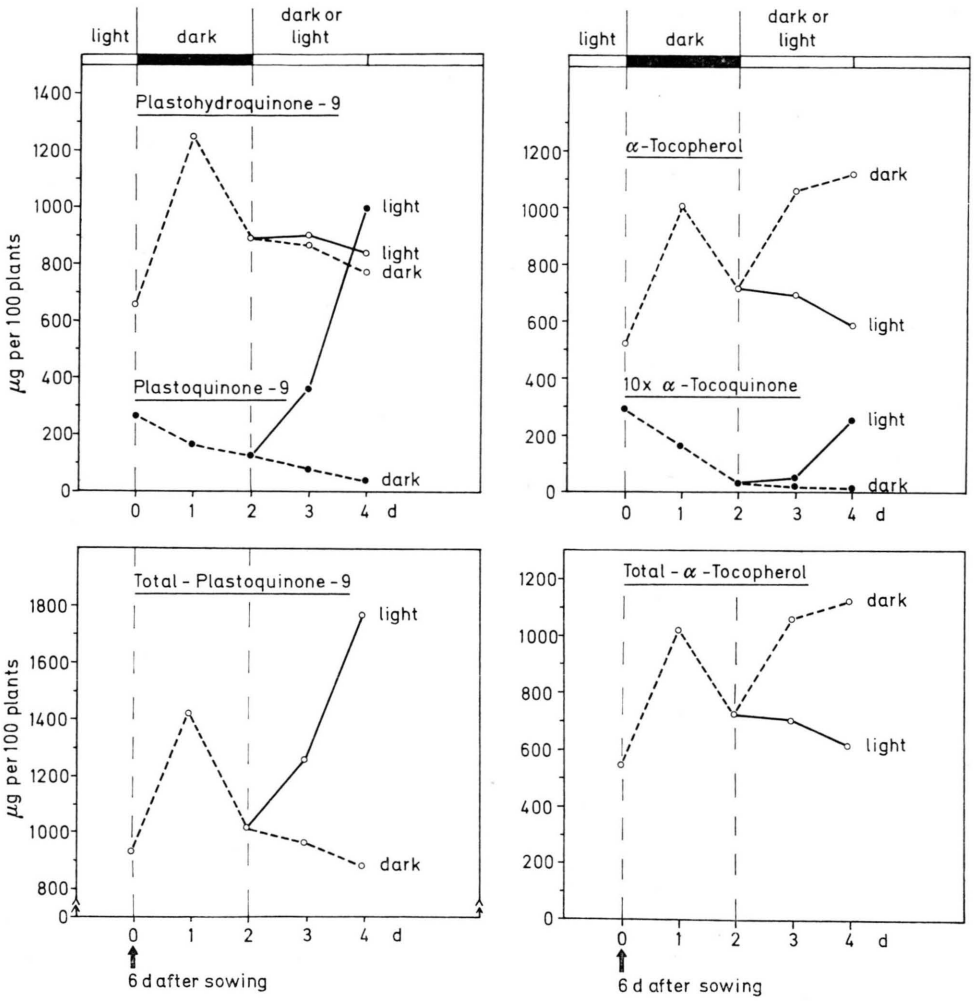

Fig. 4. Changes in the benzoquinone content of green *Hordeum* seedlings in darkness and upon re-illumination.

Table IV. Increase and decrease in the level of C_{20}-, C_{40}- and C_{45}-prenyl-chains in green 6 day old *Hordeum* seedlings in a prolonged dark period and upon re-illumination.

	Light	Dark		Dark or Light		
	6 d	+1 d	+2 d	+1 d	+2 d	
sum of C_{20} chains	981.2	1263.7	970.5	1141.6	1140.3	dark
				1012.1	*988.1*	light
C_{20} in chlorophylls	591.2	568.5	476.4	427.8	395.3	dark
				526.7	*548.8*	light
C_{20} in α-tocopherol (+α-tocoquinone)	360.2	669.8	471.2	696.4	731.4	dark
				457.5	*403.0*	light
C_{20} in vitamin K_1	29.8	25.4	22.9	17.4	13.6	dark
				27.9	*35.3*	light
C_{45} chains (plastoquinone-9 +hydroquinone)	765.6	1173.7	844.0	793.7	726.0	dark
				1039.5	*1460.3*	light
C_{40} chains (carotenoids)	1422.0	2023.5	2456.0	3603.0	2540.0	dark
				2388.0	*2602.0*	light
sum of prenyl-chains	3168.8	4459.9	4270.5	5538.3	4406.3	dark
				4439.6	*5048.4*	light

thesis. It should be considered in this respect that only the side chains bound to prenyllipids are determined in this investigation but not free side chains which may origniate, *e. g.* from the decomposition of chlorophylls.

The total prenyl chain content of chloroplasts increases up to the first day of darkness parallel to the dark formation of plastohydroquinone-9, α-tocopherol and carotenoids (Table IV). There is a small decrease in total prenyl content from the first to the second day of darkness, which is a result of the decomposition of chlorophylls, vitamin K_1, plastoquinone-9, α,tocopherol and α-tocoquinone, and which does not get compensated by the simultaneous accumulation of carotenoids. With the strongly increased formation of carotenoids from the second to the third day of darkness which parallels the re-accumulation of α-tocopherol there is a considerable increase in the total prenyl content. The latter decreases thereafter by decomposition. Re-illumination, on the other hand, leads as expected, to a continuous augmentation of the prenyl content, which parallels the light induced re-accumulation of chlorophylls, vitamin K_1, plastoquinone-9 and α-tocoquinone. The individual side chains, however, show a different behaviour. The level of the C_{45}-chain declines continuously after the first day of darkness and is increased upon re-illumination. The phytyl chain concentration shows after initial augmentation, which parallels the increase in the α-tocopherol level (first day of darkness), a marked decline on the second day, and increases once more to a constant level in further darkness. Re-illumination does not influence total phytyl chain content since the increase in chlorophyll, K_1 and α-tocoquinone level is compensated in this respect by the decomposition of α-tocopherol.

Discussion

The results of our investigation with a prolonged dark phase followed by re-illumination shows that the total pigment and lipoquinone metabolism of chloroplasts is regulated by light. Darkness, which inhibits the formation of thylakoids, changes the relative prenyllipid concentrations of chloroplasts to that of etioplasts. The concentration of chlorophylls, vitamin K_1 and of the oxidized form of the benzoquinone derivatives (plastoquinone-9, α-tocoquinone) decreases immediately in darkness. The destruction of these prenyllipids is strong indication for thylakoid breakdown. The reduced benzoquinone forms α-tocopherol and plastohydroquinone in turn, which are located only to a small extent in thylakoids and get mainly deposited in the osmiophilic plastoglobuli [9, 10], are strongly enriched in the first 24 hours of darkness. This is similar to the natural breakdown of thylakoids. In contrast to natural chloroplast breakdown, where carotenoids get decomposed in a kinetic similar to that of chlorophylls [6], they are enriched up to three days of darkness with a predominant formation of xanthophylls and less β-carotene. Thus, as seen from the prenyllipid analysis, the chloroplasts of *Hordeum* seedlings show after two days of darkness all signs of thylakoid breakdown whereby the remaining capacity for prenyllipid synthesis corresponds to that of etioplasts.

Re-illumination of the *Hordeum* seedlings, which were kept two days in darkness, regenerates the photosynthetic apparatus by new formation of thylakoids. The accumulation of chlorophylls, vitamin K_1, of solely oxidized benzoquinones (plastoquinone-9, α-tocoquinone) and of a higher portion of β-carotene are indication for active thylakoid formation. The relative chloroplast lipid concentrations of the darkened seedlings are reconverted to that of intact chloroplasts within 24 hours of re-illumination. The kinetic of prenyllipid formation upon re-illumination resembles very much that during the first greening of dark grown, etiolated seedlings [3].

The chromanol α-tocopherol, which becomes accumulated in continued darkness at high rates, is not synthesized upon re-illumination. From this one may conclude that α-tocopherol has no primary function in photosynthetic processes and plays only a role as potential lipid antioxidant [11] in the preservation of the photosynthetic biomembrane. The level of plastoquinone-9 (oxidized form), the terminal electron acceptor of the photosynthetic light reaction II which also promotes cyclic electron flow [12], in turn is raised considerably upon re-illumination. In view of the well established biosynthesis route of isoprenoid benzoquinones [8, 16], our observations thus indicate that the common benzoquinone precursor, homogentisic acid, is bound in the light mainly to the C_{45}-chain to form plastoquinone-9, and in darkness predominantly to the phytyl chain to yield α-tocopherol. This light

induced preferential synthesis of plastoquinone-9 is always found during active thylakoid formation and is further enhanced by the phytohormones kinetin [13] and β-indolacetic-acid (IAA) [14], while darkness and gibberellic acid (GA_3) [13] result in a preferential α-tocopherol accumulation.

Vitamin K_1 and α-tocoquinone, which are present in chloroplasts in minor concentration, are accumulated upon re-illumination in a similar pattern as the chlorophylls. This makes their participation as electron carriers in photosynthetic reactions very probable, though their exact position in the photosynthetic electron transport chain is not yet known. Vitamin K_1 which is bound within the chloroplasts to the photosystem I particles [15] is a possible canditate for the unknown endogenous promoter of cyclic electron flow.

Between light and darkness there is also a particular difference in the formation of the individual chloroplast prenyl chains. Though the level of chlorophylls and vitamin K_1 is decreased continuously in darkness, there seems to occur phytyl chain formation in dark as is indicated by the dark accumulation of α-tocopherol, in which the phytyl chain is built in, too Fig. 5). Since it can not be ex-

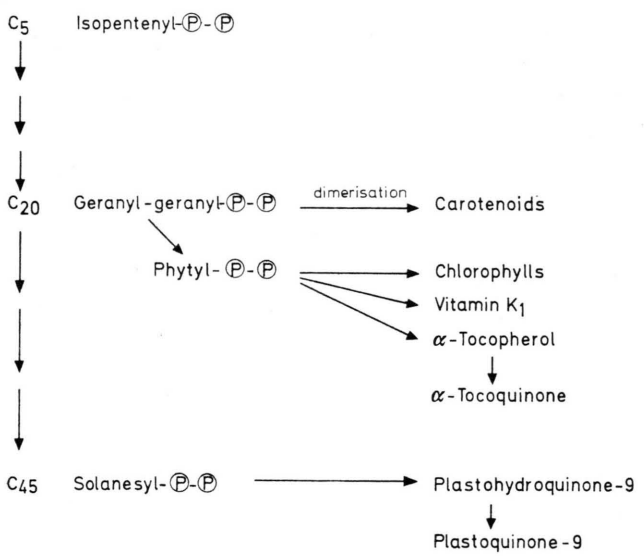

Fig. 5. Scheme of prenyl-biosynthesis (isoprenoid side chains and carotenoids) [8].

cluded that some phytyl chains, which may derive from chlorophyll breakdown, are used for α-tocopherol formation, the synthesis rate of the phytyl chain in darkness appears to be, however, much lower than in the light. The synthesis of the C_{45}-prenyl chain (solanesyl-chain of plastoquinone-9) apparently ceases after 24 hours of darkness. The formation of geranyl-geranyl-pyrophosphate, as common precursor of all chloroplast prenyl chains, and its dimerisation to the tetraterpenoid chain of carotenoids continues, however, in the dark. Re-illumination in turn gives more phytyl chains (chlorophylls, vitamin K_1), induces again the formation of the solanesyl chain (plastoquinone-9) and reduces the rate of carotenoid formation. It has also been shown in some other cases that enhanced chlorophyll synthesis is accompanied by a decrease in the accumulation rate of carotenoids [13].

The results of this investigation show that all chloroplast prenyllipids tested here undergo destruction. Thus the chloroplasts must possess the various enzymes for breakdown and metabolisation of such different prenyllipids as naphtho- and benzoquinones as well as chlorophylls and carotenoids. The destruction of these chloroplast lipids is, however, only visualized in a prolonged dark phase, when the light induced new formation of chloroplast prenyllipids is cancelled out. We thus conclude from our results with the prolonged dark phase that turnover of thylakoid prenyllipids and possibly all other thylakoid components must exist in chloroplasts of young and adult leaves.

From the decrease rate of the prenyllipids in continuous darkness one can calculate in a rough approximation their turnover rate. For the chlorophylls a und b one obtains a turnover rate of about 8% per day which corresponds to a biological half life time $\tau\ ^1/_2$ of six days. It is of interest in this respect that the turnover rate for chlorophylls of other plants, as estimated from isotopic studies, were found in the range of 5 to maximal 10% per day [17]. Vitamin K_1 seems to have a much higher turnover rate of about 13% per day ($\tau\ ^1/_2 = 4$ days). Its rate of re-accumulation upon re-illumination of the darkened seedlings is also faster than that of chlorophylls. For the oxidized benzoquinone forms plastoquinone-9 and α-tocoquinone, which are mainly localized in the thylakoids, the calculation yields turnover rates of about 20% per day ($\tau\ ^1/_2 = 2.5$ days). The reduced benzoquinone forms (α-tocopherol and plastohydroquinone-9), which are preferentially deposited in the osmiophilic plastoglobuli of the chloroplast stroma [9], seem to have lower turnover rates than their oxidized forms. Since both reduced benzoquinone derivatives exhibit an initial

fast destruction, which then slows down, one can calculate two turnover rates. These are for plastohydroquinone-9, 7 or 13% per day ($\tau\,^1/_2 = 7$ or 4 days) and for α-tocopherol 8.5 or 13% per day ($\tau\,^1/_2 = 6$ or 4 days). Since carotenoids are accumulated even in darkness and in the light it is not possible from our results to estimate their destruction rate.

In any case, this rough approximation shows that the different thylakoid prenyllipids exhibit individual half life time ($\tau\,^1/_2$) which amount to six days or less, and which may be similar for other thylakoid components. It is of particular interest that a faster destruction rate corresponds to a faster rate of re-accumulation of the individual prenyllipid in the light. This shows the usefulness of this system with prolonged dark phase and re-illumination for the investigation of the turnover of thylakoids and their lipid and protein components. Further experiments to determine turnover rates of thylakoid lipids with ^{14}C labelling are in progress.

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