Biochem. J. (1974) **142**, 437–440
Printed in Great Britain

Synthesis of Polyprenyltoluquinols from Homogentisate and Polyprenyl Pyrophosphates in Particulate Fractions of *Euglena* and Sugar Beet

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(*Received* 28 *May* 1974)

Chloroplast-rich particles of sugar beet and *Euglena gracilis* are able to carry out the light-, H_2O_2- and O_2-independent syntheses of a nonaprenyltoluquinol, an octaprenyltoluquinol and a phytyltoluquinol from homogentisate and nonaprenyl, octaprenyl and phytyl pyrophosphate. The formation of these compounds probably takes place by the concomitant polyprenylation (or phytylation) and non-oxidative decarboxylation of homogentisate.

It is now well established that homogentisate provides a toluquinone unit in the biosynthesis of plastoquinones and tocoquinones and a toluquinol-derived unit in the biosynthesis of plastochromanols, tocochromanols and 1-*O*-methyl ethers of phytylplastoquinol and 2-demethylphytylplastoquinol (see Threlfall, 1971). The natures and actual sequences of the enzymic steps on the pathways leading from homogentisate to the various quinones, chromanols and ethers have still to be elucidated, although it is clear that the enzymes on these pathways must catalyse: (i) the formation of a *C*-methyl group from the α-carbon atom of the acetic acid side chain of homogentisate by either oxidative decarboxylation followed by reduction of the resultant oxygenated carbon atom or non-oxidative decarboxylation, (ii) polyprenylation or phytylation and, if required, (iii) *C*-methylation, (iv) *O*-methylation, (v) hydrogenation, (vi) cyclization, (vii) hydroxylation and (viii) esterification (see Threlfall, 1971).

At the present time the most interesting steps are undoubtedly those that are concerned with the formation of the *C*-methyl group from the α-carbon atom of homogentisate and the introduction of the polyprenyl or phytyl side chain into the ring system. Isotope-competition experiments and ^{14}C-tracer studies with whole plants have provided some evidence that C_6–C_1 compounds such as gentisic acid, gentisaldehyde, gentisyl alcohol, toluquinol and their glucosides are not involved in the pathway (Whistance & Threlfall, 1968, 1970). These findings, together with the knowledge that the α-carbon atom of homogentisate gives rise in the plastoquinones and biogenetically related compounds to the nuclear *C*-methyl group *meta* to the polyprenyl side chain (Whistance & Threlfall, 1971), suggested that the first step(s) on each of the pathways may be either the sequential or concomitant polyprenylation (or phytylation) and non-oxidative decarboxylation of homogentisate to give 2-demethylplastoquinol (3-polyprenyltoluquinol) (or 3-phytyltoluquinol) or the sequential or concomitant polyprenylation (or phytylation) and oxidative decarboxylation of homogentisate followed by subsequent reduction of the oxygenated carbon atom.

In the present paper we report the demonstration that chloroplast-rich particles of sugar beet and *Euglena gracilis* are able to carry out the light-, H_2O_2- and O_2-independent synthesis of compounds with the properties expected of 2-demethylplastoquinol-9, 2-demethylplastoquinol-8 and 2-demethylphytyltoluquinol from homogentisate and nonaprenyl, octaprenyl and phytyl pyrophosphate.

Experimental

[U-^{14}C]Homogentisic acid (8.9mCi/mmol), [α-^{14}C]homogentisic acid (6.9mCi/mmol) and [*carboxy*-^{14}C]homogentisic acid (48.8mCi/mmol) were synthesized by the procedure described by Whistance & Threlfall (1970). Trilithium isopentenyl pyrophosphate, *trans*-geranyl pyrophosphate, *trans-trans*-farnesyl pyrophosphate and *trans*-phytyl pyrophosphate were synthesized from the appropriate alcohols by the methods described by Cornforth & Popják (1969).

E. gracilis strain Z was grown for 3 days under the conditions described by Whistance & Threfall (1968). After being harvested and washed by resuspension in 0.05M-potassium phosphate buffer, pH7.1, the cells from 3 litres of medium were taken up in 10ml of buffer and exposed to ultrasound for 2×20s at 2°C (Dawe Soniprobe Automatic Type 7532A: sonic converter fitted with 1.27cm-diameter tip; generator adjusted to an output of 70W). The resultant preparation was centrifuged first at 270*g* for 5min to remove whole cells, and then at 5000*g* for 10min to sediment a chloroplast-rich particulate fraction. The chloroplast-rich particles were washed and then resuspended in 5ml of buffer.

Sugar-beet (*Beta vulgaris*) leaves (30g; 12 weeks old) were deveined, sliced with a razor blade and homogenized in 200ml of ice-cold 0.2M-Tris–HCl

buffer, pH7.5, containing 0.5M-sucrose for 30s in an MSE homogenizer. The homogenate was then filtered through one layer of Miracloth and centrifuged at 1000*g* for 3min, followed by 6000*g* for 8min. The resulting chloroplast-rich particles were resuspended in 5ml of 0.05M-potassium phosphate buffer, pH7.2, and exposed to ultrasound for 15s at 2°C.

The standard incubation mixture consisted of 2.5ml of 0.05M-potassium phosphate buffer, pH7.1, 2ml of *Micrococcus lysodeikticus* extract that had been prepared and preincubated with 11.4μmol of trilithium isopentenyl pyrophosphate under the conditions described by Raman *et al.* (1969), 0.5ml of chloroplast-rich particles [2–4mg of chlorophyll (determined by the procedure of Arnon, 1949)/ml] and 1μCi of [^{14}C]homogentisate. The mixture was incubated in either daylight or the dark for 75min at 30°C with gentle agitation. In some incubations the second component was replaced by 2ml of buffer containing 2μmol of an alcohol pyrophosphate.

At the end of the incubation period the reaction was stopped by the rapid addition of 20ml of chloroform–methanol (1:2, v/v) and, after the mixture had been left for 2h in the dark, the lipids were extracted by the method of Galliard *et al.* (1965). The light-petroleum (b.p. 40–60°C)-soluble fraction of the lipid extract, after removal of a small sample for the assay of ^{14}C content in a Beckman liquid-scintillation spectrometer (Thomas & Threlfall, 1974), was divided into two: one portion was chromatographed on thin layers of silica gel H (E. Merck, Darmstadt, Germany) developed with acetone–light petroleum (b.p. 40–60°C) (3:7, v/v) and the other portion on thin layers of silica gel G (E. Merck) developed with benzene. After development the thin layers were scanned for radioactivity in a Panax TL chromatogram scanner system. Any ^{14}C-labelled materials were eluted with diethyl ether, assayed for ^{14}C content and rechromatographed on thin layers of paraffin-impregnated silica gel G (E. Merck) developed with aq. 95% (v/v) acetone.

Results and discussion

Preliminary studies had shown that chloroplast preparations of higher plants and algae are unable to synthesize any ^{14}C-labelled polyprenyl quinones, quinols or chromanols when incubated with [U-^{14}C]-homogentisate, isopentenyl pyrophosphate and *S*-adenosylmethionine, owing to the inability of the preparations to make the necessary polyprenyl pyrophosphates from isopentenyl pyrophosphate. To circumvent this problem, it was decided to use an approach that had proved useful in studying the first step on the pathway leading from *p*-hydroxybenzoate to ubiquinone, i.e. the provision of a range of protein-bound polyprenyl pyrophosphates (Raman *et al.*, 1969; Winrow & Rudney, 1969; Thomas & Threlfall, 1973). Accordingly, chloroplast-rich particles of sugar beet and *E. gracilis* were incubated with [U-^{14}C]homogentisate, Mg^{2+} and an *M. lysodeikticus* extract that had been preincubated with isopentenyl pyrophosphate, and the incubation mixture was analysed for the presence of light-petroleum-soluble ^{14}C-labelled lipids. The results of these analyses showed that irrespective of the conditions of illumination and of the presence or absence of Mg^{2+} the preparations produced ^{14}C-labelled lipids with the t.l.c. properties expected for poly-

Table 1. *Incorporation of radioactivity from [U-^{14}C]homogentisate into polyprenyltoluquinols, phytyltoluquinol and chromanols*

Unless otherwise stated, chloroplast-rich particles were incubated under the conditions described in the text with either 0.5μCi (*E. gracilis* incubations) or 1μCi (sugar-beet incubations) of [U-^{14}C]homogentisate and *M. lysodeikticus* extract that had been preincubated with isopentenyl pyrophosphate

Details of incubation			$10^{-3}\times$ Incorporation of radioactivity into toluquinols and chromanols (d.p.m.)	
Illumination	Chlorophyll (mg)	Variations from standard incubation procedure	Polyprenyltoluquinols* or phytyltoluquinol	Chromanols*
E. gracilis				
Light	0.9	+100μmol of Mg^{2+}	33	36
Dark	0.9	+100μmol of Mg^{2+}	33	39
Dark	0.9	None	34	40
Dark	0.9	*M. lysodeikticus* supplement boiled	34	42
Dark	0.9	+Catalase and N_2	36	38
Dark	0.9	*M. lysodeikticus* supplement replaced by 2μmol of phytyl pyrophosphate	2	0
Sugar beet				
Light	1.35	None	16	0
Dark	1.35	None	15	0

* Equimolar mixture of nona- and octa-prenyl homologues.

prenyltoluquinols and chromanols (only produced in the *E. gracilis* incubation) (Table 1). Thus they co-chromatographed with 2-demethylphytylplastoquinol (3-phytyltoluquinol) (R_F 0.14) on silica gel G developed with benzene (previous experience had shown that phytylquinols and phytylquinones usually co-chromatograph with their corresponding deca-, nona- and octa-prenyl homologues in the adsorptive t.l.c. systems used in this study), were resolved into polyprenyltoluquinols (R_F 0.62) and chromanols (R_F 0.81), which co-chromatographed with 2-demethylphytylplastoquinol and 2-demethylphytylplastoquinone respectively on silica gel H developed with acetone–light petroleum (b.p. 40–60°C) (3:7, v/v), and behaved as mixtures of two long-chain polyprenyl homologues on reversed-phase t.l.c. Subsequent studies showed that both groups of compounds are labelled from [α-^{14}C]homogentisate but not [*carboxy*-^{14}C]homogentisate, and that their production is unaffected if the *M. lysodeikticus* extract that had been preincubated with isopentenyl pyrophosphate is boiled before being added to the incubation mixture. These results establish that the chloroplast preparations are carrying out the decarboxylation and polyprenylation of homogentisate and that the *M. lysodeikticus* preparation is only responsible for providing polyprenyl pyrophosphates.

Further evidence in support of the identities of the polyprenyltoluquinols was provided by the demonstrations that (*a*) they could be oxidized with AgO to compounds which co-chromatographed with 2-demethylphytylplastoquinone in the adsorptive systems described above, and which could either be reduced back to quinols by treatment with $Na_2S_2O_4$ or be converted by refluxing in pyridine into compounds (R_F 0.20) that ran just behind plastochromenol-8 (R_F 0.25) on silica gel G developed with benzene, and (*b*) the R_F values of their quinones on reversed-phase t.l.c. are those expected for the nonaprenyl (R_F 0.32) and octaprenyl (R_F 0.43) forms of a polyprenyltoluquinone, cf. mobility of plastoquinone-9 (R_F 0.19) relative to that of nonaprenyltoluquinone (R_F 0.32) with mobility of phytylplastoquinone (R_F 0.4) relative to that of 2-demethylphytylplastoquinone (R_F 0.52). Although all the evidence just cited is consistent with the polyprenylquinols being polyprenyltoluquinols, there was a possibility that they could be polyprenylgentisaldehydes, since studies with toluquinol, gentisylaldehyde, gentisyl alcohol and gentisic acid had shown that it is difficult to distinguish between the first two compounds by t.l.c. To explore this possibility, and also the more unlikely possibility that they could be either polyprenylgentisyl alcohols or polyprenylgentisates, the polyprenylquinones were treated with either $LiAlH_4$ to convert any –CHO and $-CO_2H$ groups into $-CH_2OH$ groups or silylating reagent to silylate any $-CH_2OH$ groups. Both reagents failed to alter the t.l.c. properties of the quinones, confirming that they are polyprenyltoluquinones.

With the techniques and reference compounds available, it was not possible to determine which of the isomers of polyprenyltoluquinol had been produced. On biogenetic grounds (see the introduction), however, it is to be expected that they are the 3-polyprenyl-substituted forms, i.e. 2-demethylplastoquinols.

The chromanols produced by *E. gracilis* have adsorptive t.l.c. properties which are similar to the t.l.c. properties expected of a demethylplastochromanol or demethylplastochromenol. The possibility that they are cyclized forms of the polyprenyltoluquinols can be discounted, however, since they cannot be oxidized with $FeCl_3$ or $HAuCl_4$. They are also non-reducible and their identification must await further study.

The demonstration that chloroplast-rich preparations synthesize a nona- and an octa-prenyltoluquinol provided good evidence that, as proposed by Threlfall & Whistance (1971), the first step(s) on the pathway leading from homogentisate to the plastoquinones and plastochromanols is the sequential or concomitant polyprenylation and non-oxidative decarboxylation of homogentisate to form 2-demethylplastoquinol (3-nonaprenyltoluquinol). The possibility still remained, however, that it could be an oxidative decarboxylation followed by reduction of the resultant oxygenated group. To investigate this, preparations were supplemented with catalase (to remove H_2O_2) and incubated in the dark with continuous purging with N_2. The nature and amounts of products produced were unaltered by this treatment, demonstrating that the decarboxylation reaction is non-oxidative (Table 1).

In contradistinction to those experiments in which it was shown that plant mitochondria on incubation with *p*-hydroxybenzoate and *M. lysodeikticus* extract supplemented with isopentenyl pyrophosphate gave rise to a range of 4-carboxy-2-polyprenylphenols (Thomas & Threlfall, 1973), the incubations described above gave rise to two polyprenylquinols only. This suggested that the polyprenyltransferase must be very specific with regard to the length of polyprenyl unit transferred. To test this *E. gracilis* preparations were incubated with [U-^{14}C]homogentisate and geranyl pyrophosphate or farnesyl pyrophosphate or phytyl pyrophosphate, a possible precursor of the side chains of phytylquinones, tocopherols and tocopherolquinones. In the incubations containing the first two substrates no ^{14}C-labelled compounds were produced. In the incubation containing the phytyl pyrophosphate, however, small amounts of a compound with t.l.c. properties identical with those of 2-demethylphytylplastoquinol were produced (Table 1).

The importance of this study is that it provides the first evidence that chloroplasts contain enzymes which are capable of producing compounds of the type that have been postulated as precursors of plastoquinones, plastochromanols, tocopherols and tocopherolquinones from homogentisate and nonaprenyl, octaprenyl and phytyl pyrophosphate. Although the precise sequence of events involved in the formation of these compounds is unknown, the facts that the process is non-oxidative and that in none of the many incubations carried out as part of this study have any radioactive compounds with the t.l.c. properties of either toluquinol or polyprenylhomogentisates been detected provide some evidence that their formation is by the concomitant polyprenylation (or phytylation) and non-oxidative decarboxylation of homogentisate.

This work was supported by a grant from the Science Research Council. We thank Miss Susan Normington for expert technical assistance.

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