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INTRACELLULAR LOCALIZATION OF TOCOPHEROL BIOSYNTHESIS IN *CALENDULA OFFICINALIS*

WIRGINIA JANISZOWSKA

Institute of Biochemistry, University of Warsaw, Żwirki i Wigury 93, 02–089 Warszawa, Poland

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Key Word Index—*Calendula officinalis*; Compositae; protoplasts; intracellular fractions; dynamics of labelling; tocopherols; phytylquinones; α-tocopherolquinone; vitamin K_1.

Abstract—[^{3}H]Phytol was administered to protoplasts from *Calendula officinalis* leaves, and in the subcellular fractions the dynamics of labelling of 7-monomethyltocol, 8-monomethyltocol (δ-tocopherol), 7,8-dimethyltocol (γ-tocopherol) and 5,7,8-trimethyltocol (α-tocopherol) and related phytylquinones, as well as those of vitamin K_1, were determined. By condensation with homogentisic acid two isomeric methylphytylquinones (2-methyl-5-phytylbenzoquinone and 2-methyl-6-phytylbenzoquinone) were formed. These compounds were cyclized to 7- and 8-methyltocol, respectively, or methylated to yield 2,3-dimethyl-5-phytylbenzoquinone. The latter appeared to be cyclized to γ-tocopherol which could be methylated to α-tocopherol. The prenylation reaction took place in the chloroplasts and microsomes. Some monomethyltocols and methylphytylbenzoquinones as well as vitamin K_1 which appeared to be formed in microsomes may have been transported to chloroplasts and mitochondria.

INTRODUCTION

In earlier studies [1] three methylated derivatives of tocol, 8-monomethyl-, 7,8-dimethyl- and 5,7,8-trimethyltocol (δ-, γ- and α-tocopherols, respectively), have been found in *Calendula officinalis* leaves. Studies on the distribution of these tocopherols in five purified cellular subfractions (chloroplast, mitochondrial, Golgi membranes, microsomal and cytosol) have shown that δ- and γ-T were present in chloroplasts, mitochondria and microsomes, whereas α-T was found only in the chloroplasts. No tocopherols have been found in Golgi membranes and cytosol [2].

Subsequently, small amounts of 7-monomethyltocol (7-MeT) have been detected in chloroplasts, mitochondria and microsomes of leaves of *C. officinalis* kept in the dark [3]. The third possible isomer of monomethyltocol (5-MeT) has been absent in all subfractions from this plant. Studies on the dynamics of labelling of tocopherols in the dark and in the light with [U-^{14}C]tyrosine have shown that in these three subcellular fractions both 7-MeT and δ-T were formed and were methylated to γ- and α-T not only in chloroplasts but also in microsomes. It has also been shown that light is not required for condensation of the aromatic ring with the phytyl chain, whereas it is indispensable for methylation of the aromatic ring of tocopherols.

Recently [4] it has been shown that tocopherolquinones and monomethylphytylbenzoquinones were present together with tocopherols in *C. officinalis*. Furthermore, it has been found that all these chromanols and quinones, except 7-MeT, were present during the whole vegetative period.

The aim of the present study was to investigate the site of biosynthesis of tocopherols and their translocation within the cell as well as to explain the role of phytylquinones in the biosynthetic sequence, using radioactive phytol as a precursor of the tocopherol chain.

RESULTS AND DISCUSSION

Newton and Pennock [5] have suggested that tocopherols can be synthesized outside the chloroplasts, and may be transported to plastids prior to or after introduction of methyl groups into the aromatic ring. Likewise, Schultz *et al.* [6] have stated that α-T was synthesized outside the chloroplasts from spinach leaves. However, these workers [7] have later shown that the chloroplast envelope membranes are the site of enzymatic prenylation and methylation in α-T synthesis. Our earlier studies [3] have indicated that tocopherols can be synthesized both in the chloroplasts and microsomal fraction. In order to conclusively elucidate whether the tocopherol synthesis really proceeds in these subfractions, in the present studies radioactive phytol (a precursor of the prenyl chain of tocopherols) was administered to *C. officinalis* leaf protoplasts, in preference to shoots, since the former are a more convenient material for administration and preparation of subcellular fractions.

It has been found earlier [3] that light is indispensable for introduction of the consecutive methyl groups into the aromatic ring. It has also been proved [8] that the uptake of radioactive precursors by protoplasts is more intense in the light than in the dark. Thus in the present studies, for the maximal uptake of radioactive precursor the protoplasts were kept for 15 hr in the dark, whereupon they were preincubated for 1 hr in the light (4.1 klx), and then under the same illumination conditions they were administered radioactive phytol (100 000 cpm per 20 mg protoplasts in 1 ml of solution). This amount of phytol was virtually completely absorbed during 1 hr.

It was found that no radioactive tocopherols and phytylquinones were formed in the cytosol and in the Golgi membrane fraction. Figures 1–4 illustrate the radioactivity changes in all investigated compounds occurring in the chloroplast, microsomal and mitochondrial fractions. The data presented in the figures are the results of five replicate experiments. The bars indicate the spread of the values and the points indicate the mean values. In these fractions neither radioactive γ-tocopherolquinone nor 2,3,6-trimethyl-5-phytylbenzoquinone were detected. The results show that the phytol administered to the protoplasts is utilized for phytylquinone synthesis and since it is clear that a pyrophosphate is required then the protoplasts must be capable of reacting phytol with ATP to form phytyl pyrophosphate.

In the microsomal fraction (Fig. 1), labelling of the investigated compounds is most rapid and most intense. 2-Methyl-5-phytylbenzoquinone and 2-methyl-6-phytylbenzoquinone (2-Me-5-PBQ and 2-Me-6-PBQ), are labelled first, 2-Me-5-PBQ being labelled to a greater extent than 2-Me-6-PBQ. The chromanol forms of these compounds (7-MeT and 8-MeT, respectively) are labelled less intensely. The radioactivity decreases very rapidly from the beginning of the experiment in 7-MeT, 2-Me-5-PBQ and in 2-Me-6-PBQ, whereas in 8-MeT it diminishes much slower, and after 4 hr it becomes stabilized. In 2,3-dimethyl-5-phytylbenzoquinone (phytyl plastoquinone, PPQ) the radioactivity attains a maximum after 1 hr and then drops, while radioactivity in γ-T gradually increases. In α-T the radioactivity increases for 4 hr, whereupon it diminishes. α-Tocopherolquinone (α-TQ) is labelled only very slightly and appears to remain in equilibrium with α-T.

In the chloroplast fraction (Fig. 2) 2-Me-5-PBQ and 2-Me-6-PBQ are labelled most rapidly, as was found in the microsomal fraction, but less intensly than in the microsomal fraction. However, in the chloroplast fraction, in contrast to the microsomal fraction, 2-Me-6-PBQ

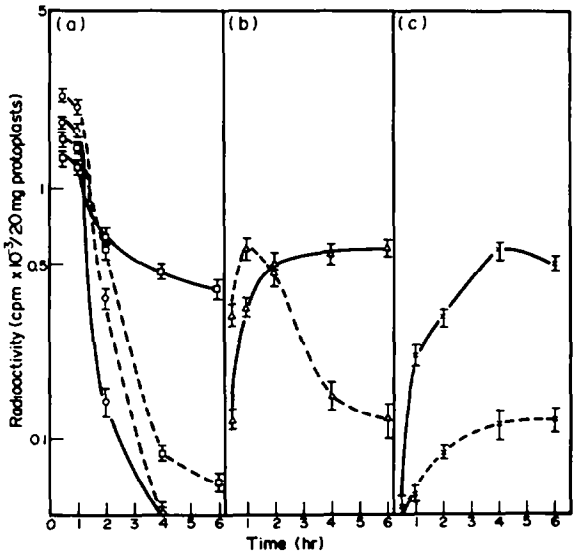

Fig. 1. Incorporation of [^{3}H]phytol into tocopherols (——) and quinones (– – –) by the microsomal fraction. (a) ○, 2-Methyl-5-phytylbenzoquinone, 7-MeT; □, 2-methyl-6-phytylbenzoquinone, 8-MeT; (b) △, PPQ, γ-T; (c) ×, α-T, α-TQ.

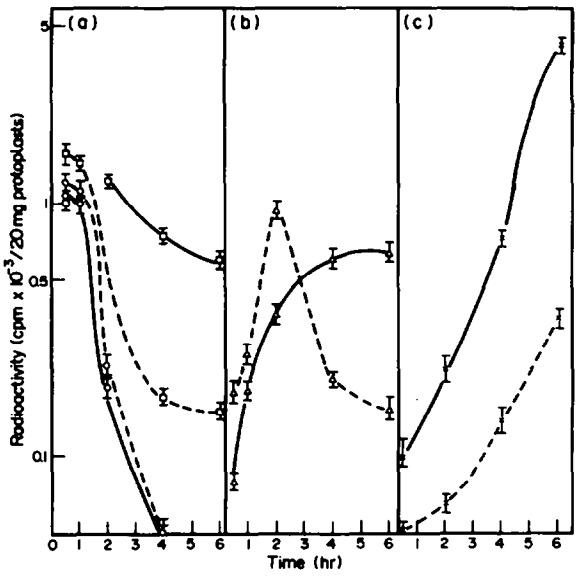

Fig. 2. Incorporation of [^{3}H]phytol into tocopherols (——) and quinones (– – –) by the chloroplast fraction. (a) ○, 2-Methyl-5-phytylbenzoquinone, 7-MeT; □, 2-methyl-6-phytylbenzoquinone, 8-MeT; (b) △, PPQ, γ-T; (c) ×, α-T, α-TQ.

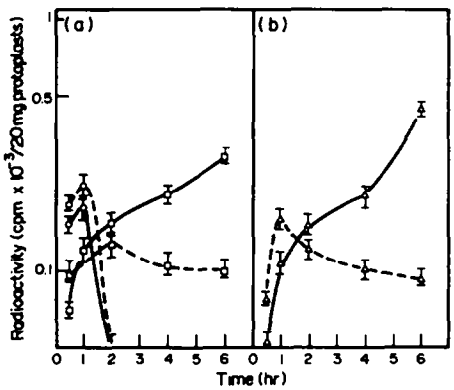

Fig. 3. Incorporation of [^{3}H]phytol into tocopherols (——) and quinones (– – –) by the mitochondrial fraction. (a) ○, 2-Methyl-5-phytylbenzoquinone, 7-MeT; □, 2-methyl-6-phytylbenzoquinone, 8-MeT; (b) △, PPQ, γ-T.

is labelled to a greater extent than 2-Me-5-PBQ. The chromanol forms, δ-T and 7-MeT, of these quinones are labelled somewhat less strongly. Subsequently in both monomethylphytylbenzoquinones and 7-MeT the radioactivity rapidly drops, and in 8-MeT and PPQ it increases. In both the latter compounds it attains a maximum after 2 hr, whereupon in 8-MeT it drops slowly and in PPQ rapidly. The drop in radioactivity in PPQ is paralleled by an increase in radioactivity in γ-T until 4 hr after precursor uptake, when both radioactivities become virtually steady. In α-T, the radioactivity at first increases slowly, and subsequently very rapidly until the end of the experiment. The α-TQ is labelled only slightly and its radioactivity increases all the time.

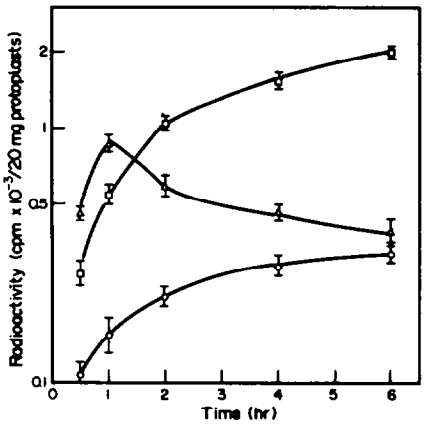

Fig. 4. Incorporation of [^{3}H]phytol into vitamin K_1 by microsomal (△), chloroplast (□) and mitochondrial (○) fractions.

In the mitochondrial fraction (Fig. 3) the labelling of all tocopherols and phytylquinones is several times less intense than in the other subfractions. In 2-Me-5-PBQ, 7-MeT and PPQ the radioactivity diminishes from a relatively high initial value, and the level in 2-Me-6-PBQ falls after 2 hr. In δ-T, as in γ-T, the radioactivity rises over the duration of the experiment.

The present results indicate that in *C. officinalis* leaf cells two sites of tocopherol biosynthesis may be present: one in the chloroplasts and another one in the microsomes. In the microsomal fraction tocopherols are labelled initially more intensely than those in the chloroplasts, perhaps because radioactive phytol, after penetration into the protoplasts, reaches the microsomes more readily. In the microsomes phytol is condensed with homogentisate to yield two isomers of phytylbenzoquinones, 2-Me-5-PBQ and 2-Me-6-PBQ. The results suggest that both quinones are methylated to the same extent to yield PPQ or are cyclized to 7-MeT and 8-MeT (δ-T), respectively. The high radioactivity in those monomethyltocols at the beginning and slower build up of PPQ suggest that cyclization of both monomethylquinones proceeds more rapidly than their methylation. The rapid fall in radioactivity in monomethylquinones could also result from transport to other cell organelles.

The radioactivity drop in 7-MeT and 8-MeT during the first hour after precursor uptake suggests that they can either be methylated to γ-T or be transported (as suggested for the monomethylquinones) to other cell organelles or perhaps it is more likely that both these processes occur simultaneously. The rapid metabolism of 7-MeT, 2-Me-5-PBQ and 2-Me-6-PBQ suggests that these are probably better substrates for methyltransferases than δ-T. After 2 hr following precursor uptake, the microsomal fraction contains no more than 10% of the radioactivity derived from phytol and from this time, methylation of 8-MeT to γ-T is very slow. The rapid formation of PPQ at the beginning of the experiment, followed by a fall in radioactivity in PPQ, with a simultaneous rise of radioactivity in γ-T, indicates that probably the second methyl group is substituted in the aromatic ring prior to cyclization. The absence of radioactive trimethylphytylbenzoquinone as well as the course of the labelling curves of α-T and α-TQ indicate that the third methyl group is substituted in the aromatic ring of γ-T, i.e after cyclization, with formation of α-T. α-TQ is not implicated in tocopherol biosynthesis but is most likely formed as a result of α-T oxidation. The relatively low labelling of PPQ, γ-T, α-T and α-TQ may suggest that these compounds, as well as their precursors (monomethylquinones and monomethyltocols) may be transported to other organelles, e.g. to the chloroplasts and mitochondria.

In the chloroplasts (Fig. 2), in contrast to the microsomes, 2-Me-6-PBQ is labelled more readily than 2-Me-5-PBQ in the initial stages. This suggests that 2-Me-6-PBQ can penetrate into chloroplasts from the microsomes more intensely than its isomer. It seems moreover, that also phytol is able to penetrate membranes to be used for phytylquinone synthesis. The further metabolism of the quinones in chloroplasts appears to be similar to that in the microsomes. Thus they may be methylated to PPQ or undergo cyclization to the related chromanols which can also be methylated to γ-T. δ-T, in contrast to 7-MeT, is metabolized more slowly. The course of the labelling of PPQ and γ-T, as well as the absence of radioactive trimethylphytylbenzoquinone, indicate that PPQ undergoes cyclization to γ-T. The latter seems to be methylated to α-T which accumulates in the chloroplasts as testified to by a steady increase in α-T radioactivity in this fraction (Fig. 2). Small amounts of α-TQ are likely to be formed as a result of α-T oxidation. These results are generally consistent with those obtained for isolated chloroplast from spinach leaves as well as for subfractions prepared from these chloroplasts [7,9]. The latter authors have shown that in the chloroplast envelope, homogentisate is prenylated to 2-Me-6-PBQ, which is methylated to PPQ; the latter undergoes cyclization to γ-T and is methylated to α-T. The α-T formed accumulates in the thylakoids.

In the mitochondrial fraction, slight labelling of tocopherols and phytylquinones and the course of their labelling curves suggests that they are transported to this fraction (mainly from the microsomes). It cannot be ruled out that some transformations of the investigated compounds may proceed also in the mitochondria. It seems probable that 2-Me-6-PBQ and PPQ may undergo cyclization to the chromanol forms. However, the course of the labelling curves of these compounds does not point to a precursor–product relationship between them. On the other hand, it seems that 2-Me-5-PBQ and 7-MeT can be methylated to PPQ and γ-T, respectively.

As stated earlier the present results confirm, on the one hand, that the chloroplasts are the site of tocopherol biosynthesis, and point, on the other, to the occurrence of another biosynthesis site of these compounds, i.e. the microsomal fraction. It is well known that homogentisate, the precursor of the aromatic ring of tocopherols, occurs in the microsomes and in the chloroplasts, whereas phytol or its pyrophosphate have not so far been detected beyond the chloroplasts. Therefore the question arises whether in fact the tocopherol biosynthesis proceeds in the microsomes. However, as earlier suggested by Newton and Pennock [5], tocopherols may be synthesized outside the chloroplasts. According to Botham and Pennock (cited in ref. [10]), methylation of γ-T to α-T can take place beyond the chloroplasts. Hutson and Threlfall [11] have administered radioactive homogentisate to lettuce leaves, and found that the biosynthesis of γ-T and α-T proceeds both in the chloroplasts and outside them. Unfortunately, these authors have analysed only the chloroplasts and the postchloroplast supernatant, and therefore they could not

identify the second site of tocopherol biosynthesis in the cell.

The possibility should also be considered that either the microsomes may contain unspecific prenaltransferases forming two isomers of monomethylquinones or that the microsomal fraction is contaminated with the chloroplast envelope where the biosynthesis of tocopherols is known to take place [9]. The latter explanation can be ruled out because it was found, using Hill's reaction to determine the percentage of envelope-free chloroplasts, that 85–95% of our chloroplasts possessed envelopes and since labelling of the investigated compounds in the microsomal fraction is 30–80% higher than in the chloroplasts initially, this radioactivity could not be derived from chloroplast envelope contamination.

In addition, a study was made of the labelling dynamics of vitamin K_1 which like the tocopherols, contains an isoprenoid side-chain derived from phytol. The results are presented in Fig. 4. As with the tocopherols, vitamin K_1 is labelled most rapidly in the microsomal fraction; the labelling attains a maximum after 2 hr, whereupon it diminishes, and from the 4th hour it remains unchanged. There is evidence of a slow accumulation of labelling in vitamin K_1 in the chloroplasts and a similar accumulation though at much lower level in the mitochondria. This is consistent with the finding of Schultz *et al.* [12] who state that the chloroplast envelope has the ability to prenylate 1,4-dihydroxy-2-naphtoic acid and to introduce the methyl group into the aromatic ring. The delay in vitamin K_1 labelling in the chloroplasts, as compared with the microsomes, is probably because the radioactive phytol penetrates the chloroplast only slowly.

EXPERIMENTAL

Isolation of protoplasts. Protoplasts were obtained according to Takebe *et al.* [13] from leaves of 5-week-old plants of *Calendula officinalis* L. cv Radio, cultivated in a lumistat [14], as described previously [8].

Preparation of radioactive phytol. A soln of $K_2Cr_2O_7$ (0.37 g) in H_2O (2 ml) was added to a soln of phytol (0.5 g) in Et_2O (20 ml) and then conc. H_2SO_4 (1 ml) was added dropwise with stirring. After 2 hr at room temp., the reaction was completed. The reaction product (1 mg) was reduced with $NaBT_4$, sp. act. 627 mCi/mM (Izinta, Budapest, Hungary) in basic MeOH for 16 hr at room temp. [2,3-^{3}H]Phytol was purified by TLC on silica gel G60, with $CHCl_3$ development.

Precursor administration. Protoplasts were treated with [2,3-^{3}H]phytol (100 000 cpm/20 mg of protoplasts), sp. act. 10.8 mCi/mM in 1% EtOH soln; they were processed 0.5, 1, 2, 4 and 6 hr after precursor uptake. Phytol which had not been taken up was removed by centrifugation according to Auguścińska and Kasprzyk [8].

Preparation of the cellular subfractions. Osmotically shocked protoplasts were centrifuged successively at 200 *g* for 1 min, 3000 *g* for 10 min, 14 000 *g* for 20 min and 105 000 *g* for 90 min. The 14 000 *g* pellet was suspended in 0.3 M sucrose containing 0.01 M Tris–HCL buffer, pH 7.6, and was further fractionated by sucrose density gradient centrifugation at 95 000 *g*, exactly as described in ref. [15]. The two membrane layers localized at the 1.0–1.25 M sucrose and 1.25–1.5 M sucrose interfaces were collected as the Golgi membrane fraction, and the pellet was the mitochondrial fraction. The chloroplast fraction was suspended in 0.3 M sucrose–5 mM $MgCl_2$–0.01 M phosphate buffer, pH 7.2, and it was purified by filtration through 1.6 M sucrose at 22 000 rpm for 60 min. All subfractions have earlier been characterized by light or electron microscopy and marker enzymes [2, 16].

Extraction. In each case, the pellet and final supernatant were extracted after Newton and Pennock [5]. Unlabelled tocopherols, phytylquinones and α-TQ were added as carrier substances (30 μg per aliquot) to each extraction soln. Chlorophylls were removed from the chloroplast extract on a Brockmann Grade 3 acidic alumina column developed with 30% Et_2O in hexane which eluted the chlorophyll-free lipid.

TLC systems. System I—silica gel with $CHCl_3$, system II—silica gel with hexane–Et_2O, 94:6; system III—the same but 4:1; system IV—the same but 3:7; system V—silica gel with C_6H_6; system VI—reversed-phase system Kieselguhr impregnated with 5% paraffin oil in hexane with Me_2CO–H_2O, 4:1; system VIII—the same but 19:1. All substances were characterized by cochromatography with markers. The chromanols were detected with the Emmerie–Engel reagent or 0.01% fluorescein in EtOH and examination under a UV at 254 nm. The quinones were detected by quenching fluorescence at 254 nm or spraying with leucomethylene blue [5, 9, 12, 15].

Isolation of tocopherols and phytylquinones. The extracts were evapd to dryness under N_2. The residue was dissolved in a minimum of Et_2O and chromatographed in system I. After recovery of the compounds from this system, phytylquinones were purified in systems II and VII, γ-T and α-T in systems III and VI, 7-MeT and 8-MeT in systems IV and VI [5, 9, 12, 17].

Nitroso-derivatives of 7-MeT, 8-MeT and γ-T were obtained according to ref. [18] and chromatographed in system V.

General methods. The percentage of envelope-free chloroplasts in intact chloroplasts was calculated from the difference in ferricyanide reduction (Hill's reaction) between intact and osmotically shocked chloroplast preparations [19].

Chlorophyll content was determined according to Arnon [20].

The radioactivity of compounds was measured with a scintillation soln containing PPO (3 g/l) and POPOP (0.3 g/l) in toluene.

Chemical synthesis. Synthesis of monomethyltocols was carried out as described by Marcinkiewicz *et al.* [21]. Monomethylquinones and PPQ were prepared from phytol and toluquinol or 2,3-dimethylbenzoquinol, respectively, and oxidized with Ag_2O according to Burnett and Thomson [22].

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