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SYNTHESIS OF 3-POLYPRENYLTOLUQUINOLS AND 4-CARBOXY-2-POLYPRENYLPHENOLS BY CELL-FREE PREPARATIONS OF *EUGLENA GRACILIS*

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Key Word Index—*Euglena gracilis*, Euglenophyceae, 3-polyprenyltoluquinols, 4-carboxy-2-polyprenylphenols, biosynthesis, intracellular distribution

Abstract—Cell-free homogenates of *Euglena gracilis* are able to carry out the light- and Mg^{2+}-independent syntheses of approximately equal amounts of a nonaprenyltoluquinol, an octaprenyltoluquinol and two uncharacterized compounds referred to as chromanols from homogentisate and a *Micrococcus luteus* extract that has been preincubated with isopentenylpyrophosphate (MLE-IPP). In addition they also synthesized substantial amounts (20%) of previously unencountered $CHCl_3$-soluble products from homogentisate and MLE-IPP, and the 2-deca-, 2-nona- (principal product) and 2-octa-prenyl forms of 4-carboxy-2-polyprenylphenol from *p*-hydroxybenzoate and MLE-IPP. The polyprenyltoluquinols were shown to be 3-polyprenyl-toluquinols, compounds postulated as intermediates on the pathway from homogentisate to plastoquinone, by determination of the ratio of $^{14}C:^{3}H$ incorporated into them from 2,5-dihydroxyphenylacetic-[U-^{14}C,4,6-$^{3}H_2$] acid. Intracellular distribution studies using green, dark-grown and streptomycin-bleached cells, established that the 3-polyprenyltoluquinols are synthesized in the chloroplasts and the etioplasts, and that 4-carboxy-2-polyprenylphenols are synthesized in the mitochondria and a particle sedimenting at 1000–15000 *g*.

INTRODUCTION

Thomas and Threlfall [1] demonstrated that chloroplast-rich preparations of sugar beet and *Euglena gracilis* are able to carry out the light-, O_2- and H_2O_2-independent syntheses of a nonaprenyltoluquinol and an octaprenyltoluquinol from homogentisate and protein-bound polyprenylpyrophosphates (MLE-IPP) produced by incubating MLE with IPP, and of a phytyltoluquinol from homogentisate and phytylpyrophosphate. The formation of these compounds, the 3-polyprenyl (or 3-phytyl) isomers of which have been postulated as precursors of plastoquinones, tocoquinones, plastochromanols and tocochromanols [2], appeared to take place by the concomitant polyprenylation (or phytylation) and non-oxidative decarboxylation of homogentisate (Scheme 1). The *E. gracilis* preparations supplemented with MLE-IPP also synthesized substantial quantities of two homologues of an uncharacterized decarboxylated and polyprenylated form of homogentisate.

Recently it has been shown that, contrary to previous findings [3], cell-free preparations of *E. gracilis* are able to synthesize 4-carboxy-2-polyprenylphenols, compounds which are putative intermediates in the biosynthesis of higher plant ubiquinones [3], from *p*-hydroxybenzoate and

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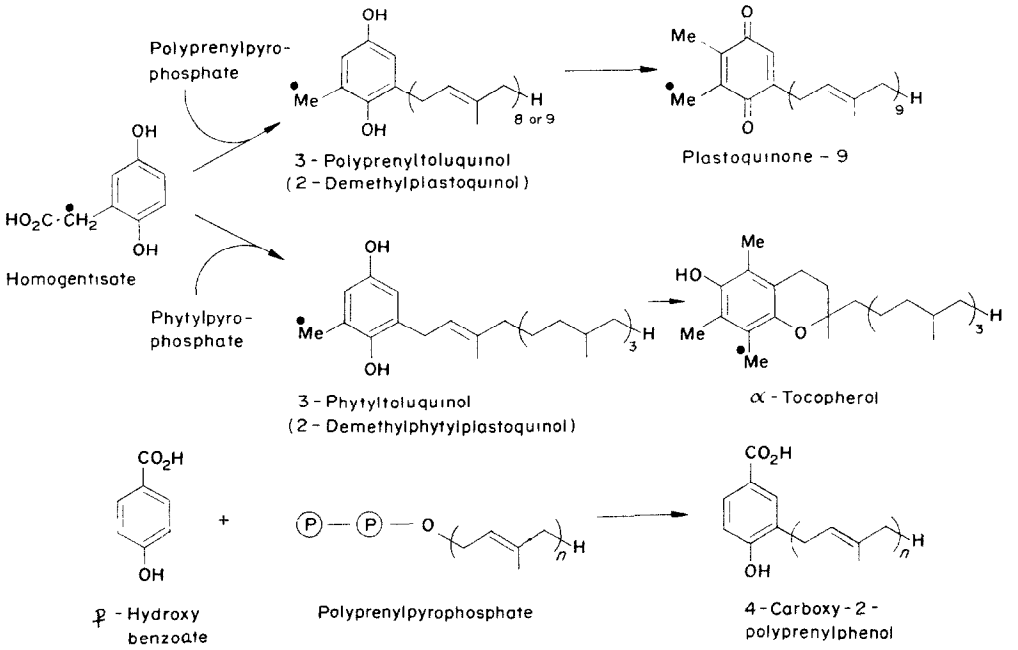
Abbreviations IPP, trilithium isopentenylpyrophosphate, MLE, *Micrococcus luteus* extract, MLE-IPP, MLE that has been preincubated with IPP, GPP, *trans*-geranylpyrophosphate, FPP, *trans-trans*-farnesylpyrophosphate



Scheme 1 Biosynthesis of polyprenyltoluquinols, phytyltoluquinol and 4-carboxy-2-polyprenylphenols

MLE-IPP (G Thomas and D R Threlfall, unpublished observations) (Scheme 1)

In the present paper we report on the properties and intracellular distribution of the enzymes concerned with the decarboxylation and polyprenylation of homogentisate and the polyprenylation of *p*-hydroxybenzoate in *E. gracilis*

RESULTS

Formation of 4-carboxy-2-polyprenylphenols, and polyprenyltoluquinols and related compounds by cell-free homogenates

We previously demonstrated that when 5000 *g* particles from green cells of *E. gracilis* are incu-

Table 1 Effect of light on the biosynthesis and isolation of polyprenyl quinones, chromanols and phenols

Conditions of illumination			Incorporation of radioactivity (% distribution)			
Incubation	Extraction	Total (10^{-3} × dpm)	Polyprenyl-toluquinols*	Polyprenyl-quinones*	Chromanols†	Unknowns‡
Experiments with homogentisate-[U-^{14}C]						
Daylight	Dark	68	37	0	46	17
Daylight	Light	71	0	30	36	34
2000 lm/ft^2	Dark	56	29	0	52	20
Dark	Dark	68	38	0	44	18
Dark	Light	66	0	28	34	38
Experiments with *p*-hydroxybenzoate-[7-^{14}C]						
Daylight	Light	69	Present in compounds with the TLC properties of 4-carboxy-2-polyprenylphenols			
Dark	Dark	66				
Experiments with *p*-hydroxybenzoate-[U-^{14}C]						
Dark	Dark	14				

Incubation mixtures consisted of 2 ml 0·05 M Pi buffer pH 7·1, 1 ml MLE-IPP (6·5 μmol), 0·5 ml cell-free homogenate (0·32 mg of chlorophyll), 100 μmol $MgCl_2$ and 10^6 dpm of homogentisate-[U-^{14}C] (8·9 μCi/μmol) or *p*-hydroxybenzoate-[7-^{14}C] (55 μCi/μmol) or *p*-hydroxybenzoate-[U-^{14}C] (7·7 μCi/μmol). The mixtures were incubated for 45 min at 30°.

* Radioactivity distributed between nona- (60%) and octa- (40%) prenyl forms.

† Radioactivity distributed equally between nona- (5%) and octa- (5%) prenyl forms.

‡ Radioactivity distributed between compounds that migrated with R_f 0·50 (50%), 0·40 (10%), 0·18 (10%) and 0·00 (30%) on Si gel H developed with Me_2CO–petrol (3:7).

bated with homogentisate-[U-^{14}C] and MLE-IPP most of the radioactivity recovered in the $CHCl_3$ soluble extracts is distributed equally between a nonaprenyltoluquinol, an octaprenyltoluquinol and two uncharacterized compounds referred to as chromanols [1]. To establish if in the course of intracellular fraction studies any other radioactive $CHCl_3$ soluble compounds would be encountered when cell-free fractions are incubated with homogentisate-[U-^{14}C] and MLE-IPP, cell-free homogenates were incubated with these two supplements under varying conditions of illumination (Table 1). At the same time the effect of light on the isolation of the radioactive products was examined, as was the ability of the homogenates to synthesize 4-carboxy-2-polyprenylphenols from *p*-hydroxybenzoate-[^{14}C] and MLE-IPP (Table 1). The results for the incubations containing homogentisate-[U-^{14}C] showed that some 20% of the radioactivity present in the $CHCl_3$ soluble extracts was distributed between four types of compound not found in significant quantities in incubations containing 5000 *g* particles [1]. The rate of synthesis of all the compounds studied was the same in both the dark and daylight; however, some inhibition of polyprenyl-toluquinol synthesis was observed at high light intensity. When the extractions were carried out in daylight most of the polyprenyltoluquinols were photooxidized to polyprenyltoluquinones, whilst the remainder along with some of the chromanols were photodegraded to a wide range of compounds which contributed to the background radioactivity in the regions of the TL chromatograms where the new unknowns ran. These findings together with a previous observation that the quinols are rapidly oxidized to quinones if there is any trace of peroxide in the ether used to elute them from developed TLC plates, serve to emphasize the care which must be exercised in the isolation and purification of these compounds. In the cases of the incubations containing *p*-hydroxybenzoate-[^{14}C], radioactivity was incorporated into compounds which had the TLC properties expected of 2-deca- (18% of the radioactivity), 2-nona- (76% of the radioactivity) and 2-octaprenyl- (6% of the radioactivity) forms of 4-carboxy-2-polyprenylphenol. Confirmation of the identities of these phenols was obtained by the same methods as those used to characterize the phenols produced by *Saccharomyces carlsbergensis* [3, 4]. The requirement by the homogenate for all of the components that were included in the incubations just described was examined. The incubations (Table 2) established that (a) MLE-IPP is acting only as a source of polyprenylpyrophosphates; (b) the crude cell-free preparation, unlike a yeast homogenate [4], cannot synthesize any suitable polyprenylpyrophosphates from IPP and cannot make use of

Table 2 Requirements for biosynthetic activity

Variations from complete reaction mixtures	Radioactivity in $CHCl_3$ soluble compounds (10^{-3} dpm)*	
	Homogentisate-[U-^{14}C] incubations	*p*-Hydroxy benzoate-[7-^{14}C] incubations
Complete	29 8	46 2
Boiled homogenate	0 2	0
− MLE/IPP or − ME/IPP + MLE	0 2	0
− MLE/IPP + IPP	0 2	0
Boiled MLE/IPP	28 1	48 0
− MLE/IPP + 1 μmol of either FPP or GPP	0 1	0
− MLE/IPP + 2 μmol of phytyl PP	2 8	0
$-Mg^{2+}$	27 4	47 3
− Homogentisate-[U-^{14}C] + 2 μCi of either *p*-hydroxyphenylacetate-[U-^{14}C] (8.9 μCi/μmol) or *p*-hydroxyphenylpyruvate-[U-^{14}C] (10 μCi/μmol)	0	—
+ 10 μmol of homogentisate	0·4	46·5
+ 10 μmol of toluquinol	29 1	47 8
+ 10 μmol of *p*-hydroxybenzoate	27 2	0 6

Complete reaction mixtures were of the same composition as incubation mixtures in Table 1, except that cell-free homogenate contained 0·9 mg chlorophyll/ml Mixtures were incubated in the dark for 45 min at 30°

* Radioactivity was distributed between the $CHCl_3$ soluble compounds in a similar manner to the way it was distributed in the experiments described in Table 1

Table 3. Incorporation of 2,5-dihydroxy phenylacetate-[U-^{14}C,4,6-$^{3}H_2$]

	Observed ^{14}C:^{3}H ratio	Atomic ratio: Corrected ^{14}C:^{3}H ratio	Atomic ratio: Expected ^{14}C:^{3}H ratio
S-Tyrosine-[U-^{14}C,3,5-$^{3}H_2$]	1:10·6	9:2	9:2
2,5-Dihydroxyphenylacetate-[U-^{14}C,4,6-$^{3}H_2$]	1:12·0	8:2	8:2
E. gracilis incubation			
Nonaprenyltoluquinol	1:12·9	7:2	7:2* or 7:1†
Octaprenyltoluquinol	1:12·8	7:2	7:2* or 7:1†
Chromanols	1:13·7	7:2	?
Maize experiment			
Plastoquinone-9	1:7·8	7:1	7:1
α-Tocopherol	1:0	7:0	7:0

The *E. gracilis* incubation consisted of 2 ml Pi buffer, pH 7·1; 1 ml MLE-IPP (6·5 μmol); 1 ml 12000 *g* preparation (0·63 mg of chlorophyll) and 1 μCi (as ^{14}C) of 2,5-dihydroxy phenylacetate-[U-^{14}C,4,6-$^{3}H_2$]. The mixture was incubated in the dark for 50 min at 30°. In the maize exp., 200 6-day-old etiolated maize shoots were excised and incubated in the light with 5 μCi (as ^{14}C) of 2,5-dihydroxyphenylacetate-[U-^{14}C,4,6,-$^{3}H_2$] for 18 hr in the manner described by Threlfall and Whistance [6]. At the end of this time plastoquinone-9 and α-tocopherol were isolated by standard procedures [6].

* 3-Polyprenyltoluquinol.

† 4- or 5-Polyprenyltoluquinol.

either FPP or GPP; (c) Mg^{2+} is not required; (d) *p*-hydroxyphenylpyruvate (the probable precursor of homogentisate), *p*-hydroxyphenylacetate (an analogue of homogentisate) and toluquinol (a possible intermediate in the synthesis of polyprenyltoluquinols from homogentisate) cannot be used in place of homogentisate or *p*-hydroxybenzoate.

Incorporation of dihydroxyphenylacetate-[U-^{14}C,4,6-$^{3}H_2$]

In our previous investigation we did not establish the substitution pattern(s) of the polyprenyltoluquinols synthesized by 5000 *g* particles from green cells of *E. gracilis*, although the assumption was made on biogenetic grounds that they were the 3-polyprenyl-substituted forms, i.e. 2-demethylplastoquinols [1]. To establish which isomers are being produced, a 12000 *g* pellet from green cells of *E. gracilis* was incubated with dihydroxyphenylacetate-[U-^{14}C,4,6-$^{3}H_2$] and MLE-IPP and the ratio of ^{3}H:^{14}C incorporated into the two polyprenyltoluquinols determined (Table 3). As a control the incorporation of the doubly labelled substrate into plastoquinone-9 and α-tocopherol in maize shoots was determined (Table 3), since the probable manner of incorporation of homogentisate into these two compounds in maize had been fairly well established by determination of the patterns of incorporation of DL-shikimate-[1,2-^{14}C] [5]. The values obtained for the observed and corrected ratio of ^{3}H:^{14}C provided unequivocal evidence that the *E. gracilis* preparations are synthesizing 3-polyprenyltolu-

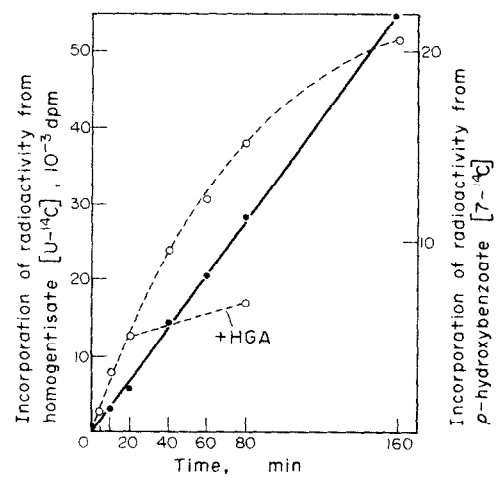


Fig. 1. Effect of time on the incorporation of radioactivity from homogentisate-[U-^{14}C] (○——○) and *p*-hydroxybenzoate-[7-^{14}C] (●——●) into $CHCl_3$ soluble compounds by cell free homogenates. Incubation mixtures consisted of 2 ml 0·05 M Pi buffer, pH 8·0, 1 ml MLE-IPP (6·5 μmol), 0·5 ml cell-free homogenate (0·41 mg chlorophyll) and 10^6 dpm of either homogentisate-[U-^{14}C] (8·9 μCi/μmol) or *p*-hydroxybenzoate-[7-^{14}C] (55 μCi/μmol). Mixtures were incubated in the dark at 30°. To one of the incubations containing homogentisate-[U-^{14}C] 10 μmol of unlabelled homogentisate (HGA) was added 20 min after the start of the incubation.

* The radioactivity was distributed between the $CHCl_3$ soluble compounds in a similar manner to the way in which it was distributed in the experiments described in Table 1.

Table 4. Intracellular distribution of polyprenyltransferase activities in 3- and 4-day-old light grown cells of *E. gracilis*

Fraction	Chlorophyll (mg)	Chlorophyll (% total)	Succinic oxidase (% total activity)	Homogentisate decarboxylase-polyprenyltransferase activities: Total (10^{-3} dpm)	3-Polyprenyltoluquinols* (10^{-3} dpm)	3-Polyprenyltoluquinols* (% total)	Chromanols* (10^{-3} dpm)	Chromanols* (% total)	Unknowns† (10^{-3} dpm)	Unknowns† (% total)	*p*-Hydroxybenzoate polyprenyltransferase activity*: (10^{-3} dpm)	*p*-Hydroxybenzoate polyprenyltransferase activity*: (% total)
3-day-old												
1000 *g*	2·34	25·2	10·9	200	146	30·2	15	7·7	39	10·7	213	19·4
12000 *g*	3·54	38·2	17·4	360	224	46·3	56	28·7	80	21·9	368	33·5
100000 *g*	2·85	30·8	65·2	311	114	23·5	72	37·0	115	31·4	484	44·2
Supernatant	0·54	5·8	6·5	184	0	0	52	26·6	132	36	32	2·9
4-day-old												
1000 *g*	4·90	47·5	13·2	225	173	71·4	32	34·9	19	13·3	257	41·7
12000 *g*	2·32	22·5	13·8	92	40	16·6	20	21·6	32	22·3	150	24·4
100000 *g*	2·50	24·2	68·0	105	29	12	40	43·5	36	24·6	203	33·0
Supernatant	0·60	5·8	5·0	58	0	0	0	0	58	39·8	5	0·9

Incubation mixtures for assay of transferase activities consisted of 2 ml Pi buffer, pH 8, 1 ml MLE-IPP (6·5 μmol), 10^6 dpm of either homogentisate-[U-^{14}C] (8·9 μCi/μmol) or *p*-hydroxybenzoate-[7-^{14}C] (55 μCi/μmol) and either 0·25 ml from 2 ml resuspended particulate fraction or 1 ml from 10–20 ml of supernatant fraction. Mixtures were incubated in the dark for 40 min at 30°. The homogentisate decarboxylase–polyprenyltransferase and *p*-hydroxybenzoate polyprenyltransferase activities were calculated by using the expression dpm incorporated × total vol (ml) of fraction ÷ vol. (ml) of fraction used in the incubation.

* Radioactivity was distributed between homologues in a similar manner to its distribution in the experiments described in Tables 1 and 2. There is no experimental evidence to support the view that formation of these compounds involves a decarboxylation reaction.

quinols. They also showed that the chromanols are unsubstituted at the positions corresponding to 4 and 6 of dihydroxyphenylacetate. In the maize experiment the plastoquinone-9, as expected, retained one ^{3}H atom, whilst the α-tocopherol contained no ^{3}H.

Effect of pH and time on the synthesis of $CHCl_3$ soluble compounds

Before proceeding to the intracellular fractionation studies, the effect of time and pH on the synthesis of homogentisate- and *p*-hydroxybenzoate-derived compounds was investigated.

The optimal pH for the incorporation of radioactivity into $CHCl_3$ soluble compounds was found to be 8 for homogentisate-[U-^{14}C] and 7·9 for *p*-hydroxybenzoate-[7-^{14}C]. In the homogentisate-[U-^{14}C] experiments the ratio of 3-polyprenyltoluquinols:chromanols:unknowns was only slightly affected by pH. The rate of incorporation of radioactivity into $CHCl_3$ soluble compounds was linear with time for *p*-hydroxybenzoate-[7-^{14}C], but decreased with time for homogentisate-[U-^{14}C] (Fig. 1). The addition of unlabelled homogentisate to a homogentisate-[U-^{14}C] incubation after 20 min decreased the rate of incorporation of radiactivity but had no effect on the ratio of 3-polyprenyltoluquinols:chromanols:unknowns. This established that there is no precursor–product relationship between these groups of compounds.

Although the above studies were all carried out using cell-free preparations, essentially similar results for the effect of Mg^{2+}, pH and time have since been obtained with individual intracellular fractions.

Intracellular distribution of homogentisate decarboxylase-polyprenyltransferase and p-hydroxybenzoate polyprenyltransferase activities in green, dark-grown and streptomycin-bleached cells

To obtain information on the intracellular sites of synthesis of the various compounds discussed above, suitably buffered and osmotically stabilized cell-free preparations obtained from green, dark-grown and streptomycin bleached cells of *E. gracilis* were fractionated by differential centrifugation, and the fractions analysed for chlorophyll content, and succinic-oxidase, *p*-hydroxybenzoate polyprenyltransferase and homogentisate decarboxylase-polyprenyltransferase activities (Tables 4–6).

The results show that homogentisate decarboxylase-polyprenyltransferase activities are highest in the fractions from green cells and lowest in the fractions from streptomycin bleached cells (Tables 4–6). The distribution of the homogentisate decarboxylase-polyprenyltransferase activity responsible for the synthesis of 3-polyprenyltoluquinols is similar to the distribution of chlorophyll in the green cells (Table 4), and to the distribution expected of etioplasts in dark-grown cells

Table 5 Intracellular distribution of polyprenyltransferase activities in 3-day-old dark grown cells of *E. gracilis*

Fraction	Succinic oxidase (% total activity)	Homogentisate decarboxylase-polyprenyltransferase activities							*p*-Hydroxybenzoate polyprenyltransferase activity	
		Total (10^{-3} dpm)	3-Polyprenyltoluquinols (10^{-3} dpm)	(% total)	Chromanols (10^{-3} dpm)	(% total)	Unknowns (10^{-3} dpm)	(% total)	(10^{-3} dpm)	(% total)
1000 *g*	3.6	38	16	23.6	1	12.5	21	27.6	228	18.1
12000 *g*	13.7	45	25	37.3	2	25	18	23.7	458	29.8
100000 *g*	75.0	44	24	36.7	1	12.5	19	25	720	47.0
Supernatant	7.7	24	2	2.8	4	50	18	23.7	78	5.0

Experimental conditions were the same as those described in Table 4

(Table 5) Its complete absence from streptomycin-bleached cells seems to confirm that it is found only in chloroplasts and etioplasts (Table 6). The chromanol synthesizing activity of the various fractions did not present a clear cut picture with regard to the intracellular distribution of the enzymes involved However, it did appear to be related to the degree of chloroplast development since it was highest in fractions from green cells but much reduced in dark-grown cells and it was undetectable in streptomycin-bleached cells (Tables 4–6). The activities of the enzymes responsible for the synthesis of the four unknown types of compound (other than chromanols) also appeared to be related to the degree of chloroplast development (Tables 4–6) As in the case of the chromanol synthesizing activity, however, it was impossible to associate these activities with specific intracellular fractions (Table 7)

The *p*-hydroxybenzoate polyprenyltransferase activity was high in all of the cells used in this study Rather unexpectedly, its intracellular distribution pattern did not follow the distribution of succinic oxidase activity as it does in animals [7], yeast [4] and broad bean seeds (J. Casey and D R Threlfall, unpublished observations), there being a substantial amount of activity in all of the 1000 *g* fractions (Tables 4–6). Several possible explanations can be advanced to account for these results Thus, *p*-hydroxybenzoate polyprenyltransferase activity may be present in both mitochondria and plastids (chloroplasts, etioplasts and streptomycin-bleached etioplasts): the decarboxylase-polyprenyltransferases responsible for 3-polyprenyltoluquinol synthesis could be using *p*-hydroxybenzoate in place of homogentisate, those polyprenyltransferases which have not been assayed could be using *p*-hydroxybenzoate and polyprenylpyrophosphates in place of their normal substrates (e g transferases which synthesize phylloquinone from 4-(2'-carboxyphenyl)-4-oxobutyrate and phytylpyrophosphate, and phytylquinones and tocopherols from homogentisate and phytylpyrophosphate). In an attempt to clarify the situation two experiments were carried out In the first, chloroplasts were isolated by a flotation procedure and assayed for homogentisate decarboxylase-polyprenyltransferase and *p*-hydroxybenzoate polyprenyltransferase activities (Table 8) In the second, 1000 *g* and 100000 *g* fractions from green cells were incubated with *p*-hydroxybenzoate-[7-^{14}C] and MLE-IPP, in the

Table 6 Intracellular distribution of polyprenyltransferase activities in 4-day-old streptomycin-bleached cells of *E. gracilis*

Fraction	Succinic oxidase (% total activity)	Homogentisate polyprenyltransferase activities (10^{-3} dpm)*	(% total)	*p*-Hydroxybenzoate polyprenyltransferase activity (10^{-3} dpm)	(% total)
1000 *g*	4.3	2	25	127	26.0
12000 *g*	26.9	2	25	140	28.7
100000 *g*	64.5	3	37.5	219	45.0
Supernatant	4.3	1	12.5	2	0.3

Experimental conditions were the same as those described in Table 4 * Present in unknowns

Table 7 Inter- and Intra-fraction distribution of the radioactivity present in the 4 unknown types of compound

Fraction	Radioactivity present in unknowns (10^{-3} dpm)*											
	Green cells								Dark-grown cells			
	3-day-old				4-day-old							
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1000 *g*	9	13	10	7	6	6	4	3	18	1	1	1
12000 *g*	23	15	19	23	20	6	4	2	9	3	3	3
100000 *g*	33	21	28	33	23	11	0	2	10	6	1	2
Supernatant	86	2	2	42	42	16	0	0	12	2	2	2

* **1**, **2**, **3**, and **4** refer to the compounds which migrate with R_f 0.50, 0.40, 0.18 and 0.00 respectively on Si gel H developed with Me_2CO–petrol (3:7)

Table 8 Polyprenyltransferase activities present in chloroplasts prepared by a flotation procedure and the effect of homogentisate on *p*-hydroxybenzoate polyprenyltransferase activity

Fraction	Homogentisate decarboxylase-polyprenyltransferase activity (dpm/mg of chlorophyll)		*p*-Hydroxybenzoate polyprenyltransferase activity	
	Polyprenyl-toluquinols	Chromanols	(dpm/mg of chlorophyll)	(dpm/incubation)
Purified chloroplast experiment				
Purified chloroplasts	40	14	6	
1000 *g* fraction (3-day-old cells Table 4)	62	6	91	
1000 *g* fraction (4-day-old cells Table 4)	35	7	52	
Homogentisate experiment				
1000 *g*				17
100000 *g*				19
1000 *g*		+ 10 μmol homogentisate		12
100000 *g*				16

In the purified chloroplast expt the incubation mixture consisted of 2 ml Pi buffer, pH 8, 1 ml MLE-IPP (6·5 μmol), 0·6 ml chloroplast preparation (0·38 mg chlorophyll) and 10^6 dpm of either homogentisate-[U-^{14}C] (8·9 μCi/μmol) or *p*-hydroxy benzoate-[7-^{14}C] (55 μCi/μmol) Mixtures were incubated in the dark for 45 min at 30°. In the homogentisate expt incubation mixtures consisted of 2 ml Pi buffer, pH 8·0, 1 ml MLE-IPP (6·5 μmol), 10^6 dpm *p*-hydroxybenzoate-[7-^{14}C] (55 μCi/μmol) ± 10 μmol of homogentisate and either 0·2 ml 1000 *g* preparation (0·31 mg of chlorophyll) or 100000 *g* preparation (0·07 mg of chlorophyll) Mixtures were incubated in the dark for 35 min at 30°.

presence or absence of unlabelled homogentisate (Table 8). The results showed that purified chloroplasts produced 3-polyprenyltoluquinols, chromanols and small amounts of 4-carboxy-2-polyprenylphenols and that homogentisate had very little effect on the incorporation of radioactivity from *p*-hydroxybenzoate-[7-^{14}C] into 4-carboxy-2-polyprenylphenols (Table 8).

DISCUSSION

In keeping with previous results obtained with 5000 *g* chloroplast-rich preparation [1], it was found that cell-free homogenates of *E. gracilis* are able to carry out the light- and Mg^{2+}-independent syntheses of approximately equal amounts of a nonaprenyltoluquinol, an octaprenyltoluquinol and two uncharacterized compounds referred to as chromanols from homogentisate and MLE-IPP (Table 1). In addition, however, it was found that they synthesized substantial amounts (20%) of previously unencountered $CHCl_3$-soluble products from homogentisate and MLE-IPP, and the 2-deca-, 2-nona-(principal product), and 2-octa-prenyl forms of 4-carboxy-2-polyprenylphenol from *p*-hydroxybenzoate and MLE-IPP (Table 1). The reason why the synthesis of 4-carboxy-2-polyprenylphenols had not been demonstrated in a previous study [3] can probably be attributed to the use of a less efficient ultrasonic disintegrator than the one used to disrupt the cells in this investigation. The enzymes responsible for the syntheses of all the above compounds appeared to be fairly specific with regard to the length of polyprenyl unit transferred, since they were unable to make use of either added short chain alcohol pyrophosphates (GPP and FPP) or FPP that had been generated from IPP *in situ*, and selected the deca-, nona- and octaprenylpyrophosphates from the source of preformed long chain polyprenylpyrophosphates provided (Table 2). The failure of the homogenates to make any compounds when supplemented with IPP showed that although they could make FPP (G. Thomas and D. R. Threlfall, unpublished observations), they did not have the ability to synthesize any long chain polyprenylpyrophosphates.

The cell-free homogenates were unable to make use of *p*-hydroxyphenylpyruvate (the probable precursor of homogentisate), *p*-hydroxyphenyl acetate (an analogue of homogentisate) and tolu-

quinol (a possible intermediate on the pathway from homogentisate to plastoquinone) in place of homogentisate (Table 2) The failure of the preparations to use toluquinol would seem to completely eliminate this compound from serious consideration as an intermediate on the pathways leading from homogentisate to plastoquinones, tocoquinones and biogenetically related compounds

No attempt was made in this study to characterize either the compounds referred to as chromanols or the new unknowns produced when cell-free homogenates are incubated with homogentisate and MLE-IPP, since their relevance to quinone and chromanol biosynthesis has yet to be established and it may well be that they are not produced in the intact cell. The polyprenyltoluquinols, however, were shown to be 3-polyprenyltoluquinols, compounds postulated as intermediates on the pathway leading from homogentisate to plastoquinone, by determination of the ratio of $^{14}C:^{3}H$ incorporated into them from dihydroxyphenylacetate-[U-^{14}C,4,6-^{3}H] (Table 3).

Intracellular distribution studies using green, dark-grown and streptomycin-bleached cells, and incubations using chloroplasts prepared by a flotation procedure established that the enzyme(s) (homogentisate decarboxylase-polyprenyltransferase) responsible for the synthesis of 2-nona- and 2-octaprenyltoluquinol is found only in the chloroplasts of green cells and the etioplasts of dark-grown cells, and is completely absent from streptomycin-bleached cells (Tables 4–6 and 8), i e it is present only in cells and organelles which contain plastoquinone-9 (Table 9) The intracellular distribution of the enzymes responsible for the synthesis of the other homogentisate-derived compounds is not clear, since, although their degree of activity in the cell appears to be directly related to chloroplast development, they are found in the chloroplast, mitochondrial, microsomal, and soluble fractions (Tables 4–7) The *p*-hydroxybenzoate polyprenyltransferase activity was associated with particulate fractions in the three cell types fractionated (Tables 4–6). It was not, however, distributed in the same way as the mitochondrial marker enzyme succinic oxidase as it is in bean seeds (J Casey and D. R Threlfall, unpublished observations), yeast [4] and rat liver cells [7], but appeared to be present in both the mitochondria and particles which sedimented at 1000–15000 *g* (Tables 4–6). One explanation which would account for these results is that the mitochondria contain the polyprenyltransferase responsible for the synthesis of the 4-carboxy-2-polyprenylphenol required for the formation of the mitochondrial quinones, ubiquinone-9 and rhodoquinone-9 (Table 9), and that the chloroplasts etioplasts or streptomycin-bleached etioplasts prepared by differential centrifugation contain polyprenyltransferases which are lost from chloroplasts prepared by flotation (Table 8), and which under the conditions of the standard assay procedure are able to synthesize 4-carboxy-2-polyprenylphenols from *p*-hydroxybenzoate and polyprenylpyrophosphates (Tables 4–6) These enzymes are not the ones that are concerned with the synthesis of 3-polyprenyltoluquinols (Table 8), but they could be those responsible for the synthesis of hydroxyphylloquinone, tocopherols, phytylquinones and ethers of phytyltoluquinols (Table 9). Alternatively, there might be a non-chloroplastidic organelle sedimenting at 1000–15000 *g* which does synthesize 4-carboxy-2-polyprenylphenols in the intact *Euglena* cell, e.g. the nucleus

Table 9 Quinones, chromanols and ethers present in light grown and streptomycin bleached cells of *E. gracilis* strain Z (Ah Law and D R Threlfall, unpublished work)

Compounds present in both types of cell	Compounds present only in light grown cells
Homogentisate derived	
2-Demethylplastoquinone	Plastoquinone-9
1-*O*-Methyl-2-demethylplastoquinone	Plastoquinone-8
Phytylplastoquinone	*o*-Succinylbenzoate derived
1-*O*-Methylphytylplastoquinone	Phylloquinone
α-Tocopherol	5′-Monohydroxyphylloquinone
p-Hydroxybenzoate derived	
Ubiquinone-9	
Rhodoquinone-9	

EXPERIMENTAL

Radiochemicals *p*-Hydroxybenzoic-[U-^{14}C] acid (7·8 mCi/mmol), homogentisic-[U-^{14}C] acid (8·9 mCi/mmol) and 2,5-dihydroxyphenylacetic-[U-^{14}C,4,6-$^{3}H_2$] acid were synthesized by our standard procedures from L-tyrosine-[U-^{14}C] hydrochloride (10 mCi/mmol) and L-tyrosine-[3,5-$^{3}H_2$] (53 Ci/mmol) [6, 8]

Synthesis of IPP, GPP and FPP Synthesis of these compounds was carried out by standard procedures [4]

Biological material *E gracilis* strain Z was obtained from The Culture Collection of Algae and Protozoa, Cambridge, U K It was grown in (a) the light under the conditions described by Ah Law *et al* [9], and (b) the dark under the same conditions as those used for the growth of *E gracilis* Y_2ZSmL (see below) *E gracilis* Y_2ZSmL, prepared by treating *E gracilis* strain Z with streptomycin, was grown in the same way as *E gracilis* Y_1ZSmL [9] Etiolated 6–7-day-old maize seedlings (*Zea mays* var South African White Horse Tooth) were grown in the manner described by Griffiths *et al* [10]

Preparation of cell-free homogenates and intracellular fractions Cells from 2–4 l of growth medium were harvested by centrifugation, washed with 0 05 M Pi buffer, pH 7 2, suspended in 10–20 ml 0 4 M sucrose–10 mM NaCl–0 05 M Pi buffer, pH 7 2, and exposed to ultrasound for 2 × 20 sec at 2° (Dawe Soniprobe Automatic Type 7532A sonic convertor fitted with a 1 27 cm tip; generator adjusted to an output of 70 W) Resultant preparation was centrifuged at 270 *g* for 5 min to remove whole cells and cell debris Cell-free homogenate obtained by the above procedure was either used for incubation studies without further treatment or fractionated by centrifugation at 1000 *g* for 10 min, 12000 *g* for 15 min and 100000 *g* for 30 min In one expt chloroplasts in the 1000 *g* pellet were purified by suspending the pellet in 0 4 M sucrose–10 mM NaCl–0 05 M Pi buffer, pH 7 2, adding 2 vol 75% (w/v) sucrose and subjecting resultant suspension to 23000 *g* for 10 min in a swing-out rotor Chloroplasts which rose to the top of the centrifuge tube were collected and resuspended in 0 05 M Pi buffer, pH 7 2

Incubation of cell-free preparations with radiochemical substrates Deails of the various incubation procedures used are given in Results In those incubations supplemented with MLE-IPP the MLE was prepared and preincubated with IPP (MLE–IPP, 100 1 5) under the conditions described by Raman *et al* [11] At the end of the incubation period the reaction was stopped by the rapid addition of 15 ml $CHCl_3$–MeOH (1 2) and, after allowing the mixture to stand for 2 hr, the $CHCl_3$-soluble lipids were extracted by the method of Gallard *et al* [12] In those incubations containing homogentisate-[^{14}C], extraction and subsequent purification of the lipid extracts was carried out in the dark Resultant lipid extract was taken up in 2 ml petrol (bp 40–60°) and a sample assayed for radioactivity Radioactivity present in the lipid extracts from incubations containing (a) *p*-hydroxybenzoate-[^{14}C] was associated entirely with 4-carboxy-2-polyprenylphenols, and (b) homogentisate-[^{14}C] was associated with a variety of compounds (see Results and below)

Identification of radioactive compounds present in lipid extracts (a) Extracts from incubations containing *p*-hydroxybenzoate-[^{14}C] Identification and determination of the chain lengths of the 4-carboxy-2-polyprenylphenols present in these extracts was carried out by a combination of adsorptive and reversed-phase TLC [3, 4] (b) Extracts from incubations containing homogentisate-[^{14}C] The extract was divided into two portions one portion was chromatographed on thin-layers of Si gel H developed with Me_2CO–petrol (bp 40–60°) (3 7) (polyprenyltoluquinones, R_f 0 81, polyprenyltoluquinols, R_f 0 62, chromanols, R_f 0 81, unknowns, R_f 0 50, 0 40, 0 18 and 0 00) and the other portion on Si gel G developed with C_6H_6 (polyprenyltoluquinones, R_f 0 38, polyprenyltoluquinols, R_f 0 14, chromanols, R_f 0 14, unknowns, R_f 0 00) After development the distribution of radioactivity between various compounds was determined by scanning Finally, the polyprenyltoluquinols and chromanols were eluted with Et_2O and, after oxidation of polyprenyltoluquinols to their corresponding quinones by treatment with Ag_2O, rechromatographed on paraffin-impregnated Si gel G developed with aq 95% Me_2CO (nonaprenyltoluquinol, R_f 0 32, octaprenyltoluquinol, R_f 0 43, nonaprenyl form (') of chromanol, R_f 0 48, octaprenyl form (') of chromanol, R_f 0 59)

Determination of chlorophyll This was carried out by the method of Arnon [13]

Succinic oxidase activity This was determined by a polarographic procedure [14]

Radioassay Samples were assayed by described procedures [4]

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