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DETERMINATION OF PHYTYL DIPHOSPHATE AND GERANYLGERANYL DIPHOSPHATE IN ETIOLATED OAT SEEDLINGS

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Key Word Index—*Avena sativa*; Gramineae; etiolated seedlings; chlorophyll biosynthesis; tetraprenyl diphosphates; phosphate determination.

Abstract—The supernatant of a homogenate from etiolated oat seedlings was analysed for geranylgeranyl diphosphate and phytyl diphosphate. Direct GLC analysis of free alcohols after treatment of the crude extract with alkaline phosphatase was not possible because of interfering compounds. New methods for the purification and determination of geranylgeranyl diphosphate and phytyl diphosphate are described.

INTRODUCTION

On illumination, dark-grown (etiolated) oat and bean seedlings accumulate chlorophyll$_{GG}$* which is then hydrogenated to chlorophyll$_P$ [1, 2]. The determination of the enzyme specificity of 'chlorophyll synthetase', the enzyme which catalyses the esterification of chlorophyllide [3], suggested that geranylgeranyl diphosphate (GGPP) should be present in these etiolated seedlings. The soluble fraction of a homogenate from etiolated oat seedlings was investigated by means of the chlorophyll synthetase reaction [4]. It was concluded that either GGPP or a biogenetic precursor thereof but no phytyl diphosphate was present in the soluble extract [4].

The presence of phytyl diphosphate in soluble fractions from homogenates of etiolated French bean [5] and barley seedlings [6] has been reported. In these studies crude extracts were treated with alkaline phosphatase and the free phytol was then identified by GLC. This method does not distinguish between phytyl diphosphate and phytyl monophosphate. The extracts were apparently not tested for the presence of geranylgeraniol. These authors also found free phytol when they extracted the plants with organic solvents. We were unable to confirm the presence of free phytol in such extracts by GLC [7].

The hydrogenation of [^{14}C]geranylgeranyl diphosphate to phytyl diphosphate was demonstrated with a membrane-bound enzyme from spinach chloroplasts [8, 9]. Phytyl diphosphate rather than GGPP is incorporated into α-tocopherol [10] and phylloquinone precursors [11]. The formation of phytyl diphosphate, therefore, seems to be an essential part of the biosynthesis of these compounds. This does not mean, however, that phytyl diphosphate must be found in the soluble fraction. The association of prenyl diphosphates with membranes has been discussed earlier [12].

We describe here the analysis of the soluble fraction from etiolated oat seedlings for GGPP, phytyl diphosphate and the corresponding monophosphates.

RESULTS AND DISCUSSION

An extraction procedure which leaves plastids intact was chosen. The soluble extract would therefore contain mainly compounds of the cytoplasm and vacuole. It had, however, been demonstrated that GGPP can easily penetrate the plastid envelope membrane [4]. So its presence was expected in the cytoplasm if it was accumulated in the plastid stroma. The incorporation of [^{14}C]phytyl diphosphate into chlorophyll in plant cell suspension cultures demonstrated that also this compound can penetrate the plastid envelope membrane (J. Benz and W. Rüdiger, unpublished work). Therefore, both soluble GGPP and phytyl diphosphate would be extracted into the supernatant by our procedure.

We tried at first to detect these compounds according to a previously described method [5, 6]. The supernatant was extracted with petrol, then incubated with alkaline phosphatase and again extracted with petrol. The second organic phase should contain only those components which became lipophilic after reaction with phosphatase. We found many peaks in the GLC of this organic phase, two of which coincided with the peaks of phytol and geranylgeraniol. However, the position of these peaks, unlike those of the authentic alcohols, was not changed on formation of the TMSi derivatives [1]. In conclusion, we were unable to detect phytyl diphosphate or GGPP by this method.

We then rigorously purified the diphosphate fraction before reaction with alkaline phosphatase. The efficiency of the single steps had been tested with synthetic phytyl diphosphate and GGPP. For the exact determination of the yield in each single step, a small amount of [1-^{3}H]geranylgeranyl diphosphate was added to the supernatant of the crude extract. The purification steps are described in the Experimental, and the results are summarized in Table 1. The final yield of 51 % was used as the basis for calculating the concentration of all tetraprenyl

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*Abbreviations: Chlorophyll$_{GG}$, geranylgeranyl chlorophyllide; chlorophyll$_P$, phytyl chlorophyllide; GGPP, geranylgeranyl diphosphate; GGMP, geranylgeranyl monophosphate.

Table 1. Purification of tetraprenyl phosphates from etiolated oat seedlings

Step No.	Purification method	Yield (% radioactivity)
1	Deproteinization with acetone	99
2	Anion exchanger DE 52	95
3	Adsorption resin XAD-2	72
4	Gel chromatography Sephadex LH-20	71
5	Chromatography, silica gel	66
6	Adsorption resin XAD-2	51

Oat shoots (1.5 kg) were homogenized and centrifuged. To the clear supernatant, [1-^{3}H]geranylgeranyl diphosphate (30 nmol, 12 000 Bq = 100%) was added. The yield of tetraprenyl phosphates in each step was determined by the recovery of radioactivity. For details of purification steps see Experimental.

phosphate in the original extract.

The solution from step 6 was analysed by TLC for phosphate esters. Only two phosphate spots were visible after staining which corresponded to GGPP and geranylgeranyl monophosphate. No inorganic phosphate was present. We therefore analysed the solution for total phosphate after acid hydrolysis of the organic phosphates. We adopted a spectrophotometric method from a sensitive staining reagent [13]. We found this method at least one order of magnitude more sensitive than the previously described methods for the determination of phosphate esters [14]. Although phosphate esters also develop some blue colour, we found the method only reliable with inorganic phosphate, i.e. after acid hydrolysis of phosphate esters. The results are shown together with those of the subsequent GLC analyses in Table 2.

The tetraprenyl diphosphates were determined by densitometry on the TLC plate after staining according to ref. [13]. The method was calibrated with known amounts of authentic GGPP. The tetraprenyl monophosphates were determined with the same calibration curve. The monophosphate fraction also contained radioactivity (*ca* 7% of that of the diphosphate fraction). Because only [1-^{3}H]geranylgeranyl diphosphate had been added to the extract, it can be concluded that the monophosphate was produced from the diphosphate during extraction and purification.

Because the TLC method does not separate GGPP from phytyl diphosphate and GGMP from phytyl monophosphate, the nature of tetraprenyl alcohols present in both fractions was determined by TLC and GLC after hydrolysis with alkaline phosphatase. Only geranylgeraniol and phytol were detected in these fractions, the ratio of which was determined by GLC. The mono- and the di-phosphate fractions contained the same ratio of geranylgeraniol to phytol. This was to be expected since the monophosphates are artefactual hydrolysis products of the diphosphates. The geranylgeraniol contained two *cis*-isomers besides the all-*trans* compound similar to the synthetic sample. We assumed that some isomerization occurred during the purification procedure.

From these data, the amount of GGPP present in the original extract was calculated as 16.0 nmol/g fr. wt including the monophosphate which was derived from GGPP by partial hydrolysis (Table 2). The corresponding value for phytyl diphosphate was 2.0 nmol/g fr. wt.

Table 2. Determination of geranylgeranyl diphosphate (GGPP), geranylgeranyl monophosphate (GGMP), phytyl diphosphate (PPP) and phytyl monophosphate (PMP) via analysis of phosphates and free alcohols

Compound	Method of determination	Amount (nmol/g) fr. wt)	Corresponding to
Total phosphate after hydrolysis	Colorimetry	17.0	GGPP, GGMP, PPP, PMP
Tetraprenyl diphosphates	Densitometry	15.4	GGPP, PPP
Tetraprenyl monophosphates	Densitometry*	2.6	GGMP, PMP
Total geranylgeraniol	GLC†	16.0	GGPP, GGMP
Total phytol	GLC†	2.0	PPP, PMP
Geranylgeranyl diphosphate	Calculated from	13.7	GGPP
Geranylgeranyl monophosphate	the data given above	2.3	GGMP
Phytyl diphosphate		1.7	PPP
Phytyl monophosphate		0.3	PMP

The compounds were determined in aliquots of the solution from purification step 6 (see Table 1). The values were corrected for the yield of added [1-^{3}H]geranylgeranyl diphosphate.

*By ratio of tetraprenyl diphosphate: tetraprenyl monophosphate.

†By ratio of geranylgeraniol: phytol, assuming a total amount of 18.0 nmol/g fr. wt.

Because the oat seedlings contain *ca* 10 nmol chlorophyllide/g fr. wt after a short irradiation, the amount of GGPP would be sufficient to esterify this pigment whereas the amount of phytyl diphosphate would not be sufficient. The added amount of [1-^{3}H]geranylgeranyl diphosphate does not interfere with this determination because it was present at a final concentration of only 20 pmol/g fr. wt.

Watts and Kekwick [5] and Liljenberg [6] found about ten times more phytyl diphosphate than we were able to detect. Besides the different plant species used, this difference could be due to several reasons: (i) We used capillary GLC whereas packed columns were used before. Some compounds (e.g. *cis*-geranylgeraniol) are not readily separated from phytol on packed columns and could, therefore, erroneously be taken as phytol. (ii) The extraction procedures used in refs. [5, 6] could solubilize phytyl diphosphate from other compartments, e.g. from membranes. We investigated only soluble compounds. (iii) Phytol esters are slowly degraded during storage of seeds. Thus seedlings grown from aged seeds contain less phytol esters than seedlings from fresh seeds [7]. If this were also true for phytol diphosphate, it could explain the observed difference: we used aged oat seeds throughout because these germinate much better than fresh oat seeds which are still dormant.

EXPERIMENTAL

Step 1. *Extraction and protein precipitation.* Seedlings of oat (*Avena sativa* L. var. Pirol, Bayerische Futter- und Saatbau GmbH, München) were grown on moist vermiculite at 27–28° for 7 days in the dark. The shoots (1.5 kg fr. wt) were homogenized with a buffer (pH 7.5) containing 0.45 M D-(−)-sorbitol, 0.2% bovine albumin, 1 mM $MgCl_2$, 8 mM KH_2PO_4, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 1 mM ascorbic acid and 1 mM NaF. The homogenate was filtered through cheesecloth. The filtrate was clarified by centrifugation (2500 *g* for 30 min, then 40000 *g* for 1 hr). For determination of the yield in each single step, [1-^{3}H]geranylgeranyl diphosphate (30 nmol, 1.2×10^4 Bq) was added to the clear supernatant. The aq. soln (2500 ml) was mixed with Me_2CO (3000 ml). The protein ppt. was removed by centrifugation (2500 *g*) and washed twice with 60% aq. Me_2CO. The soln and combined washings were extracted several times with EtOAc to remove free phytol, geranylgeraniol, and yellow pigments. The aq. phase was partly evapd under red. pres. (40°) to remove Me_2CO and then lyophilized.

Step 2. *Anion exchange.* The lyophilisate was dissolved in H_2O (200 ml) and divided into three batches. To each batch 500 g exchange resin (Whatman DE 52) was added and the mixture slowly stirred for 90 min. The mixture was then transferred to a Büchner funnel. The resin was washed with 0.01 N NH_3 until all neutral contaminants were removed (Fehling test for reducing sugars). The aq. filtrate and washings did not contain any radioactivity and were discarded. The resin was then washed with Et_2O–EtOH–MeOH (1:1:1, 2 × 250 ml). This eluted only 4% of the radioactivity. The resin was resuspended with 0.01 N NH_3 and the organic phosphates were eluted with 0.2 M $(NH_4)_2CO_3$ (yield of radioactivity 90%). The eluate was concd under red. pres. to remove traces of organic solvents and NH_3, and then lyophilized.

Step 3. *Adsorption resin* (*modified after ref.* [15]). The lyophilisate of each batch of step 2 (corresponding to 500 g fr. wt of seedlings) was dissolved in H_2O, adjusted to pH 10.0 with 1 N NaOH and added to the mixture of 150 g activated XAD-2 resin [15] in 100 ml 0.01 N NH_3. The mixture was slowly stirred for 90 min. The resin was washed on a Büchner funnel with 0.01 N NH_3 until the filtrate was colourless. The organic phosphates were then eluted with *n*-PrOH–25% NH_3–H_2O (6:3:1) until no more radioactivity was eluted (yield 76%). With MeOH–NH_3 used in ref. [15] for elution, the yield never exceeded 25%.

Step 4. *Gel chromatography.* The conc. solns of all batches of step 3 were combined and placed onto a column of Sephadex LH-20 (7 × 15 cm) which had been equilibrated with 0.01 N NH_3. The column was eluted with the same solvent. The radioactivity of each fraction (5 ml) was determined. Fractions 16–44 which contained 98% of the applied radioactivity were combined and lyophilized after addition of 2–3 drops of 1 N NaOH. This step removed many low MW compounds because the organic phosphates apparently form micelles.

Step 5. *Column chromatography* (*modified after ref.* [16]). The residue of step 4 was dissolved in 5 ml PrOH–25% NH_3 (12:1), clarified with a few drops of H_2O, and applied onto a column (4 × 25 cm) of silica gel G-60 (Merck) which had been equilibrated with the same solvent. The column was eluted as follows: 125 ml PrOH–25% NH_3 (12:1); 125 ml PrOH–25% NH_3–H_2O (83:14:3); 125 ml PrOH–25% NH_3–H_2O (79:17:4); 450 ml PrOH–25% NH_3–H_2O (6:3:1). Fractions of 8–9 ml were collected. Fractions 23–29 contained free alcohols (4% of applied radioactivity); fractions 57–79 contained the organic phosphates (93% of applied radioactivity). Fractions 57–79 were combined. The solvent was removed by evapn and lyophilization as above.

Step 6. *Adsorption resin.* Step 3 with adsorption resin XAD-2 (150 g for combined eluates of step 5) was repeated. Before elution of the organic phosphates, the resin was washed with MeOH–H_2O (2:3) containing 0.01 N NH_3. This removed most of the yellow contaminants, but only 10% of radioactivity. The yield of radioactivity by subsequent elution with PrOH–25% NH_3–H_2O (6:3:1) was 75%.

Step 7. *TLC* [17] *and GLC.* Aliquots of the eluate of step 6 were applied to silica gel HPTLC plates which had been washed with MeOH and dried before use. The chromatograms were developed with PrOH–25% NH_3–H_2O (6:3:1). The organic phosphates were stained with the reagent of Bochner *et al.* [13]. The R_f values were: organic diphosphates, 0.41; organic monophosphates, 0.53; tested with authentic mono- and di-phosphates of geranylgeraniol, phytol and farnesol. The (unstained) mono- and di-phosphates were eluted with PrOH–25% NH_3–H_2O (6:3:1). After removal of PrOH and NH_3 by partial evapn (see above), the aq. soln was adjusted to pH 10.5 with 0.1 N NaOH and incubated with alkaline phosphatase (2.2 units/ml, Sigma) at 21° for 12 hr and at 37° for 1 hr. This resulted in 68% hydrolysis to free alcohols as monitored by radioactivity. The free alcohols were extracted with petrol (40–60°) and purified by TLC on silica gel plates with petrol (60–80°)–Et_2O (11:9). R_f values of authentic compounds: farnesol, 0.36; geranylgeraniol, 0.37; phytol, 0.40; solanesol, 0.48; geranyllinalool, 0.59. The zones between R_f 0.35 and 0.42 were eluted with Me_2CO. The eluted compounds were investigated by GLC after transfer into *n*-hexane. Detector: FID, He at 60 ml/min, isothermal at 186°, injector and detector 225°, WCOT glass capillary column OV 1 (0.3 mm × 20 m) splitless, sample vol. 1 μl.

Step 8. *Spectrophotometric estimation of phosphate esters.* The sample (250 μl) containing GGPP or other allylic phosphates was acidified with HCl (final concn 1 M) and heated for 40 min at 60°, or 10 min at 100°. After cooling, the hydrolysate was incubated with 500 μl of the reagent of Bochner *et al.* [13] for 1 hr. Then 2 ml H_2O was added and A at $\lambda_{max} = 815$ nm determined immediately. The calibration curve was determined with $Na_4P_2O_7$ (0.2–7 ng P). Compounds of crude plant extracts can cause a decrease of A_{815} nm, therefore calibration was performed with the particular extract.

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