Mevalonate-derived isopentenyl diphosphate is the biosynthetic precursor of ubiquinone prenyl side chain in tobacco BY-2 cells

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Study of the incorporation of ¹³C-labelled glucose or pyruvate into the isoprenoids of tobacco BY-2 cells allowed the biosynthetic origin of isopentenyl diphosphate to be determined. Sterols synthesized in the cytoplasm and the prenyl chain of ubiquinone Q10 located in mitochondria were derived from the same isopentenyl diphosphate pool, synthesized from acetyl-CoA through mevalonate, whereas the prenyl chain of

plastoquinone was obtained from the mevalonate-independent glyceraldehyde 3-phosphate/pyruvate route, like all chloroplast isoprenoids from higher plants. These results are in accord with the compartmentation and complete enzymic independence of the biosynthesis of long-chain all-*trans* polyprenols in mitochondria and chloroplasts.

INTRODUCTION

Two distinct pathways are involved in the biosynthesis of plant isoprenoids (Scheme 1). The classical well-known mevalonate pathway (Scheme 1, pathway A) for the formation of sterols and triterpenoids has largely been documented over the last couple of decades [1,2]. This metabolic route does not, however, allow satisfactory interpretation of labelling studies for the formation of many other plant isoprenoids [3–6]. Only a few important features will be highlighted here. Mevalonate was always found to be poorly incorporated, if at all, into monoterpenoids, diterpenoids and chloroplast isoprenoids such as carotenoids or the phytol chain of chlorophylls [7,8]. Mevinolin, a potent inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the key enzyme of this pathway, strongly inhibits sterol biosynthesis in the cytoplasm but has essentially no effect on carotenoid or chlorophyll formation in the chloroplasts [9,10]. An alternative mevalonate-independent route to isopentenyl diphosphate (IPP) (Scheme 1, pathway B) was recently discovered in bacteria and partially elucidated [11–15]. It involves a rearrangement reaction [12] and starts from pyruvate and glyceraldehyde 3-phosphate (GAP) [15]. D-1-Deoxyxylulose 5-phosphate, the first C₅ intermediate in this metabolic route [13,14], results from condensation of (hydroxyethyl)thiamine derived from pyruvate decarboxylation on the carbonyl group of GAP. The subsequent transposition yields the branched skeleton of the isoprenic unit from this straight-chain deoxypentulose. 2C-Methyl-D-erythritol, or rather its mono- or di-phosphate, is probably among the first intermediates with such a branched skeleton. The free tetrol or the corresponding lactone is found in many higher plants, and its 2,4-cyclodiphosphate is accumulated by some bacteria in oxidative stress conditions ([16] and references cited therein). The carbon skeletons of 2C-methyl-D-erythritol and the isoprenic units from dihydromenaquinones were both shown to arise in *Corynebacterium ammoniagenes* via the mevalonate-independent GAP/pyruvate route [16]. Deuterium-

labelled 2C-methyl-D-erythritol was found to be incorporated into ubiquinone and menaquinone in *Escherichia coli* [17]. No other intermediates have been identified.

This alternative metabolic route to IPP was later found to be widespread in all higher plants. The first proof was found in the extensive and careful study of Schwarz and Arigoni on isoprenoid biosynthesis in ginkgo embryos [18]. Sterols and sesquiterpenoids were synthesized as expected from mevalonate, whereas diterpenoids of the ginkgolide series were synthesized by the mevalonate-independent route. This alternative pathway was also found to be involved in the formation of ubiquitous and essential chloroplast isoprenoids such as carotenoids, plastoquinone and phytol [19], diterpenoids of more restricted distribution [20,21], monoterpenes [22] and isoprene [23], as well as for the formation of all isoprenoids in the green alga *Scenedesmus obliquus* [24].

No reliable data were, however, available for the early steps in the formation of the prenyl chain of ubiquinone found in plant mitochondria. Mevalonate 5-diphosphate was, for instance, not incorporated by isolated mitochondria from potato tubers, spinach leaves and daffodil petals into this isoprenoid, whereas IPP was efficiently incorporated, pointing to the formation of IPP at another site [25]. The keys to the successful elucidation of isoprenoid biosynthesis in the chloroplast were utilization of labelled glucose as a precursor instead of acetate, and the use of heterotrophic growth conditions in which no photosynthesis occurred. This last feature avoided recycling of ¹³CO₂ derived from glucose catabolism by photosynthesis, which gives scrambling and uniformly labelled metabolites [26]. Tobacco BY-2 cell cultures [27,28] were therefore found to be a perfect tool for work on the very early steps of the biosynthesis of the IPP involved in the formation of the ubiquinone prenyl chain. They are devoid of functional chloroplasts and contain only proplastids, are able to grow heterotrophically on glucose as sole carbon source and lastly contain a large number of mitochondria, resulting in adequate ubiquinone concentrations. ¹³C-

Abbreviations used: Ac-CoA, acetyl-CoA; GAP, glyceraldehyde 3-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl diphosphate.

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Scheme 1 Glucose catabolism via glycolysis, the mevalonate pathway (A) and the GAP/pyruvate pathway (B) for isoprenoid biosynthesis

NMR spectroscopy could therefore be performed on ubiquinone using reasonable amounts of biological material and labelled precursors. This condition is rarely fulfilled by other plant tissues, and allowed us to decipher the origin of the IPP involved in ubiquinone biosynthesis.

EXPERIMENTAL

Cell culture and labelling experiments

TBY-2 cells were cultured in a modified Murashige-Skoog medium [29] (obtained from Duchefa, Haarlem, The Netherlands). The concentration of KH_2PO_4 was increased to 540 mg/l. Other additives were as described by Nagata et al. [30], except the further inclusion of 100 mg/l *myo*-inositol [31]. Cell cultures (80 ml in 250 ml Erlenmeyer flasks) were kept in the dark at 26 °C and shaken at 174 rev./min. Cells were subcultured weekly (2 ml to 80 ml of new medium). In a preliminary series of experiments, sucrose was replaced by increasing amounts of glucose and/or other precursors. Growth was determined on the basis of the fresh weight of the biomass to optimize the composition of the culture medium to obtain sufficient amounts of isoprenoids with significant ^{13}C labelling. Whereas cell growth was optimal at 3.5 % (w/v) sucrose, growth in the presence of glucose was optimal at 2 % (w/v) and achieved on average 43 % of the sucrose value. Glucose in a highly concentrated solution

was autoclaved separately to prevent degradation and was added to the culture medium under sterile conditions. Initial experiments using D-[6- ^{14}C]glucose were performed to make sure that appreciable amounts of label appeared in isoprenoid products such as sterols and ubiquinone. The labelling experiments with [^{13}C]glucose required a 1:10 dilution with unlabelled substrate [1.5 % (w/v) final concentration]. Eight Erlenmeyer flasks (250 ml) with 80 ml cultures were used for each experiment. After 8 days, cells were harvested to yield about 15 g of fresh weight per culture flask. When sodium [2- ^{13}C]pyruvate [0.15 % (w/v); 99 % isotopic enrichment] was fed to the cells, it was combined with 1.35 % (w/v) sucrose. Acetate at concentrations required for ^{13}C labelling, even in combination with sucrose, was found to be toxic to the cells.

Analytical methods and evaluation of isotopic abundances

[1- ^{13}C]-, [2- ^{13}C]-, [3- ^{13}C]-, [4- ^{13}C]-, [5- ^{13}C]- and [6- ^{13}C]-D-glucose (99 % isotopic abundance) were obtained from Omicron Biochemicals Inc. (South Bend, IN, U.S.A.). Sodium [2- ^{13}C]pyruvate (99 % isotopic abundance) was purchased from Isotec Inc. (Plaisir, France) and from Eurisotop (Gif sur Yvette, France). NMR spectra were recorded on a Bruker WP 400 spectrometer in [^2H] chloroform, and ^{13}C isotopic abundances were determined

Figure 1 Isoprenoids from *Nicotiana tabacum* BY-2 cell cultures

Campesterol (24 α) and 22,23-dihydrobrassicasterol (24 β) (I), sitosterol (II), stigmasterol (III), ubiquinone Q10 (IV) and plastoquinone (V).

as reported previously [11]. For steryl acetates, the methyl singlet of the acetoxy group (1.1 % isotopic abundance) was utilized as internal reference. In the case of ubiquinone and plastoquinone spectra, no signal could be utilized as internal standard. Isotopic abundances were therefore estimated in comparison with signals of carbon atoms that were supposed to be unlabelled, i.e. the signal of carbon atoms derived from C-3 of IPP at 134.97 p.p.m. for the incubations of [1- ^{13}C]- or [6- ^{13}C]glucose and that of carbon atoms derived from C-5 of IPP at 16.05 p.p.m. for all other incubations.

For the incubation of [2- ^{13}C]glucose, carbon atoms derived from C-2, C-4 and C-5 of IPP were slightly, but significantly, labelled in steryl acetates (isotopic abundance 1.8–2.1 %) by comparison with the natural isotopic abundance (1.1 %) of the acetoxyethyl group introduced by chemical derivatization. As labelling patterns were identical in isoprenic units from sterols and ubiquinone in each labelling experiment, we assumed this was also the case for the incorporation of [2- ^{13}C]glucose. The 1.8 % isotopic abundance found for sterol carbon atoms derived from C-4 of IPP was therefore also assigned to those of ubiquinone derived from C-4 of IPP, the signal of which served as a reference signal for determination of isotopic abundance (Table 1).

Isolation and identification of sterols, ubiquinone Q10 and plastoquinone

Lyophilized cells (about 4 g, from a 640 ml culture medium) were extracted three times for 1 h under reflux with chloroform/methanol [2:1 (v/v); 250 ml]. The combined extracts were taken to dryness and thoroughly washed at room temperature with hexane in order to recover sterols (I–III), ubiquinone Q10 (IV) and plastoquinone (V) (Figure 1). The hexane extract was separated by TLC (dichloromethane) yielding plastoquinone (R_f 0.71), pure ubiquinone Q10 (R_f 0.48; 250 $\mu\text{g/g}$; lyophilized cells) and sterols (R_f 0.16). Plastoquinone (75 $\mu\text{g/g}$; lyophilized cells) was further purified by TLC [hexane/dichloromethane, 1:1 (v/v); R_f 0.45]. Sterols were acetylated overnight at room temperature using a mixture of acetic anhydride and pyridine [1:1 (v/v); 400 μl]. Steryl acetates (R_f 0.48) were purified by TLC [cyclohexane/ethyl acetate, 9:1 (v/v)]. Stigmasteryl acetate (R_f 0.28) was separated from the mixture of campesteryl acetate, 22,23-dihydrobrassicasteryl acetate and sitosteryl acetate (R_f 0.41)

on AgNO₃-impregnated TLC plates [32] using ethanol-free chloroform as eluent. Campesteryl acetate, 22,23-dihydrobrassicasteryl acetate and sitosteryl acetate were separated by reverse-phase HPLC on a Dupont Zorbax ODS column (250 mm $\times$ 21.2 mm) using methanol (15 ml/min) as eluent and a differential IOTA 2 refractometer as a detector. The residue left after hexane extraction was saponified by refluxing for 1 h with a 6 % KOH solution in methanol (15 ml). After the addition of 2 vol. of water and acidification to pH 1–2 with conc. HCl, fatty acids were extracted with hexane and methylated with a diazomethane solution in ether. The fatty acid methyl esters (R_f 0.50) were purified by TLC [cyclohexane/ethyl acetate, 9:1 (v/v)]. Pure saturated fatty acid methyl esters (R_f 0.68) corresponding mainly to methyl palmitate (76 %) containing some methyl stearate (24 %) were obtained after TLC on AgNO₃-impregnated silica gel (dichloromethane).

Steryl acetates and ubiquinone were identified by ^1H - and ^{13}C -NMR as well as by MS (electron impact ionization; 70 eV; GC-MS for steryl acetates and direct inlet for ubiquinone). Plastoquinone was identified by TLC and MS (direct inlet; electron impact ionization; 70 eV). All data were compared with those obtained on reference compounds. A large-scale culture (8 g; lyophilized cells from a 1280 ml culture) performed with [1- ^{13}C]glucose as carbon source yielded enough plastoquinone (about 500 μg) for ^{13}C -NMR analysis.

RESULTS

Labelling of isoprenic units in sterols and ubiquinone after incorporation of ^{13}C -labelled glucose

Labelling patterns of isoprenic units observed after feeding of each glucose isotopomer were identical in the sterols (I–III) and ubiquinone (IV) (Table 1). They corresponded exactly to those expected from the mevalonate pathway, with acetyl-CoA (Ac-CoA) obtained from glucose catabolism via glycolysis with some participation of the pentose phosphate pathway (Scheme 1, pathway A and Scheme 2). The direct involvement of Ac-CoA was demonstrated by the identical labelling found in each experiment for acetyl units in fatty acids and in isoprenic units of sterols and ubiquinone (Tables 1 and 2). Feeding of glucose labelled at C-1 or C-6, at C-2 or C-5 and at C-3 or C-4 resulted in qualitatively identical labelling patterns in the isoprenic units. These labelling patterns were consistent with glucose being converted into fructose 1,6-bisphosphate, which was cleaved by aldolase into dihydroxyacetone phosphate and GAP, and with the two resulting triose phosphates being interconverted by the isomerase. Such a catabolism results in the metabolic equivalence of C-1 and C-6, C-2 and C-5, and C-3 and C-4 of the hexose derivatives. Ac-CoA resulted from the action of pyruvate dehydrogenase on pyruvate obtained from GAP. Carbon atoms corresponding to C-3 and C-4 of glucose were probably lost as CO₂ by the oxidative decarboxylation of pyruvate and were therefore not incorporated, and those corresponding to C-2 and C-5 or C-1 and C-6 of glucose yielded C-1 or C-2 of acetate respectively.

Less efficient incorporation of C-1 of glucose compared with that of C-6 was probably due to an early loss of the C-1 of glucose as CO₂ via the oxidative pentose phosphate pathway (Scheme 2). Similar losses have been reported for isoprenoid biosynthesis in *E. coli*, *Alicyclobacillus acidoterrestris* [12] and the green alga *S. obliquus* [24], which all synthesize their isoprenoids via the mevalonate-independent route.

Incorporation of [2- ^{13}C]glucose also pointed to the participation of the pentose phosphate pathway in glucose catabolism. The strong labelling of carbon atoms corresponding to C-1 and

Table 1 Isotopic abundances in isoprenic units from sterols (I, II and III), ubiquinone Q10 (IV) and plastoquinone (V) in *N. tabacum* BY-2 cell cultures after feeding of ^{13}C -labelled glucose isotopomers or pyruvate (isotopic abundance of all labelled carbon sources 10%)

In each isoprenoid analysed, all isoprenic units were identically labelled. For the sake of clarity, labelling patterns are therefore only indicated as mean values for all carbon atoms corresponding to the same carbon atom of IPP. Numbering of IPP is given in Scheme 1. Ubiquinone and plastoquinone signals labelled with an asterisk were assumed to correspond to the natural ^{13}C abundance (1.1%) and were utilized as standards. ^{a,b}Two feeding experiments were carried out with $[1-^{13}\text{C}]$ glucose. Entries with the same superscript correspond to the same experiment. The values in bold highlight the strong labelling.

Substrate	Isotopic abundances of IPP carbon atoms (%)				
	C-1	C-2	C-3	C-4	C-5
$[1-^{13}\text{C}]$ Glucose					
(I) ^a	1.0	2.4	1.0	2.5	2.5
(II) ^a	1.5	3.4	1.3	3.1	3.7
(III) ^a	1.2	2.8	1.2	2.8	2.7
(IV) ^a	1.3	2.4	1.1*	2.4	2.8
(IV) ^b	1.4	2.9	1.1*	2.9	3.1
(V) ^b	3.0	1.1	1.1*	1.6	2.9
$[2-^{13}\text{C}]$ Glucose					
(I)	3.9	1.9	4.2	2.1	1.9
(II)	4.2	2.0	4.1	1.8	2.1
(III)	3.8	1.8	3.7	1.8	1.9
(IV)	3.9	1.8	4.0	1.9	1.9*
$[3-^{13}\text{C}]$ Glucose					
(I)	1.7	1.2	1.6	1.4	1.3
(II)	1.5	1.3	1.7	1.2	1.3
(III)	1.8	1.4	1.8	1.5	1.5
(IV)	1.3	1.1	1.3	1.4	1.1*
$[4-^{13}\text{C}]$ Glucose					
(I)	1.1	1.0	1.1	1.1	1.1
(II)	1.6	1.5	1.4	1.4	1.4
(III)	1.3	1.2	1.2	1.3	1.2
(IV)	1.1	1.1	1.3	1.3	1.1*
$[5-^{13}\text{C}]$ Glucose					
(I)	3.8	1.3	4.1	1.3	1.3
(II)	4.4	1.6	4.9	1.3	1.5
(III)	3.6	1.3	3.6	1.2	1.3
(IV)	3.0	1.2	3.2	1.1	1.1*
$[6-^{13}\text{C}]$ Glucose					
(I)	1.3	3.9	1.2	4.2	4.3
(II)	1.4	4.9	1.2	4.7	5.4
(III)	1.5	5.1	1.4	5.1	5.2
(IV)	1.2	3.3	1.1*	3.3	3.4
$[2-^{13}\text{C}]$ Pyruvate					
(I)	8.3	0.8	8.4	0.9	0.9
(II)	7.8	1.0	7.8	1.0	1.1
(III)	11.6	0.9	11.3	0.8	1.2
(IV)	9.3	1.1	9.5	0.9	1.1*

C-3 of IPP (isotopic abundance 3.7–4.2%) corresponded to incorporation of $[1-^{13}\text{C}]\text{Ac-CoA}$ directly obtained from $[2-^{13}\text{C}]\text{pyruvate}$ via glycolysis (Scheme 1, pathway A). The weak labelling (isotopic abundance 1.8–2.1%) probably resulted from the participation of additional steps of the pentose phosphate pathway. $[2-^{13}\text{C}]\text{Glucose}$ yields pentose derivatives labelled at C-1 via the oxidative pentose phosphate route, which are converted by transketolase and transaldolase into $[1,3-^{13}\text{C}]\text{fructose 6-phosphate}$ (Scheme 2). Incorporation of the latter hexose through glycolysis yields $[3-^{13}\text{C}]\text{pyruvate}$, from which $[2-^{13}\text{C}]\text{Ac-CoA}$ is obtained, resulting in labelling of carbon atoms derived from C-2, C-5 and C-4 of IPP. No detectable scrambling was observed

for the incorporation of the other glucose isotopomers. Additional steps of the pentose phosphate pathway and recycling of the metabolites were therefore not significantly involved in the formation of Ac-CoA from glucose.

Labelling of isoprenic units in sterols and ubiquinone after incorporation of $[2-^{13}\text{C}]\text{pyruvate}$

Direct confirmation of the involvement of the mevalonate pathway in ubiquinone biosynthesis could not be obtained by examining incorporation of acetate, as this carbon source proved to be toxic at the concentrations required to perform the labelling experiments. An incubation was therefore attempted with $[2-^{13}\text{C}]\text{pyruvate}$ in the presence of unlabelled sucrose. Labelled pyruvate was expected to be converted by the pyruvate dehydrogenase into $[1-^{13}\text{C}]\text{Ac-CoA}$. The labelling patterns observed in isoprenic units from the sterols (I–III) and ubiquinone (IV) were again qualitatively identical and corresponded to those expected via the mevalonate pathway from the incorporation of $[1-^{13}\text{C}]\text{acetate}$ derived from $[2-^{13}\text{C}]\text{pyruvate}$ (Tables 1 and 2). Differences were observed in the labelling intensities of the isoprenoids analysed. Significantly lower incorporation was observed into campesterol (I) and sitosterol (II) (isotopic abundance 7.8–8.4%) than into stigmasterol (III) and ubiquinone (IV) (isotopic abundance 10.3–11.4%). This feature reflects a more significant contribution of the unlabelled carbon source (sucrose) than of $[2-^{13}\text{C}]\text{pyruvate}$ in the formation of the cellular Ac-CoA pool involved in the formation of campesterol and sitosterol. This differential incorporation points to the possibility that all isoprenoids are not synthesized at the same rate and/or at the same stage of the growth cycle. Similarly, the labelling patterns of fatty acids from $[2-^{13}\text{C}]\text{pyruvate}$ (Table 2) clearly indicate that Ac-CoA originated directly from pyruvate, but the much lower incorporation reflects an even more significant contribution of the unlabelled sucrose to fatty acid biosynthesis than to isoprenoid biosynthesis. Such a discrimination is frequently observed when several carbon sources, and consequently different metabolic routes, are in competition for the formation of a metabolite. Striking differences were observed, for instance, in labelling experiments with *Methylobacterium fujisawaense* using $[3-^{13}\text{C}]\text{pyruvate}$ in the presence of unlabelled acetate or glucose between ubiquinone and triterpenoids of the hopane series on the one hand, and even between biogenetically very closely related hopanoids on the other [12].

Labelling of isoprenic units in plastoquinone after incorporation of $[1-^{13}\text{C}]\text{glucose}$

After incorporation of $[1-^{13}\text{C}]\text{glucose}$, the labelling patterns of isoprenic units in plastoquinone (V) on the one hand and those of ubiquinone (IV) and sterols (I–III) on the other differed completely (Table 1). IPP involved in the formation of the plastoquinone prenyl chain was formed via the mevalonate-independent route via GAP and pyruvate (Scheme 1, pathway B). Such a labelling pattern was previously found after feeding of $[1-^{13}\text{C}]\text{glucose}$ for the isoprenoids of *E. coli* and *A. acidoterrestris* [12], those of *S. obliquus* [24] or the chloroplast isoprenoids from higher plants [19]. All of them were shown to be formed via the GAP/pyruvate route. Isotopic abundances found on the labelled positions of plastoquinone were nearly identical with those found in ubiquinone and sterols, in accordance with glucose catabolism predominantly via glycolysis and formation of Ac-CoA from pyruvate (Scheme 1).

Scheme 2 Glucose catabolism via the pentose phosphate pathway showing the loss of C-1 of glucose via the oxidative pathway and the possible incorporation of C-2 of glucose into C-1 of Ac-CoA by the non-oxidative pathway

TK, transketolase; TA, transaldolase.

Table 2 Isotopic abundances of methyl palmitate obtained from *N. tabacum* BY-2 cell cultures after feeding of ^{13}C -labelled glucose or pyruvate

* The signal of the ester methyl group (isotopic abundance 1.1%) was utilized as reference for the evaluation of the isotopic abundances. The values in bold highlight strong labelling.

Carbon atom	[1- ^{13}C]-Glucose	[2- ^{13}C]-Glucose	[3- ^{13}C]-Glucose	[4- ^{13}C]-Glucose	[5- ^{13}C]-Glucose	[6- ^{13}C]-Glucose	[2- ^{13}C]-Pyruvate
C-1	1.6	6.9	1.7	1.5	5.9	1.9	5.4
C-2	3.2	2.1	1.0	1.1	1.6	5.4	1.2
C-3	1.1	4.6	1.3	1.1	5.9	1.3	3.6
C-4	3.0	2.8	1.1	1.1	1.8	5.0	1.2
C-5	1.3	5.6	1.6	1.3	5.6	2.0	4.1
C-6	3.1	3.0	1.1	1.2	2.1	5.5	1.3
C-7 to C-12	—	—	—	—	—	—	—
C-13	1.1	5.0	1.3	1.1	5.0	1.5	3.7
C-14	3.3	2.4	1.0	1.1	1.6	5.6	1.0
C-15	1.2	6.1	1.6	1.3	5.9	1.4	4.4
C-16	3.4	2.8	1.1	1.2	1.3	5.7	1.0
OMe*	1.1	1.1	1.1	1.1	1.1	1.1	1.1

DISCUSSION

According to all the labelling experiments performed in this study with ^{13}C -labelled D-glucose isotopomers and pyruvate, TBY-2 cells utilized only the mevalonate pathway for the formation of sterols and ubiquinone. No evidence for the

participation of the plastidic mevalonate-independent GAP/pyruvate route could be detected, at least within the limits of detection of the ^{13}C -NMR spectroscopy. These experiments represent the first direct unambiguous proof that IPP involved in the formation of the ubiquinone prenyl chain is synthesized in a plant system from Ac-CoA through mevalonate. Comparison of the labelling patterns found in fatty acids and isoprenoids confirmed that they were all derived from a single Ac-CoA pool (Tables 1 and 2). Ubiquinone biosynthesis in TBY-2 cells is therefore probably essentially dependent on a supply of IPP from the cytoplasm. It is however, necessary to check whether the TBY-2 cell culture is representative of all plant systems before generalizing such an assertion.

Small amounts of plastoquinone were still detected in TBY-2 cells. In *Lemna gibba*, plastoquinone was shown to be synthesized via the GAP/pyruvate pathway [19]. According to the results of [1- ^{13}C]glucose incorporation, the vestigial proplastids of the TBY-2 cells maintained the capacity to use this metabolic pathway, despite the absence of detectable amounts of other plastidic isoprenoids (carotenoids, phytol from chlorophylls).

TBY-2 cells originally derived from tobacco plants (var. Bright Yellow) were first characterized by their 3 to 4 times higher ubiquinone Q10 content than other plant cell lines [33,34]. From this initial culture, further clones were isolated for their even more superior production of ubiquinone [28]. However, this increase negatively affected growth rate. As ubiquinone was produced in a constant ratio to other components of the mitochondrial respiration chain (cytochrome *aa3*, cytochrome *b* and *c*, coupling factor, mitochondrial proteins), the high ubi-

quinone content was apparently due to an increase in mitochondrial volume and/or number [28]. If we assume that biosynthesis of ubiquinone and phytosterol shares a common mevalonate-derived precursor pool, possibly IPP, then diverging a putatively limiting substrate into an end product other than phytosterols might lead to limitation of cell division activity because of some lack of material for membrane synthesis. The original cell line TBY-2 was further selected for rapid proliferation [30], and therefore it cannot be ruled out that further point mutations might have occurred. Although the TBY-2 cells used by us are still characterized by a high number of mitochondria per cell [30], this might explain why the relative ubiquinone content that we found was slightly below that initially reported by Ikeda et al. [33,34]. Nevertheless, taking advantage of the rapid-division cell cycle (about 14 h under standard conditions), enough material could be produced for extraction of amounts of ubiquinone suitable for NMR analysis. Cells grew sufficiently well when glucose replaced sucrose, but at a lower rate.

The major pathway to ubiquinone in mammalian and bacterial systems was reviewed previously [35]. For mammalian tissues and cells, evidence was presented that availability of mevalonate plays a limiting role in ubiquinone biosynthesis, and that the inner mitochondrial membrane is capable of synthesis and lengthening of the polyprenyl phosphate via prenyltransferases utilizing IPP [36]. This also implies that systems must exist that are responsible for the transport of IPP into the mitochondria, but it was not ruled out that preformed polyprenyl chains synthesized in the cytosol might contribute to the mitochondrial polyprenyl pyrophosphate pool [36]. Some evidence has been presented that ubiquinone, usually in its reduced form and therefore capable of acting as a powerful scavenger of radicals [37,38], is present in practically all cell membranes and is not exclusively confined to mitochondria. In mammalian cells, the Golgi apparatus appears to play an important role in ubiquinone biosynthesis [39]. In studies with the unicellular alga *Euglena gracilis*, it was concluded that 4-hydroxybenzoate polyprenyltransferase activity, which catalyses an important reaction in ubiquinone biosynthesis, did not parallel exactly the mitochondrial marker enzyme, succinic acid oxidase [40]. This might be indicative of ubiquinone synthesis occurring at several sites in the cell. By using fractionation methods for the isolation of subcellular membranes and organelles from spinach leaves similar to those described for rat tissues, it was even suggested that the Golgi apparatus is responsible for synthesizing not only ubiquinone, but also plastidic components such as plastoquinone [41]. Membrane fractions enriched in endoplasmic reticulum and Golgi particles have been reported to contain most of the cellular capacity to catalyse the prenylation of 4-hydroxybenzoate (precursor of ubiquinone) and 2-methylquinol (precursor of plastoquinone) [41]. Final products, predominantly found in their reduced form, should then be transported to the mitochondria and plastids respectively [42]. This contradicts the observations that plastoquinone in TBY-2 cells and *L. gibba* as well as all other prenyl lipids present in chloroplasts are clearly and practically derived exclusively from the alternative pathway [19]. Compartmentation of all-*trans*-polyprenol biosynthesis is therefore likely in plant cells. The nonaprenyl chain of plastoquinone arises from the GAP/pyruvate route to IPP, whereas the decaprenyl chain from ubiquinone Q10 is derived from the mevalonate pathway. This suggests that prenyltransferases catalysing the same reaction yielding all-*trans*-polyprenols should be present at least in chloroplasts and mitochondria. As in animal tissues ([43] and references cited therein), *cis*- and *trans*-prenyltransferases at the entry of the elongation pathways were found in plant cells,

with the former being soluble and the latter being membrane-bound [44]. In animals cells, *trans*-geranylgeranyl diphosphate synthase provides the substrate for geranylgeranylation of proteins, and after appropriate elongation of farnesyl diphosphate by *trans*-prenyltransferases, the side chain of ubiquinone [39,43,45,46]. In this way, interference with the formation of *cis*-polyprenyl pyrophosphates starting from farnesyl diphosphate, with dolichol phosphates as prominent ubiquitous end products, might be prevented.

Mevalonate 5-diphosphate was not incorporated into ubiquinone by intact plant mitochondria, whereas IPP and *p*-hydroxybenzoate were readily incorporated [25]. This implies that the biosynthesis of IPP required for ubiquinone formation occurs outside the mitochondria in the cytoplasm and that a translocation system must exist. The strict dependence of ubiquinone prenyl chain formation on a source of IPP has also been shown for mitochondria from rat liver [47,48] and yeast [49]. In this respect, it is interesting to note that *Arabidopsis thaliana* contains at least two genes coding for farnesyl diphosphate synthase (FPS) (*FPS1* and *FPS2*), which are differentially regulated. The *FPS1* gene generates two FPS isoforms (FPS1L and FPS1S mRNAs) which differ only by the presence or absence of an N-terminal extension of 41 amino acid residues in FPSL [50]. The functionality of this extension in the FPS1L as a mitochondrial transit peptide has been demonstrated [50]. However, to our knowledge enzymes responsible for chain elongation of farnesyl diphosphate with IPP units have not yet been characterized further, nor have the corresponding genes been cloned from plants. In fact, isoprenoid biosynthesis involves movement of Ac-CoA precursors between mitochondria and cytoplasm. Ac-CoA utilized in the cytoplasm for IPP biosynthesis derives from citrate synthesized in the mitochondria from pyruvate, which comes from glycolysis. This citrate is transferred to the cytoplasm and cleaved there to oxaloacetate and Ac-CoA, which can then be incorporated into mevalonate, and thus into IPP [51,52].

From earlier studies with mevinolin (also referred to as lovastatin), a highly specific inhibitor of HMG-CoA reductase [53], using cell suspension cultures of *Silybum marianum* [54], it was concluded that inhibition of cytosolic mevalonate biosynthesis led to an immediate effect on sterol accumulation, whereas at inhibitor concentrations below the limit of general cytotoxicity, ubiquinone content of the cells did not decrease below 50 % of the normal concentration. Similar differential effects on ubiquinone and sterol accumulation were found with intact mevinolin-treated radish seedlings [55]. Thus a complete dependence of sterol biosynthesis on cytosolic mevalonate production and an apparently only partial dependence of ubiquinone side-chain formation suggested some independent precursor-product relationship, and some contribution of the alternative pathway could be possible. However, the data presented in the present study indicate a common IPP precursor pool for sterols and ubiquinone, since no involvement of the GAP/pyruvate route for the formation of the prenyl chain of ubiquinone was observed. Differential effects might also be explained by greatly differing affinities of the enzymes, e.g. prenyltransferases. In mammalian tissues, squalene synthase has a considerably lower affinity for farnesyl diphosphate than other branch-point enzymes; this would explain why inhibition of HMG-CoA reductase primarily affects cholesterol synthesis [42]. Mevalonate-derived IPP units would therefore be preferentially shuttled into the ubiquinone branch. At this stage therefore we cannot exclude the possibility that, under specific stress conditions, e.g. complete blocking of cytosolic IPP synthesis from HMG-CoA, some rescue mechanisms are activated that would overcome the lack of

mevalonate-derived IPP units for ubiquinone biosynthesis and protein isoprenylation and that thereby the two pathways somehow 'conspire'. In contrast with the model proposed by Kreuz and Kleinig [56,57], it has been suggested that, under certain circumstances, IPP may be formed exclusively in plastids and then distributed to other cell compartments; however, in their study the authors assumed that IPP made in the plastids would be derived from mevalonate [52] and not via the GAP/pyruvate route as shown later [19].

In conclusion, this study again demonstrates the predominant role of glucose rather than acetate as carbon source in labelling experiments for isoprenoid biosynthesis in phototrophic organisms. These results are consistent with the occurrence of at least two sites of IPP biosynthesis in plant cells: one located in the cytoplasm producing IPP via the mevalonate pathway, which is distributed in the endoplasmic reticulum and mitochondria for the formation of triterpenoids (including sterols), sesquiterpenoids and the prenyl chain of ubiquinone, and a second in the chloroplasts producing IPP via the GAP/pyruvate route for the formation of all isoprenoids involved in photosynthesis (phytol, plastoquinone, carotenoids).

We thank M. Jean-Daniel Sauer for all NMR measurements and M. Patrick Wehrung for recording the mass spectra. We are indebted to Professor T. Nagata (Tokyo) for permission to use TBV-2 cells. A.D. was supported by grants from the French government (Centre National des Deuvres Universitaires et Scolaires) and from DAAD (Deutscher Akademischer Austauschdienst).

REFERENCES

- Spurgeon, S. L. and Porter, J. W. (1981) In *Biosynthesis of Isoprenoid Compounds*, vol. 1 (Porter, J. W. and Spurgeon, S. L., eds.), pp. 1–46, John Wiley and Sons, New York.
- Qureshi, N. and Porter, J. W. (1981) In *Biosynthesis of Isoprenoid Compounds*, vol. 1 (Porter, J. W. and Spurgeon, S. L., eds.), pp. 47–94, John Wiley and Sons, New York.
- Kleinig, H. (1989) *Annu. Rev. Plant Physiol.* **40**, 39–59.
- Bach, T. J. (1995) *Lipids* **30**, 191–202.
- Chappell, J. (1995) *Annu. Rev. Plant Physiol.* **46**, 521–547.
- Lichtenthaler, H. K., Rohmer, M. and Schwender, J. (1997) *Physiol. Plant.* **101**, 643–652.
- Treharne, K. J., Mercer, E. I. and Goodwin, T. W. (1966) *Biochem. J.* **99**, 239–245.
- Banthorpe, D. V., Charlwood, B. V. and Francis, M. J. (1972) *Chem. Rev.* **72**, 115–155.
- Bach, T. J. and Lichtenthaler, H. K. (1987) *Am. Chem. Soc. Symp. Ser.* **325**, 109–139.
- Bach, T. J., Motel, A. and Weber, T. (1990) *Recent Adv. Phytochem.* **24**, 1–82.
- Flesch, G. and Rohmer, M. (1988) *Eur. J. Biochem.* **175**, 405–411.
- Rohmer, M., Knani, M., Simonin, P., Sutter, B. and Sahm, H. (1993) *Biochem. J.* **295**, 517–524.
- Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A. and Zenk, M. H. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10600–10605.
- Rosa Putra, S., Lois, L.-M., Campos, N., Boronat, A. and Rohmer, M. (1998) *Tetrahedron Lett.* **39**, 23–26.
- Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S. and Sahm, H. (1996) *J. Am. Chem. Soc.* **118**, 2564–2566.
- Duvold, T., Bravo, J.-M., Pale-Grosdemange, C. and Rohmer, M. (1997) *Tetrahedron Lett.* **38**, 4769–4772.
- Duvold, T., Cali, P., Bravo, J.-M. and Rohmer, M. (1997) *Tetrahedron Lett.* **38**, 6181–6184.
- Schwarz, M. K. (1994) Ph.D. Thesis Nb ETH 10951, Eidgenössische Technische Hochschule, Zürich.
- Lichtenthaler, H. K., Schwender, J., Disch, A. and Rohmer, M. (1997) *FEBS Lett.* **400**, 271–274.
- Eisenreich, W., Menhard, B., Hylands, P. J., Zenk, M. H. and Bacher, A. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6431–6436.
- Knöss, W., Reuter, B. and Zapp, J. (1997) *Biochem. J.* **326**, 449–454.
- Eisenreich, W., Sagner, S., Zenk, M. H. and Bacher, A. (1997) *Tetrahedron Lett.* **38**, 3889–3892.
- Zeidler, J. G., Lichtenthaler, H. K., May, H. V. and Lichtenthaler, F. W. (1997) *Z. Naturforsch.* **52c**, 15–23.
- Schwender, J., Seemann, M., Lichtenthaler, H. K. and Rohmer, M. (1996) *Biochem. J.* **316**, 73–80.
- Lütke-Brinkhaus, F., Liedvogel, B. and Kleinig, H. (1984) *Eur. J. Biochem.* **141**, 537–541.
- Seemann, M. (1995) Ph.D. Thesis, Université de Haute Alsace, Mulhouse.
- Matsumoto, T., Kanno, N., Ikeda, T., Obi, Y., Kisaki, T. and Noguchi, M. (1981) *Agric. Biol. Chem.* **45**, 1627–1633.
- Ikeda, T., Matsumoto, T., Obi, Y., Kisaki, T. and Noguchi, M. (1981) *Agric. Biol. Chem.* **45**, 2259–2263.
- Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473–479.
- Nagata, T., Nemoto, Y. and Hasegawa, S. (1992) *Int. Rev. Cytol.* **132**, 1–30.
- Reichheld, J.-P., Sonobe, S., Clément, B., Chaubet, N. and Gigot, C. (1995) *Plant J.* **7**, 245–252.
- Aitzetmüller, K. and Gueraldo Goncalves, L. A. (1987) *J. Chromatogr.* **519**, 349–358.
- Ikeda, T., Matsumoto, T. and Noguchi, M. (1976) *Phytochemistry* **15**, 568–569.
- Ikeda, T., Matsumoto, T. and Noguchi, M. (1978) *Phytochemistry* **17**, 1879–1883.
- Rudney, H. (1977) In *Biochemical and Clinical Aspects of Coenzyme Q*, vol. 1 (Folkers, K. and Yamamura, Y., eds.), pp. 29–45, Elsevier/North-Holland Biomedical Press, Amsterdam.
- Rudney, H., Nambudiri, A. M. D. and Ranganathan, S. (1981) In *Biochemical and Clinical Aspects of Coenzyme Q*, vol. 3 (Folkers, K. and Yamamura, Y., eds.), pp. 279–290, Elsevier/North-Holland Biomedical Press, Amsterdam.
- Forsmark-Andrée, P., Dallner, G. and Ernster, L. (1995) *Free Radic. Biol. Med.* **19**, 749–757.
- Ernster, L. and Dallner, G. (1995) *Biochim. Biophys. Acta* **1271**, 195–204.
- Teclebrhan, H., Olson, J., Swiezewska, E. and Dallner, G. (1993) *J. Biol. Chem.* **268**, 23081–23086.
- Threlfall, D. R. (1982) In *Biochemistry and Metabolism of Plant Lipids* (Wintermans, J. F. G. M. and Kuiper, P. J. C., eds.), pp. 527–536, Elsevier Biomedical Press B.V., Amsterdam.
- Swiezewska, E., Dallner, G., Andersson, B. and Ernster, L. (1993) *J. Biol. Chem.* **258**, 1494–1499.
- Osowska-Rogers, S., Swiezewska, E., Andersson, B. and Dallner, G. (1994) *Biochem. Biophys. Res. Commun.* **205**, 714–721.
- Ericsson, J. (1992) Doctoral Dissertation, Arrhenius Laboratories of Natural Sciences, Akademiktryk AB, Edsbruk, Sweden (ISBN-91-7153-061-4).
- Cornish, K. (1993) *Eur. J. Biochem.* **218**, 267–271.
- Teclebrhan, H., Jakobsson-Borin, A., Brunck, U. and Dallner, G. (1995) *Biochim. Biophys. Acta* **1256**, 157–165.
- Runquist, M., Ericsson, J., Thelin, A., Chojnacki, T. and Dallner, G. (1994) *J. Biol. Chem.* **269**, 5804–5809.
- Momose, K. and Rudney, H. (1972) *J. Biol. Chem.* **247**, 3930–3940.
- Trumpower, B. L., Housser, R. M. and Olson, R. E. (1974) *J. Biol. Chem.* **249**, 3041–3048.
- Casey, J. and Threlfall, D. R. (1978) *Biochim. Biophys. Acta* **530**, 187–502.
- Cunillera, N., Boronat, A. and Ferrer, A. (1997) *J. Biol. Chem.* **272**, 15831–15838.
- Hanson, J. B. (1985) *Encycl. Plant Physiol. New Ser.* **18**, 248–280.
- McCaskill, D. and Croteau, R. (1995) *Planta* **197**, 49–56.
- Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monagan, R., Curries, S., Stapley, E., Albers-Schönberg, G., Hensens, O., Hirshfield, J., Hogsteen, K., Liesch, J. and Springer, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3957–3961.
- Döll, M., Schindler, S., Lichtenthaler, H. K. and Bach, T. J. (1984) In *Structure, Function and Metabolism of Plant Lipids* (Siegenthaler, P.-A. and Eichenberger, W., eds.), pp. 277–280, Elsevier Science Publishers, B.V., Amsterdam.
- Schindler, S., Bach, T. J. and Lichtenthaler, H. K. (1985) *Z. Naturforsch.* **40c**, 208–214.
- Kreuz, K. and Kleinig, H. (1981) *Planta* **153**, 578–581.
- Kreuz, K. and Kleinig, H. (1984) *Eur. J. Biochem.* **141**, 531–535.