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ON THE INTRACELLULAR SITE OF BIOSYNTHESIS OF α-TOCOPHEROL IN *HORDEUM VULGARE*

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Key Word Index—*Hordeum vulgare*; Gramineae; site of biosynthesis; α-tocopherol; plastoquinone.

Abstract—When barley seedlings were exposed to $^{14}CO_2$ for 1–3 hr, ^{14}C was preferentially incorporated into plastoquinone, but only in low amounts into α-tocopherol. On the other hand, on treating excised shoots with DL-tyrosine-[3-^{14}C], it was observed that ^{14}C was incorporated in equal amounts into both plastoquinone and α-tocopherol. From these results it is concluded that α-tocopherol is synthesized outside the chloroplast.

INTRODUCTION

The chloroplast membrane (envelope) is relatively impermeable to mevalonate and its pyrophosphate [1]. As a result of this phenomenon ^{14}C from $^{14}CO_2$ is incorporated preferentially into intraplastidic isoprenoids while ^{14}C from mevalonate-[2-^{14}C] is incorporated into extraplastidic compounds. By making use of this differential incorporation it was demonstrated that plastoquinone and phylloquinone are synthesized intraplastically, whereas ubiquinone is formed extraplastically [2]. In the case of tocopherols there is some uncertainty as to the site of biosynthesis [2, 3]. In the present work, an improved method for treating barley seedlings was developed in order to obtain an indication of the site of biosynthesis of α-tocopherol.

RESULTS

Incorporation of $^{14}CO_2$

After fractionating the lipids and isoprenoids of barley seedlings (in the stage of primary leaf) exposed to $^{14}CO_2$ for 60 min, considerable radioactivity was found not only in the lipid zones but also in the isoprenoid zones such as those of the carotenoids, chlorophylls, and prenylquinones. The investigation of prenylquinones by TLC with system I (Fig. 1a) and subsequent rechromatography of the plastoquinone zone with reversed phase system II (Fig. 1b) indicated the incorporation of ^{14}C into plastoquinone. On the other hand, no similar observation could be made in the case of α-tocopherol. The activity contaminating the α-tocopherol zone (Fig. 1a) was separated by the subsequent rechromatography (Fig. 1c). Only a small part of the separated substance was identified as plastoquinol. α-Tocopherolquinone and α-tocopherolquinol (indicated by arrows in Fig. 1c) were found to be inactive. As shown in Table 1 the ratio of ^{14}C incorporated into plastoquinone and that into α-tocopherol shifted from 20:1 after treatment for 80 min (ex-

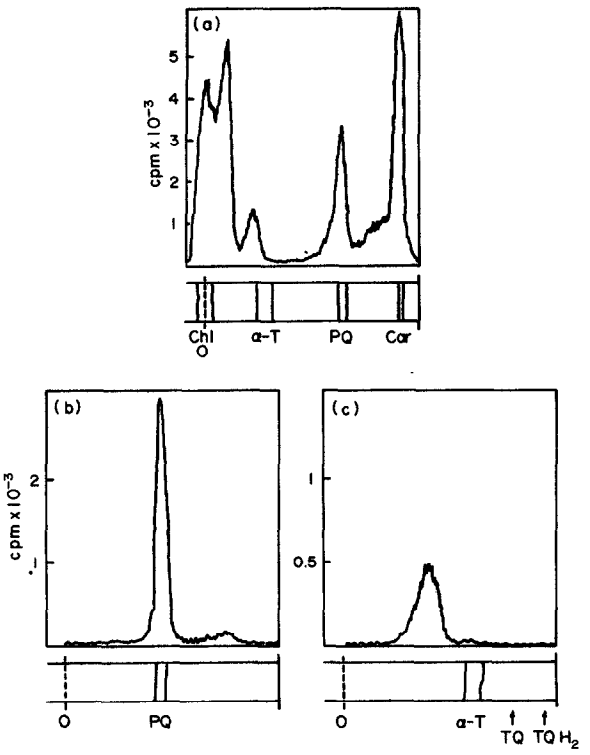

Fig. 1. Radioscans of thin layer chromatograms of the lipids obtained from barley exposed to $^{14}CO_2$. 1a: TLC of the lipids with system I. 1b: Rechromatography of the plastoquinone zone with system II. 1c: Rechromatography of the α-tocopherol zone with system II. Experimental conditions: time of exposure 60 min; temperature 22–23°; 175 seedlings (6.6 g fr. wt) were treated with 500 μCi $^{14}CO_2$ (sp. act. 1 μCi/μmol); for light intensity, and CO_2 concentration see the Experimental section. Car, carotene; Chl, chlorophyll; PQ, plastoquinone; α-T, α-tocopherol; TQH_2, α-tocopherolquinol; TQ, α-tocopherolquinone; O, origin.

‡ This forms a part of the experimental work for the state examination of Edeltrudis Schoensee.

periment 1) to 12.8:1 after 150 min (experiment 2). From these results it might be concluded that CO_2 was incorporated predominantly into plastoquinone in the early stages and also into α-tocopherol in the later stages.

Incorporation of DL-*tyrosine*-[3-^{14}C]

DL-Tyrosine-[3-^{14}C] was added to the lower part of the excised shoots for 90 min without light followed by 270 min with light. TLC and subsequent rechromatography with the systems mentioned above confirmed the incorporation of ^{14}C into plastoquinone and α-tocopherol (Fig. 2a–c) as described previously in detail by Threlfall *et al.* [2].

Two peaks of radioactivity with lower R_f-values than that of α-tocopherol accompanied the zones of α-tocopherolquinone and α-tocopherolquinol, but these were not investigated. As expected, only negligible radioactivity was found in chlorophylls and carotenoids.

In experiment 4 (Table 1), the incorporation of ^{14}C into plastoquinone as well as into α-tocopherol was in the same magnitude after a period of treatment of 60 min without light followed by 150 min with light. In experiment 3 (Table 1), the time of exposure was shortened by vacuum infiltration of the labelled substance into the lower part of the shoot. α-Tocopherolquinol and α-tocopherolquinone were found only in traces in this experiment.

When DL-mevalonate-[2-^{14}C] was added to shoots, low radioactivity was observed in the zones of chlorophylls, carotenoids, and prenylquinones. This might be due to the fact that mevalonate was incorporated predominantly into sterols and their esters [2]. Therefore, no further experiments were performed.

Biosynthesis of other phenols

Though synthesis of plastoquinone took place under photosynthetic conditions with low CO_2-partial pressure (<10 μmol/l. ≙ <0.02 vol. %) and moderate light in-

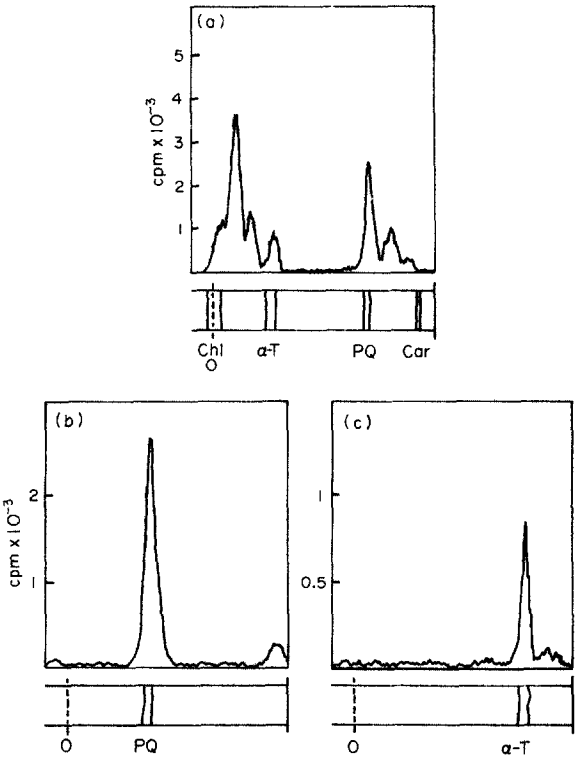

Fig. 2. Radioscans of thin layer chromatograms of the lipids obtained from barley incubated with DL-tyrosine-[3-^{14}C]. 2a: TLC of the lipids with system I. 2b: Rechromatography of the plastoquinone zone with system II. 2c: Rechromatography of the α-tocopherol zone with system II. Experimental conditions: time of exposure 90 min without light followed by 270 min with light; temperature 28–32°; 175 excised shoots (6.2 g fr. wt) were treated with 14 μCi DL-tyrosine-[3-^{14}C] (sp. act. 15.2 μCi/mol); for light intensity, and CO_2 concentration see the Experimental section; for abbreviations see Fig. 1.

Table 1. Incorporation of ^{14}C from $^{14}CO_2$ and DL-tyrosine-[3-^{14}C] into plastoquinone (PQ) and α-tocopherol (α-T) of barley seedlings and excised shoots, respectively

	Time of exposure (min)		TLC system I Amount (nmol)	Sp. act. (dpm/nmol)	Re-TLC system II Amount (nmol)	Sp. act. (dpm/nmol)	Incorporation (dpm)†	Ratio (dpm_{PQ})/($dpm_{\alpha-T}$)
$^{14}CO_2$								
Expt 1	80 L*	PQ	140.8	1575	55	1537	216400	20.6:1
		α-T	268	1261	11	39	10450	
Expt 2	150 L	PQ	144		46.5	1132	162000	12.8:1
		α-T	108.5		28.8	117	12680	
DL-Tyrosine-[3-^{14}C]								
Expt 3	75 L	PQ	98‡	458	34.6	570	55900‡	0.7:1
		α-T	220‡	160	34.5	364	80100‡	
Expt 4	60 D + 150 L	PQ	177	809	108	595	105000	1.46:1
		α-T	495	216	199	156	72000	

Experimental conditions: temperature 25–28°; for light intensity, and CO_2 concentrations see the Experimental section; expt 1: 175 seedlings (6.3 μg fr. wt) were treated with 1000 μCi $^{14}CO_2$; expt 2: 175 seedlings (7.4 g fr. wt) were treated with 400 μCi $^{14}CO_2$; expt 3: 175 excised shoots (8.1 g fr. wt) were vacuum-infiltrated with 16.6 μCi DL-tyrosine-[3-^{14}C] (15.2 μCi/μmol); expt 4: 175 excised shoots (9.1 g fr. wt) were treated with 16.6 μCi DL-tyrosine-[3-^{14}C] (15.2 μCi/μmol).

* L, with light; D, without light. † Specific radioactivity in system II × amount in system I. ‡ Values estimated.

tensities (7500 lx, white light), synthesis of the flavonoids of barley could not be carried out under these conditions [4]. The flavone saponarin (7-glucoside of 6-C-glucosyl-apigenin) was synthesized only under optimum conditions as described in the Experimental section. Figure 3 illustrates the incorporation of ^{14}C from $^{14}CO_2$ into saponarin which was chromatographed with system III (Fig. 3a) and subsequently rechromatographed with system IV (Fig. 3b). However, the distribution of radioactivity between the aglycone and the C- and O-glycosidic moieties has not been determined.

DISCUSSION

From the results of the experiments presented above, it might be concluded that an extraplastidic site of biosynthesis of α-tocopherol exists which is actively operating under the conditions of these experiments. That might be valid also for synthesis of α-tocopherolquinol and α-tocopherolquinone as constituents of a redox system. On the other hand, the results of fractionation procedures on *Fucus spiralis* with α-, β-, γ- and δ-tocopherol, on leaves of *Phaseolus vulgaris*, on fruits of *Lycopersicum esculentum* with α- and γ-tocopherol [5], and on leaves of *Vicia faba* with α-tocopherol [6] indicated that δ-, but also β- and γ-tocopherol were present in the supernatant fraction representing the non-plastidic fraction, whereas α-tocopherol was found only in the fraction precipitated at higher *g*-values containing plastid particles. To account for this apparent discrepancy, Newton and Pennock [5] have suggested that tocopherols may be synthesized outside the chloroplast and transported into the plastid before or after the final methylation step.

As described in the following paper [7], the biosynthesis of plastoquinone could be demonstrated in intact, isolated chloroplasts, but not that of α-tocopherol. Since plastoquinone and tocopherol are formed via the *p*-hydroxyphenylpyruvate-homogentisate pathway [8], it might be assumed that the initial stages of this pathway are located inside as well as outside the chloroplast. Shortening the period of treatment from 1–2 days to *ca* 1–3 hr requires an optimization of photosynthetic conditions. Though intraplastidic and extraplastidic sites are separated by the chloroplast membrane, an exchange of metabolites has to be assumed within this period. According to Heber [9] the metabolites of photosynthesis are transported at relatively high rates. Therefore, no depletion of substrate can be expected for extraplastidic synthesis of mevalonate (via acetyl-S-CoA and acetoacetyl-S-CoA). Thus it might be assumed that differences of mevalonate synthesis at the two sites are attributable to some regulatory effect.

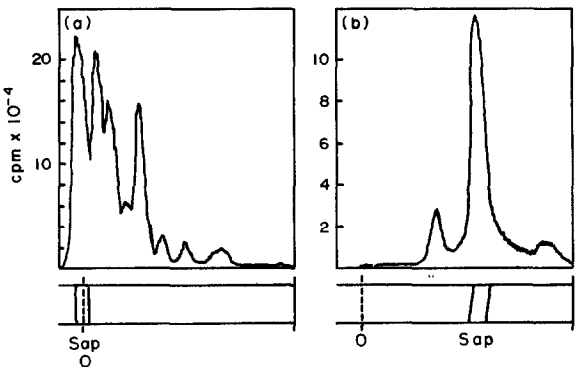

Fig. 3. Radioscans of the thin layer chromatogram of the water phase containing saponarin (Sap) obtained from the $^{14}CO_2$ experiment: 3(a): TLC with system III. 3(b): Rechromatography with system IV. Experimental conditions are described in Fig. 1.

EXPERIMENTAL

Cultivation of seedlings of barley (*Hordeum vulgare* cv Nota) is described in Ref. [10].

Vessels for treating barley seedlings with radioactive substances. Vessels consisted of a perforated PVC-base plate and an upper chamber composed of a PVC-cylinder with a fixed glass plate. The cylinder (diam. 12 cm; ht = 7 cm) contained (a) a water cooling system, (b) an external gas circulation system with a peristaltic pump, and (c) a measuring unit (with a micro photo-electric cell and a thermistor). Determination of CO_2-partial pressure was carried out photometrically [11] in a cuvette incorporated in the gas circulation system. Light intensities were maintained at 14000–15000 lx with halogen lamps.

Application of $^{14}CO_2$ to rooted seedlings. In the $^{14}CO_2$ experiments, the shoots of the rooted seedlings (n = 88 per vessel) were inserted through the perforated plate and affixed with cold glue. Simultaneously with switching-on the light, $^{14}CO_2$ was released by injecting 50 μl 0.5 M $NaH^{14}CO_3$ into the side vessel of the gas circulation system containing H_3PO_4 at time intervals of 10 min (for sp. act. see Figures and Table). The concentration of CO_2 was adjusted to 40 ± 10 μmol/l. The rate of photosynthesis ranged from 45 to 67 μmol CO_2/mg chlorophyll/hr.

Application of DL-*tyrosine*-[3-^{14}C] *and* DL-*mevalonate*-[2-^{14}C] *to the upper part of the excised shoots.* Shoots were inserted from above through the performed plate into micro vessels fitted below. Each micro vessel contained 20 μl of aq. radioactive soln per shoot. Unlabelled CO_2 was used at the concentration mentioned above.

Isolation and determination of prenylquinones. Extraction of the plant material. Shoots (7–9 g fr. wt) of 175 seedlings were homogenized in 70 ml Me_2CO and filtered under red. pres. After adding 70 ml petrol (40–60°) to the filtrate, it was washed free of Me_2CO with 2 portions of 700 ml H_2O. The petrol phase was dried with Na_2SO_4 and fractionated over Si gel with petrol (60–80°)–Et_2O (4:1). *TLC.* (All layers with fluorescence indicator F254) system I: Si gel with petrol (60–80°)–Et_2O (10:1); rechomatography on system II: cellulose (impregnated with paraffin oil) with Me_2CO–MeOH (2:1) [12]. *Detection (a) and determination (b).* Plastoquinone—(a) quenching of fluorescence at 254 nm; (b) at 255 nm according to Ref. [13]. Plastoquinol—(a) and (b) as for plastoquinone after oxidizing (1 day; air) the plastoquinol zone on Si gel layer developed with system I. α-Tocopherol—(a) by spraying 1/20 of the TLC plate with Emmerie–Engel reagent; (b) at 546 nm after treatment with the same reagent. α-Tocopherolquinone—(a) quenching of fluorescence at 254 nm; (b) at 262 nm according to Ref. [14]. α-Tocopherolquinol—(a) and (b) as for α-tocopherolquinone after oxidizing the tocopherolquinol zone on Si gel layer as mentioned above.

Isolation and determination of C-glycosyl-flavones (*i.e. saponarin*). Isolation, adsorption on polyamide, and determination are described in Ref. [10]. TLC with system III: Si gel with C_6H_6–MeOH–HOAc (45:8:4); rechromatography on system IV: cellulose with *n*-BuOH–HOAc–H_2O (4:1:2.2).

Assays of ^{14}C-radioactivity. The scintillator was a soln consisting of 4.5 ml Unisolve (W. Zinser, Frankfurt) + 4.5 ml 96% EtOH containing the sample.

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