Phytochemistry, 1971, Vol. 10, pp. 79 to 84. Pergamon Press. Printed in England.

PROMOTION OF VITAMIN K_1-SYNTHESIS BY NAPHTHOQUINONES

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(*Received* 21 *January* 1970, *in revised form* 3 *April* 1970)

Abstract—Dark grown barley seedlings possess only a low capacity for the synthesis of vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone). It is shown that several synthetic naphthoquinones (1,4-naphthoquinone, menadion, phthiokol) induce an increased vitamin K_1-synthesis in the dark. This promotion is specific. The synthesis of the plastidic benzoquinones and carotenoids is not affected by the applied naphthoquinones. The latter are to be regarded as possible intermediates in the biosynthesis of vitamin K_1. α-Naphthol does not, however, promote K_1 synthesis. From the results it appears that the low K_1 synthesis in the dark is due to a limited synthesis of the naphthoquinone nucleus. The phytyl moiety is apparently formed as the naphthoquinone nucleus becomes available.

INTRODUCTION

CHLOROPLASTS contain, besides several lipophilic benzoquinone derivatives, the lipid-soluble phylloquinone, vitamin K_1 (2-methyl-3-phytyl 1,4-naphthoquinone).[1] This compound is ubiquitously distributed in the photosynthetic apparatus of algae, mosses, ferns and seed plants.[2] Vitamin K_1 is localized within the chloroplasts almost quantitatively in the photochemical active thylakoids.[3,4] In the latter, it is associated nearly exclusively with the particles of the photosynthetic pigment system I.[3,5] From this and other data[6] it seems likely that the functional position of phylloquinone is in the electron transport chain catalyzed by light reaction I.

The thylakoid-free etioplasts of dark grown seedlings contain vitamin K_1 only in very low concentration.[7] Illumination of etiolated seedlings results in a strong vitamin K_1 synthesis, which parallels the formation of the chlorophylls.[8]

Etioplasts contain, besides phylloquinone, two further quinone compounds, α-tocopherol and α-tocoquinone, containing a phytyl chain. Both compounds are present in the dark tissue in 20 times higher concentration than vitamin K_1.[8] Thus the formation of the phytyl chain appears not to be a limiting factor in the dark synthesis of phylloquinone. It is more likely that the low vitamin K_1 synthesis in the dark is regulated by a limited synthesis of the naphthoquinone nucleus. If so, it should be possible to promote vitamin K_1 synthesis in the dark by feeding potential precursors of the naphthoquinone nucleus.

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[2] H. K. LICHTENTHALER, *Planta* **81**, 140 (1968).
[3] K. H. LICHTENTHALER, *Progress in Photosynthesis Research* (edited by H. METZNER), Vol. I, p. 304, H. Laupp, Tübingen (1969).
[4] H. K. LICHTENTHALER, *Z. Naturforsch.* **24b**, 1461 (1969).
[5] M. TEVINI and H. K. LICHTENTHALER, *Z. Pflanzenphysiol.* **62**, 16 (1970).
[6] H. K. LICHTENTHALER and M. TEVINI, *Z. Naturforsch.* **24b**, 764 (1969).
[7] H. K. LICHTENTHALER, *Z. Pflanzenphysiol.* **59**, 195 (1968).
[8] H. K. LICHTENTHALER, *Biochim. Biophys. Acta* **184**, 164 (1969).

The route of biosynthesis of vitamin K_1 is not yet absolutely clear. Shikimic acid functions as precursor for the naphthoquinone nucleus.[9] A further intermediate might be 1,4-naphthoquinone. Naphthoquinones have been shown to be intermediates in the biosynthesis of several other naphthoquinone derivatives, including those of the vitamin K_2-type.[10,11] Whether naphthoquinones induce an increased vitamin K_1 synthesis and promote K_1 formation in a specific way, has been investigated by feeding different synthetic naphthoquinones to etiolated seedlings.

RESULTS

Etiolated seedlings in the dark exhibit a distinct vitamin K_1 and carotenoid synthesis which is, however, much lower than synthesis in light.[7,8] This dark synthesis proceeds even when the etiolated shoots are excised at the base and the cut ends are dipped in water or 20% methanol.[12] The formation of carotenoids and vitamin K_1 continues in excised barley shoots in the dark for at least 40 hr or even longer. The rate of synthesis is only little lower than in intact dark seedlings. We have used this system to investigate the effect of naphthoquinones on the formation of vitamin K_1.

Excised 6-day-old etiolated barley seedlings were kept in the dark with the cut ends in aq. methanol. Within 20 hr the vitamin K_1-level increased about 20–30%. Application of methanolic solutions of various naphthoquinones (1,4-naphthoquinone, menadion, phthiokol) resulted in a considerable promotion of vitamin K_1 synthesis. After 20 hr in the dark the K_1-level in all cases was about twice that of the controls (Table 1). The best promotion of K_1 synthesis was obtained by application of either of the naphthoquinones in a concentration of between 1×10^{-4} to 4×10^{-4} mole/l. At higher concentrations the stimulating effect is smaller.

The carotenoid level of the treated plants corresponds to that of the controls (Table 1). This shows that the synthesis of carotenoids is neither stimulated nor decreased by the presence of the naphthoquinones. Thus the promotion of phylloquinone synthesis by naphthoquinones seems to be specific.

As a further test for a possible specific promotion of vitamin K_1 synthesis by naphthoquinones, we investigated their influence on the synthesis of the plastid benzoquinones and the individual carotenoids. The lipophilic benzoquinone derivatives plastoquinone 45, α-tocopherol and α-tocoquinone are present in the dark and their synthesis continues in excised shoots standing in 25% aq. methanol. Also all the individual carotenoids are formed under these conditions (Table 2). Application of naphthoquinones causes no promotion or inhibition of the synthesis of benzoquinone compounds or individual carotenoids. The level of the various isoprenoid lipids corresponds, within the variability range of the plant material, to that of the control (Table 2). At higher naphthoquinone concentrations (2×10^{-3} M) the synthesis of benzoquinones and carotenoids is somewhat depressed as compared to the control (Table 3). Evidently the naphthoquinones, applied in too high a concentration, suppress the general metabolism in some way. However, even under these conditions we find a low stimulation of vitamin K_1 synthesis by the three naphthoquinones.

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[11] C. MARTIUS and W. LEUZINGER, *Biochem. Z.* **340**, 304 (1964).
[12] H. K. LICHTENTHALER and K. BECKER, *Phytochem.* **9**, 2109 (1970).

TABLE 1. STIMULATION OF VITAMIN K_1-SYNTHESIS BY SYNTHETIC NAPHTHOQUINONES IN EXCISED ETIOLATED BARLEY SHOOTS IN THE DARK

Treatment	Conc. mM	Vitamin K_1	Carotenoids
		μg/100 shoots	
6-Day-old etiolated seedlings		1·2–1·5*	96–115*
6-Day-old etiolated seedlings + 20 hr darkness (controls)		1·4–1·7*	126–147*
6-Day-old etiolated seedlings standing 20 hr in the dark in:			
1,4-Naphthoquinone	0·1	3·6	137
	0·4	3·4	132
	2·0	2·0	129
Menadion	0·1	2·8	147
	0·4	2·6	126
	1·0	2·4	137
	2·0	2·1	127
Phthiokol	0·4	2·6	128
	1·0	3·1	141
	2·0	2·0	134
α-Naphthol	0·4	1·7	143
	1·0	1·8	141

* Range from four separate experiments.

TABLE 2. CAROTENOID AND LIPOQUINONE CONTENT OF ETIOLATED 7-day-old BARLEY SHOOTS, SHOWING THE SPECIFIC PROMOTION OF VITAMIN K_1-SYNTHESIS BY APPLICATION OF NAPHTHOQUINONES

	7-Day-old shoots	7-Day-old shoots standing 20 hr in the dark in:*			
		Control	1,4-Naphtho-quinone	Menadion	Phthiokol
		μg/100 shoots			
Vitamin K_1	1·4	1·9	3·0	4·0	3·7
Plastoquinone 45	30·0	36·0	39·0	44·0	37·0
α-Tocopherol	18·0	22·0	24·0	22·0	21·0
α-Tocoquinone	2·8	3·1	3·8	3·3	3·7
β-Carotene	10·0	14·0	15·0	14·0	13·0
Lutein	51·0	90·0	80·0	96·0	74·0
Violaxanthin	30·0	42·0	32·0	50·0	45·0
Neoxanthin	4·0	6·0	6·0	4·0	4·0
Antheraxanthin	31·0	37·0	38·0	31·0	28·0
Zeaxanthin	6·0	6·0	9·0	8·0	7·0
Carotenoids	132·0	197·0	180·0	203·0	171·0

* 1×10^{-4} M in 25% methanol.

TABLE 3. CAROTENOID AND LIPOQUINONE CONTENT OF ETIOLATED 6-day-old BARLEY SHOOTS BY APPLICATION OF NAPHTHOQUINONES

	6-Day-old shoots	6-Day-old shoots standing 20 hr in the dark in:*			
		Control	1,4-Naphtho-quinone	Menadion	Phthiokol
			μg/100 shoots		
Vitamin K_1	1·4	1·7	2·0	2·1	2·0
Plastoquinone 45	26·0	38·0	28·0	32·0	30·0
α-Tocopherol	18·0	24·0	21·0	18·0	20·0
α-Tocoquinone	2·4	2·7	3·2	3·1	3·0
Carotenoids	112·0	146·0	129·0	127·0	134·0

* 2×10^{-3} M in 25% methanol.

DISCUSSION

In summarizing the results from the different experiments, we can say that the three naphthoquinones (1,4-naphthoquinone, menadion, phthiokol) cause an effective and specific promotion of phylloquinone synthesis. Though the actual incorporation of the applied naphthoquinones into the vitamin K_1 nucleus has not been proved with ^{14}C-marked compounds, the specific promotion indicates that the plant cell can utilize all three naphthoquinones to synthesize vitamin K_1.

The possible synthesis route of phylloquinone from the applied naphthoquinones is shown in Fig. 1. In the case of 1,4-naphthoquinone, it cannot be judged from the results obtained here whether methylation occurs before or after the attachment of the phytyl group. The promotion of K_1 synthesis by menadion shows that route II is possible. The

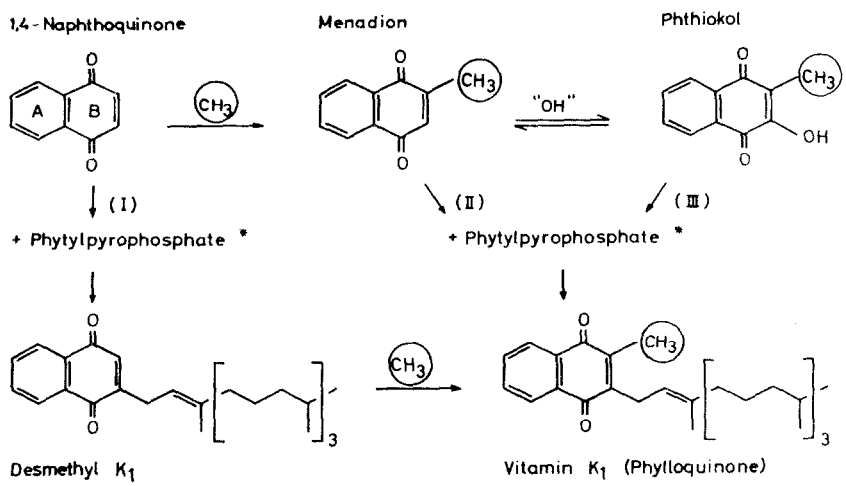

FIG. 1. POSSIBLE BIOSYNTHESIS ROUTES OF VITAMIN K_1 FROM APPLIED NAPHTHOQUINONES.
* As indicated in Ref 13.

[13] D. R. THRELLFALL, in *Terpenoids in Plants*, p. 202 (edited by J. B. PRIDHAM), Academic Press, London (1967).

hydroxynaphthoquinone phthiokol presumably reacts direct with the phytyl chain (with phytyl pyrophosphate)[13] to yield vitamin K_1 (Fig. 1, III). Another possibility would be its dehydroxylation to menadion.

Shikimic acid functions as precursor of the naphthoquinone nucleus of K_1.[9] It is possible that, similar to the biosynthesis of lawsone,[14] it combines with succinyl-TPP to yield the B ring which then gives rise to the naphthoquinone nucleus. The effective promotion of K_1 synthesis shows that the plant cell is adapted for turnover of naphthoquinones. Thus the three compounds 1,4-naphthoquinone, menadion and phthiokol are to be regarded as potential precursors in the biosynthesis of vitamin K_1. It is of interest in this respect that 1,4-naphthoquinone and menadion are intermediates in the biosynthesis of vitamin K_2 quinones.[11] In addition 1,4-naphthoquinone serves as precursor in the synthesis of juglone (5-hydroxy-1,4-naphthoquinone) and lawsone (2-hydroxy-1,4-naphthoquinone).[10] α-naphthol, which had been discussed as possible intermediate in the biosynthesis of menaquinone 9[15] is certainly not a direct precursor of the naphthoquinone nucleus of vitamin K_1, since it does not promote K_1 synthesis (Table 1).

Final proof as to which of the applied naphthoquinones are on the obligate biosynthetic pathway will come from tracer experiments which are now being carried out. There are some indications, which favour route I (Fig. 1) as the natural biosynthesis pathway for vitamin K_1. Chloroplasts from spinach and bean leaves contain a second vitamin K_1 which is thought to be desmethyl vitamin K_1.[16] Methionine functions as donor for the methyl group of K_1.[17] Etiolated barley seedlings also contain a second lipophilic naphthoquinone which disappears upon illumination and seems to function as a precursor of vitamin K_1.[18] From its chromatographic behaviour it could be identical with the desmethyl vitamin K_1. Elucidation of the chemical structure of this unknown naphthoquinone would give further information on the final step in the biosynthesis of vitamin K_1.

EXPERIMENTAL

Cultivation of Seedlings

Barley seedlings (*Hordeum vulgare* L.) were grown in water culture in the absolute dark at 20°. Each experiment was carried out with between 50 and 150 seedlings. The 6- or 7-day-old shoots were excised at the bases and batches of 10 shoots dipped with the cut ends in either 10 ml of 25% aq. MeOH (controls) or 2×10^{-3} to 1×10^{-4} M solutions of 1,4-naphthoquinone, menadion or phthiokol in 25% MeOH. The whole procedure was carried out under a very dim green safety light.[12] The excised barley shoots were kept for 20 hr in the dark and then harvested.

Extraction and Estimation of Isoprenoid Lipids

The shoots were homogenized with quartz sand and extracted with acetone and light petrol. Vitamin K_1 was separated from β-carotene and plastoquinone by two-dimensional TLC and estimated by means of its light green fluorescence under u.v. light (254 nm) as described earlier.[12] In control experiments with the different naphthoquinones applied in the investigation and with α-naphthol it was shown that these compounds are completely separated from vitamin K_1. In addition none of these compounds give any green fluorescence as does K_1.

The carotenoids were separated by TLC according to Hager and Bertenrath.[19] The bands were eluted with EtOH and then estimated spectrophotometrically. An extinction factor of $E^{1\%}_{1\,cm} = 2500$ at λ_{max} was used for all carotenoids.

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[15] E. LEISTNER, J. H. SCHMITT and M. H. ZENK, *Biochem. Biophys. Res. Commun.* **28**, 845 (1967).
[16] M. MCKENNA, M. D. HENNINGER and F. L. CRANE, *Nature* **203**, 524 (1964).
[17] D. R. THRELFALL, G. R. WHISTANCE and T. W. GOODWIN, *Biochem. J.* **106**, 107 (1968).
[18] H. K. LICHTENTHALER and K. BECKER, *Z. Naturforsch.* (in preparation).
[19] A. HAGER and TH. BERTENRATH, *Planta* **69**, 198 (1966).

The lipophilic benzoquinone compounds were separated by TLC, eluted with EtOH and estimated from the absorbance difference at λ_{max} before and after reduction with $NaBH_4$.[8] The plastoquinone values, given in the tables, represent total plastoquinone 45 consisting of quinone and hydroquinone. The latter was oxidized to the quinone by a 0·2% EtOH solution of $K_3Fe(CN)_6$ α-Tocopherol was measured quantitatively by the method of Emmerie and Engel.[20]

Acknowledgements—We are indebted to the Deutsche Forschungsgemeinschaft for financial support and to Mrs. M. Gahn for technical assistance.

[20] A. EMMERIE and C. ENGEL, *Z. Vitaminforsch.* **13,** 259 (1943).