Distribution of the mevalonate and glyceraldehyde phosphate/pyruvate pathways for isoprenoid biosynthesis in unicellular algae and the cyanobacterium *Synechocystis* PCC 6714

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Isopentenyl diphosphate, the universal isoprenoid precursor, can be produced by two different biosynthetic routes: either via the acetate/mevalonate (MVA) pathway, or via the more recently identified MVA-independent glyceraldehyde phosphate/pyruvate pathway. These two pathways are easily differentiated by incorporation of [1-^{13}C]glucose and analysis of the resulting labelling patterns found in the isoprenoids. This method was successfully applied to several unicellular algae raised under heterotrophic growth conditions and allowed for the identification of the pathways that were utilized for isoprenoid biosynthesis. All isoprenoids examined (sterols, phytol, carotenoids) of the green algae *Chlorella fusca* and *Chlamydomonas reinhardtii* were synthesized via the GAP/pyruvate pathway, as in another previously investigated green alga, *Scenedesmus obliquus*, which was also shown in this study to synthesize ubiquinone by the same MVA-independent route. In the red alga *Cyanidium caldarium* and in the Chrysophyte *Ochromonas danica* a clear dichotomy was observed: as in higher plants, sterols were formed via the MVA route, whereas chloroplast isoprenoids (phytol in *Cy. caldarium* and *O. danica* and β-carotene in *O. danica*) were synthesized via the GAP/pyruvate route. In contrast, the Euglenophyte *Euglena gracilis* synthesized ergosterol, as well as phytol, via the acetate/MVA route. Similar feeding experiments were performed with the cyanobacterium *Synechocystis* PCC 6714 using [1-^{13}C]- and [6-^{13}C]-glucose. The two isoprenoids examined, phytol and β-carotene, were shown to have the typical labelling pattern derived from the GAP/pyruvate route.

INTRODUCTION

For the last five decades, the formation of isoprenyl diphosphate (IPP), which is the common precursor for all isoprenoids, was studied in many organisms [1]. The acetate/mevalonate (MVA) pathway (see Scheme 1, pathway A) was described as the main pathway for IPP formation in animals, plants, fungi and bacteria [1–3]. In this pathway, the specific precursor of IPP (**VI**), MVA (**VII**), is formed from acetyl-CoA (**IV**). MVA is transformed into IPP by two phosphorylation steps at C-5 of MVA and a decarboxylation/elimination step [1].

An alternative and totally different pathway for IPP formation (see Scheme 1, pathway B) was first detected and partially elucidated by Rohmer and coworkers in eubacteria [4–7], and later found in the green alga *Scenedesmus obliquus* [8], in a liverwort [9] and in many higher plants [10–17]. Two main steps of this novel route have been identified. First, D-1-deoxyxylulose 5-phosphate is formed from (hydroxyethyl)thiamine (**III**), derived from pyruvate (**II**) decarboxylation and glyceraldehyde 3-phosphate (GAP) (**I**) [7,18]. This pentulose is incorporated efficiently into the menaquinone and ubiquinone prenyl chains of *Escherichia coli* [18]. The gene of the corresponding synthase was cloned recently from *E. coli* and overexpressed in this bacterium [19,20]. Secondly, an intramolecular rearrangement of D-1-deoxyxylulose 5-phosphate or one of its straight-chain derivatives yields the branched-chain carbon skeleton of IPP (**VI**), as shown by the incorporation of multiply labelled [^{13}C]glucose [6–8] or D-1-deoxyxylulose [21,22] into the isoprenoids of bacteria, algae and plants.

In the unicellular green alga *S. obliquus*, all investigated isoprenoids (the plastid-bound carotenoids, the phytyl chain of chlorophyll and plastoquinone, as well as the cytosolic sterols) were formed via the GAP/pyruvate route [8]. This suggested that *S. obliquus* uses this pathway exclusively. In contrast, the two pathways for isoprenoid biosynthesis were found in parallel in higher plants. In three higher plants, the plastid-bound essential isoprenoids (carotenoids, phytyl chain of chlorophylls and plastoquinone) were formed via the GAP/pyruvate pathway, whereas the cytoplasmic sterols were derived from the acetate/MVA pathway [10]. Evidence for the involvement of the GAP/pyruvate pathway was also found for the formation of other plant isoprenoids of more restricted distribution, or of more marginal physiological significance: isoprene [11,17], monoterpenes [12] and diterpenes [13–15].

The aim of this study was to find out which pathway is involved for isoprenoid biosynthesis in other species of algae, as well as in a cyanobacterium, *Synechocystis* PCC 6714.

EXPERIMENTAL

Cultures and labelling experiments

Strains of *Chlorella fusca* 211–8b, *Chlamydomonas reinhardtii* strain 11–31, *S. obliquus* strain 276–3d, *Ochromonas danica* 933–7 and *Euglena gracilis* 1224–5/9 were obtained from the Sammlung von Algenkulturen (Pflanzenphysiologisches Institut der Universität, Göttingen, Germany). The *Cyanidium caldarium* mutant CDP (green at dark culture) and *Synechocystis* PCC 6714

Abbreviations used: GAP, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; MVA, mevalonate.

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were provided by Professor S. Beale (Brown University, Providence, RI, U.S.A.) and Professor F. König (Universität Bremen, Bremen, Germany) respectively.

All micro-organisms were grown heterotrophically for 4 to 5 days (until the end of the exponential-growth phase, yielding about 0.5–0.6 $g \cdot l^{-1}$, dry weight) in 5 l flasks under low illumination, as previously described [8] using synthetic media (5 l) described in the literature: *Chlor. fusca*, *Chlam. reinhardtii* and *S. obliquus* [8], *Cy. caldarium* [23], *O. danica* and *Eu. gracilis* [24] and *Synechocystis* [25]. A large-scale culture (15 l) was required for the isolation of ubiquinone from *S. obliquus*. In all cases, ^{13}C-labelled glucose [0.1 % (w/v), 10 % isotopic abundance in all cases but one *Synechocystis* culture] was the carbon source (see Table 1). The culture media of *O. danica* and *Eu. gracilis* contained appreciable amounts of sodium citrate (0.8 $g \cdot l^{-1}$) and that of *Synechocystis* contained Hepes (1.43 $g \cdot l^{-1}$); these organic buffers apparently did not hamper the incorporation of the labelled carbon source. A culture of *S. obliquus* in presence of 0.1 % (w/v) glucose and 0.005 % (w/v) sodium [3-^{13}C]pyruvate (99 % isotopic abundance) was performed in order to check the incorporation of pyruvate into the isoprenoids. [1-^{13}C]- and [6-^{13}C]-glucose were from Omicron Biochemicals Inc. (South Bends, IN, U.S.A.), and sodium [3-^{13}C]pyruvate from Promochem GmbH (Wesel, Germany).

Analytical methods and evaluation of isotopic abundances

Most analytical methods were as described previously [4,8]. All NMR spectra were recorded on a Bruker WP 400 spectrometer in [^2H]chloroform solution. ^{13}C-Isotopic abundances in the labelled compounds were determined as described previously [4,8]. Unlabelled reference compounds of natural abundance were available for all isoprenoids. For the fatty acids, methyl esters and the acetates of aliphatic long-chain alcohols, the major compound, i.e. methyl palmitate, methyl myristate or docosanyl acetate was utilized as the reference. For steryl acetates, phytyl acetate and long-chain alcohol acetates, the methyl singlet of the acetoxy group (1.1 % isotopic abundance) was utilized as an internal reference. For the methyl esters of fatty acids, the ester methyl group served as a reference. In the cases of carotenoids and ubiquinone, such a signal could not be utilized as a reference and isotopic abundances were therefore estimated in comparison with signals of carbon atoms that were supposedly not labelled, i.e. the signal of the carbon atoms derived from C-3 of IPP at 134.97 p.p.m. for ubiquinone, from C-2 of β-carotene at 39.70 p.p.m. or lutein at 48.47 p.p.m. For the incubation of [1-^{13}C]glucose with *O. danica*, all carbon atoms were labelled in phytol, β-carotene and myristic acid. Assuming that phytol and β-carotene were issued from the same geranylgeranyl diphosphate pool, the same 2.3 or 2.4 % isotopic abundance (depending on the experiments, see Table 1) found for phytyl acetate was assigned to the signal of the carbon atoms derived from C-2 of IPP in the spectrum of β-carotene, which was utilized as a reference signal.

Isolation and identification of isoprenoids

In all experiments, the freeze-dried cells were extracted with methanol and chloroform/methanol (2:1, v/v) as described previously [8]. The extracts from *S. obliquus* (pyruvate-labelling experiment), *Chlor. fusca*, *Chlam. reinhardtii*, *Cy. caldarium*, *O. danica* and *Eu. gracilis* were saponified overnight at room temperature under a N_2 atmosphere using a 3 % (w/v) KOH solution in aqueous methanol. After addition of water, neutral lipids were extracted with hexane/ether (1:1, v/v). After acidification of the aqueous phase with concentrated HCl (pH 1), fatty acids were extracted with hexane, methylated with diazomethane, and the methyl esters were separated by argentation TLC (methylene chloride) to give the saturated fatty acid methyl esters (R_F 0.68).

Neutral lipids were separated by preparative TLC (methylene chloride, two migrations) yielding fractions containing β-carotene (R_F 0.90), phytol and eventually saturated long-chain alcohols (R_F 0.27), sterols (R_F 0.12) and xanthophylls on the baseline. Pure β-carotene was obtained after an additional TLC [cyclohexane/toluene (8:2, v/v), R_F 0.48]. A TLC separation of the xanthophyll fraction from *Chlam. reinhardtii* (anhydrous peroxide-free diethyl ether) afforded lutein (R_F 0.36). Phytol and sterols were acetylated overnight at room temperature in the presence of acetic anhydride/pyridine (1:1). After removal of the reagents under a nitrogen stream, the acetates were purified by TLC [cyclohexane/ethyl acetate (9:1, v/v), R_F 0.50). Phytyl acetate from *Chlor. fusca* and *Chlam. reinhardtii* was accompanied by acetates of aliphatic long-chain alcohols. They were separated from each other by argentation TLC [cyclohexane/toluene (17:3, v/v), three migrations] yielding the mixture of the long-chain alcohol acetates (R_F 0.38) and phytyl acetate (R_F 0.27). Steryl acetates from the two green algae were further purified by argentation TLC (absolute chloroform) yielding a mixture of 22,23-dihydrochondrillasteryl acetate and ergost-7-enyl acetate (R_F 0.46) and pure chondrillasteryl acetate (R_F 0.33). The two steryl acetates from the least polar fraction were isolated by reversed-phase HPLC on a Dupont Zorbax ODS column (250 mm × 21.2 mm or 250 mm × 4.6 mm) using methanol as the eluent (15 $ml \cdot min^{-1}$ or 1 $ml \cdot min^{-1}$) and an IOTA 2 differential refractometer as detector. Poriferasteryl acetate (R_F 0.43) from *O. danica* was separated from the mixture of 7-dehydroporiferasteryl acetate and ergosteryl acetate (R_F 0.10) by argentation TLC (absolute chloroform).

The chloroform/methanol extract from *S. obliquus* cells (7.5 g) obtained from the large-scale culture was separated on a silica-gel column using hexane/ether (1:1, v/v) as eluent. TLC of the least polar fraction (methylene chloride, one migration) afforded a ubiquinone-containing fraction (R_F 0.45). Pure ubiquinone Q-10 was obtained after an additional TLC [hexane/anhydrous peroxide-free diethyl ether (75:25, v/v), R_F 0.43].

After evaporation of the solvents, the dry chloroform/methanol extracts from the three *Synechocystis* samples (0.7 g, 2.0 g or 0.8 g, dry weight) were washed with hexane, as described above. The first TLC (methylene chloride) yielded β-carotene (R_F 0.84) and a fraction containing chlorophylls and xanthophylls ($R_F < 0.20$). Pure β-carotene was obtained after an additional TLC [cyclohexane/toluene (8:2, v/v), R_F 0.49]. The chlorophyll fraction was saponified, and pure phytyl acetate was obtained as described above without argentation TLC. Acetylation of the hexane-insoluble material afforded after TLC [cyclohexane/ethyl acetate (3:7, v/v), R_F 0.15] acetylated bacteriohopanetetrol glycoside [26], although in insufficient amounts for ^{13}C-NMR analysis.

Δ^7-Sterols found in *Chlor. fusca* and *Chlam. reinhardtii* were identical with those we had found previously in *S. obliquus* [8]. For *Chlor. fusca*, they corresponded to those reported in the literature [27,28]. $\Delta^{5,7}$-Sterols were, however, described as the major sterols for *Chlam. reinhardtii* [29,30]. The difference might arise from the utilization of a different strain, or might be linked to the rather unusual heterotrophic growth conditions. Such changes in sterol composition depending on growth conditions were previously reported for *Eu. gracilis* [31]. *Eu. gracilis* contained, as expected, ergosterol [32] and *O. danica* contained poriferasterol and 7-dehydroporiferasterol [33]. In *Cy. caldarium*,

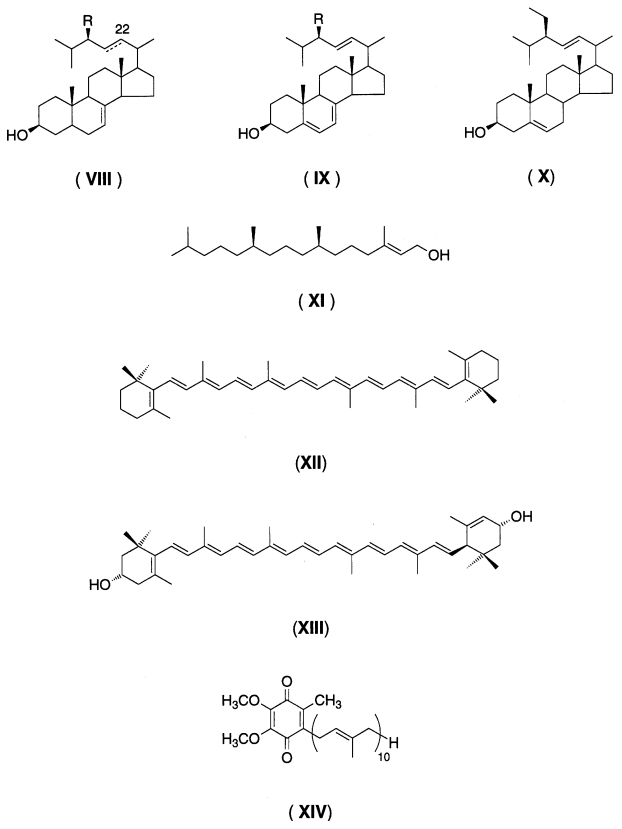


Figure 1 Isoprenoids from algae and the cyanobacterium *Synechocystis* PCC 6714

(**VIII**) ergost-7-enol (R = CH_3), 22,23-dihydrochondrillasterol (R = C_2H_5), chondrillasterol (R = C_2H_5, Δ^{22}); (**IX**) ergosterol (R = CH_3), 7-dehydroporiferasterol (R = C_2H_5); (**X**) poriferasterol; (**XI**) phytol; (**XII**) β-carotene; (**XIII**) lutein; (**XIV**) ubiquinone Q-10.

a mixture of Δ^7-sterols with $\Delta^{5,7}$-sterols only as minor components was expected [34]. Our *Cy. caldarium* mutant strain contained instead mainly ergosterol (95%). All isoprenoids were identified by GC, GC–MS, ^{1}H- and ^{13}C-NMR and by comparison of the data with those found in the literature [35–40] or with data obtained from available reference compounds (e.g. fatty acids, long-chain alcohols, phytol, ergosterol). Configuration at C-24 in sterols corresponds to that assigned in the literature for these algal sterols. ^{13}C-NMR spectra were assigned as published for phytyl acetate, ergost-7-enyl acetate, chondrillasteryl acetate and 22,23-dihydrochondrillasteryl acetate [8,36], poriferasteryl acetate [36–38], ergosteryl acetate [39,40] and β-carotene and lutein [41]. The assignment of the ^{13}C-NMR spectrum of 7-dehydroporiferasteryl acetate was on the basis of those of the spectra of ergosteryl and poriferasteryl acetates. The structures of these isoprenoids are presented in Figure 1.

RESULTS AND DISCUSSION

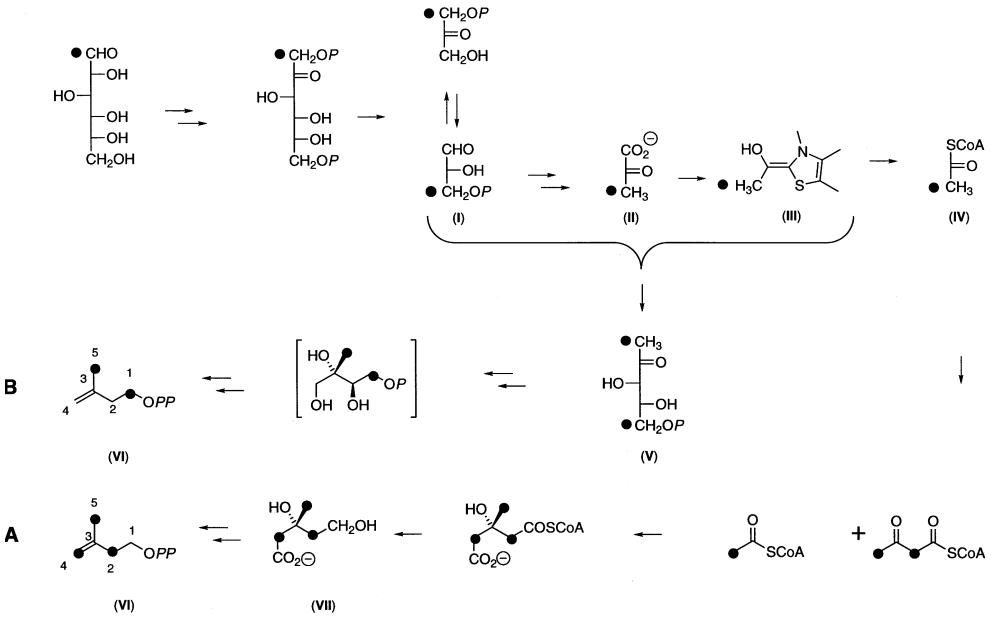
The MVA and GAP/pyruvate pathways can be differentiated unambiguously by the labelling patterns observed in isoprenoids after feeding the organisms on ^{13}C-labelled carbon sources (Scheme 1). Both acetate [4,6,8] and glucose [6,8] can be used, but glucose is a preferred substrate because it is utilized by a broader range of organisms as a sole carbon source and easily gives rise to the first precursors for both isoprenoid biosynthetic routes. In all labelling experiments performed to date with a view to the elucidation of isoprenoid biosynthesis, labelling patterns obtained from ^{13}C-labelled glucose could be explained by glucose main catabolism either via the Entner-Doudoroff pathway in bacteria [6] or via the glycolytic pathway in bacteria or phototrophic eukaryotes [6,8,10]. These glucose catabolic routes yield GAP and pyruvate, which are the first two precursors in the MVA-independent route, or acetyl-CoA, which is the starting material of the MVA pathway and is the product of pyruvate decarboxylation (Scheme 1). In the organisms investigated in this study, the labelling patterns of acetyl-CoA were deduced from those observed for the saturated fatty acids (see Table 2) and docosanol (see Table 3), which are directly obtained from the condensation of acetyl-CoA units. All labelling patterns observed in this study resulted from glucose catabolism via glycolysis and triose phosphate metabolism. The oxidative pentose phosphate pathway most probably contributed to glucose catabolism, as shown by the rather low isotopic abundances obtained after feeding [1-^{13}C]glucose of usually 10% isotopic abundance. These incorporations were significantly lower than the 5% theoretical value expected via metabolism by glycolysis alone.

Possible general occurrence of the GAP/pyruvate pathway in green algae

After growth on [1-^{13}C]glucose, the isoprenic units of all sterols, phytol and carotenoids were identically labelled in *Chlam. reinhardtii* and *Chlor. fusca* (Table 1). They were identical with those previously found for another unicellular green alga, *S. obliquus* [8], and were fully in accordance with the GAP/pyruvate pathway [8]. Label was found on carbon atoms corresponding to C-1 and C-5 of IPP, whereas, according to the acetate/MVA pathway, it would be found on those derived from C-2, C-4 and C-5 (Scheme 1) with isotopic abundances identical with that found for the even-numbered carbon atoms of palmitic acid (Table 2), which is directly derived from acetyl-CoA. A large culture of *S. obliquus* allowed enough ubiquinone Q-10 to be isolated for its labelling pattern to be analysed. Its prenyl chain was also synthesized via the MVA-independent route, like all other analysed isoprenoids of this alga (sterols, phytol, carotenoids and plastoquinone) [8].

Additional data on the presence of the GAP/pyruvate route in *S. obliquus* were obtained by the incorporation of [3-^{13}C]pyruvate (99% isotopic abundance, 50 mg·l^{-1}) in the presence of unlabelled glucose (1 g·l^{-1}) (Table 1). Isoprenic units were mostly synthesized from unlabelled glucose. Label (5% isotopic abundance) was only found on the carbon atoms derived from C-5 of IPP. Thus labelled pyruvate was only incorporated into the (hydroxyethyl)thiamine-derived C_2 moiety, and was not significantly converted into GAP. From the labelling pattern observed on methyl palmitate, pyruvate made a similar contribution to the production of acetyl-CoA, and therefore to fatty acid biosynthesis (Table 2). No evidence for incorporation via the MVA pathway of [2-^{13}C]acetate, synthesized by the decarboxylation of [3-^{13}C]pyruvate, was found. This rules out the operation of the MVA pathway and is in accordance with the results of a former incorporation of [1-^{13}C]acetate into the isoprenoids of *S. obliquus* [8]. Indeed, acetate was not directly incorporated via the acetate/MVA route, but entered the glyoxylate- and tricarboxylic-acid cycles to yield triose phosphate derivatives, which were then utilized as isoprenoid precursors in the GAP/pyruvate pathway [8].

In all experiments with green algae, there was no evidence for the MVA pathway. This was in accordance with feeding experiments run in parallel with *S. obliquus* and *Chlam. reinhardtii* using methyl [1-$^2H_1$]deoxyxyluloside and [2-^{13}C]mevalolactone



Scheme 1

[1-^{13}C]Glucose catabolism via glycolysis and incorporation of the resulting metabolites into isoprenoids via the MVA (**VII**) pathway [route A, starting from acetyl-CoA (**IV**)] or via the GAP (**I**)/pyruvate (**II**) pathway (route B).

[17]. Label was only found using GC–MS detection in phytol or sterols from both algae in the presence of the pentulose derivative. No labelling was observed in the presence of MVA. Although it is dangerous to draw definitive conclusions from the study of only three species, the exclusive occurrence of the non-MVA pathway for the biosynthesis of plastidic isoprenoids and of sterols might represent a general feature of many green algae.

Dichotomy of isoprenoid biosynthesis between cytoplasm and chloroplasts in the Rhodophyte *Cy. caldarium* and the Chrysophyte *O. danica*

Incorporation of [1-^{13}C]glucose by both *Cy. caldarium* and *O. danica* gave a similar labelling to that found for the corresponding labelling experiments with higher plants [10]. In the sterols of both algae, the carbon atoms derived from C-2, C-4 and C-5 of IPP were all labelled, indicating that the IPP involved in sterol biosynthesis was derived solely from the MVA route (Scheme 1). In contrast, in phytol from *Cy. caldarium* or in phytol and β-carotene from *O. danica* only those corresponding to C-1 and C-5 of IPP were labelled: this was the signature of the GAP/pyruvate pathway.

The results of the labelling experiment with *Cy. caldarium* using [1-^{13}C]glucose were clear-cut. In phytol, only carbon atoms derived from C-1 and C-5 of IPP were labelled, and in ergosterol, those derived from C-2, C-4 and C-5 of IPP. In both terpenoids, a 1.1 % isotopic abundance characterized all unlabelled carbon positions. These results were also in accordance, at least partially, with experiments using labelled MVA and methyl deoxyxyluloside [17]. Methyl deoxyxyluloside was utilized efficiently for phytol biosynthesis, whereas mevalolactone was not significantly incorporated. Ergosterol was labelled from both precursors, but there was a clear preference for MVA incorporation. These feeding experiments clearly indicated that both pathways were able to contribute to the synthesis of the same isoprenoid, probably depending on the growth conditions and the physiological state of the cells. Such a dichotomous biosynthetic origin for isoprenic units was reported repeatedly in other phototrophic eukaryotes [13,21,42] and its general significance should be investigated. In this study, [1-^{13}C]glucose was incorporated by *Cy. caldarium* in heterotrophic growth conditions, whereas in an earlier study labelled MVA and methyl deoxyxyluloside were fed under autotrophic growth conditions. Furthermore, addition of a precursor normally not present in large concentrations to a culture medium may disturb the normal metabolic pathways [43].

In *O. danica*, all carbon positions were significantly ^{13}C-enriched above the 1.1 % natural abundance in phytol and β-carotene (Table 1), which are both chloroplast isoprenoids, as well as in methyl myristate (Table 2). Whereas the carbon atoms derived from C-1 and C-5 of IPP and the even-numbered carbon atoms of methyl myristate were labelled with an isotopic abundance of the expected order of magnitude from the GAP/pyruvate pathway, all remaining carbon atoms, derived from C-2, C-3 and C-4 of IPP (Table 1), as well as the odd-numbered carbon atoms of fatty acids (Table 2), were significantly and equivalently labelled (average isotopic abundance of 2.3 %). Such a labelling pattern does not result from the superposition of two labellings arising from MVA and from GAP/pyruvate pathways, because the carbon atoms derived from C-3 of IPP should not be labelled in this case. It was only observed in chloroplast isoprenoids and not in sterols; it is therefore possibly linked to photosynthesis, which might occur in *O. danica* even under low light intensities. Indeed, photosynthetic recycling in the chloroplasts of $^{13}CO_2$, released by [1-^{13}C]glucose oxidation via the oxidative pentose phosphate pathway, would result in uniform labelling of GAP and pyruvate and, subsequently, of phytol and β-carotene. This was corroborated by the labelling pattern found for myristic acid. Plastids are the site of *de novo* biosynthesis of fatty acids: uniformly labelled pyruvate resulting from photosynthesis therefore yielded uniformly labelled acetyl-CoA via pyruvate dehydrogenase. In *O. danica*, pyruvate was

Table 1 Isotopic abundances in isoprenic units from algae and from the cyanobacterium *Synechocystis* PCC 6714 after incorporation of ^{13}C-labelled glucose or pyruvate

Isotopic abundance is indicated next to the carbon source. Individual isoprenic units were all identically labelled in each isoprenoid analysed. Labelling patterns were therefore indicated, for the sake of clarity, as mean values for all carbon atoms corresponding to the same carbon atom of IPP. Values in bold highlight the strong labelling. Numbering of IPP is indicated in Scheme 1.

		Isotopic abundances of IPP carbon atoms				
Micro-organism and substrate used	Isoprenoid	C-1	C-2	C-3	C-4	C-5
Cyanobacteria:						
Synechocystis PCC 6714						
[1-^{13}C]Glucose (10%)	Phytol	**2.0**	1.2	1.3	1.4	**2.3**
[1-^{13}C]Glucose (15%)	β-Carotene	**1.5**	0.9	0.9	1.3	**1.8**
	Phytol	**1.8**	1.0	1.1	1.3	**1.9**
[6-^{13}C]Glucose (10%)	Phytol	**7.0**	0.9	1.2	0.8	**4.7**
Chlorophyta:						
Chlam. reinhardtii						
[1-^{13}C]Glucose (10%)	Lutein	**3.3**	1.1	1.2	1.6	**3.7**
	Phytol	**3.3**	1.1	1.1	1.4	**3.1**
	22,23-Dihydrochondrillasterol	**4.2**	1.6	1.6	1.8	**4.1**
	Ergost-7-enol	**2.8**	0.9	1.0	1.4	**2.9**
	Chondrillasterol	**2.5**	1.0	1.1	1.3	**2.4**
Chlor. fusca						
[1-^{13}C]Glucose (10%)	β-Carotene	**2.9**	0.9	0.9	1.2	**3.1**
	Phytol	**3.5**	1.0	1.0	1.3	**3.4**
	22,23-Dihydrochondrillasterol	**3.5**	1.2	1.2	1.4	**3.5**
	Ergost-7-enol	**3.9**	1.1	1.1	1.2	**3.3**
	Chondrillasterol	**4.0**	1.1	1.1	1.4	**4.2**
S. obliquus						
[3-^{13}C]pyruvate (99%) + Glucose	Phytol	1.2	1.1	1.1	1.0	**4.7**
[1-^{13}C]Glucose (10%)	Ubiquinone	**3.1**	1.0	1.1*	1.5	**3.4**
Euglenophyta:						
Eu. gracilis						
[1-^{13}C]Glucose (10%)	Phytol	1.3	**4.8**	1.2	**4.7**	**4.7**
	Ergosterol	1.7	**4.6**	1.3	**5.0**	**4.3**
Chrysophyta:						
O. danica						
[1-^{13}C]Glucose (10%) (experiment 1)	β-Carotene	**4.6**	2.4*	2.6	2.3	**4.4**
	Phytol	**3.9**	2.4	2.5	1.9	**4.0**
	Poriferasterol	1.4	**4.3**	1.3	**4.4**	**4.5**
	7-Dehydroporiferasterol	1.5	**4.7**	1.4	**4.7**	**5.1**
[1-^{13}C]Glucose (10%) (experiment 2)	β-Carotene	**3.9**	2.3*	2.1	2.0	**4.2**
	Phytol	**3.9**	2.3	2.4	1.9	**3.8**
	Poriferasterol	1.6	**5.1**	1.3	**5.2**	**5.5**
	7-Dehydroporiferasterol	1.1	**3.5**	1.1	**3.6**	**3.8**
Rhodophyta:						
Cy. caldarium						
[1-^{13}C]Glucose (10%)	Phytol	**3.6**	0.9	0.8	0.9	**3.5**
	Ergosterol	1.2	**3.6**	0.9	**3.6**	**3.2**

* Ubiquinone and carotenoid signals denoted by * were utilized as standards for the evaluation of isotopic abundances.

derived from two sources with the growth conditions we utilized. First, a pyruvate pool resulted from the glycolytic pathway; it yielded acetyl-CoA, which was utilized for both sterol biosynthesis in the cytoplasm and, to a lesser extent, fatty acid biosynthesis in the plastids. Pyruvate was also incorporated with GAP into the isoprenoids of the plastids via the GAP/pyruvate pathway. Secondly, another pyruvate pool arose from photosynthesis, and this was incorporated into the chloroplast isoprenoids and fatty acids, and did not significantly contribute to the formation of sterols.

Putative role of the MVA pathway as the sole pathway for isoprenoid biosynthesis in *Eu. gracilis*

According to the labelling patterns found in ergosterol synthesized in the cytoplasm or phytol formed in the chloroplasts, *Eu. gracilis* synthesized both isoprenoids via the MVA pathway. Isotopic abundances of enriched carbon atoms of around 5% were found in the isoprenoids (Table 1). These values corresponded, within the limit of experimental error, to glucose catabolism via glycolysis and acetate formation from pyruvate, without any significant contribution made by other catabolic pathways. Our result confirms directly previous data obtained by incorporation of ^{14}C-labelled acetate and MVA into the β-carotene of *Eu. gracilis*, with localization of the radioactivity by chemical degradation [44]. This Euglenophyte is therefore the only phototrophic eukaryote for which no evidence for the GAP/pyruvate pathway could be found, even for the formation of chloroplast isoprenoids. The presence of this biosynthetic route cannot, at present, be completely excluded. More detailed investigations are required, especially with respect to the characterization of the genes and enzymes involved in isoprenoid biosynthesis.

Table 2 Isotopic abundances of saturated fatty acid methyl esters from algae and from the cyanobacterium *Synechocystis* PCC 6714 after incorporation of ^{13}C-labelled glucose or pyruvate

In all algae but *O. danica*, where methyl myristate was the major compound (90%), methyl palmitate was the major derivative representing 90% of the saturated fatty acid fraction. Values in bold highlight the strong labelling.

Micro-organism and substrate used	Derivative	Isotopic abundances of carbon atoms													
		C-1	C-2	C-3	C-4	C-5	C-6	C-7 to C-10	C-11	C-12	C-13	C-14	C-15	C-16	OMe*
Synechocystis PCC 6714 [6-^{13}C]Glucose (10%)	Methyl palmitate	1.9	**4.9**	1.1	**4.7**	1.5	**4.9**	–	–	–	1.3	**5.0**	1.3	**5.3**	1.1
Synechocystis PCC 6714 [1-^{13}C]Glucose (10%)	Methyl palmitate	1.3	**2.0**	1.0	**1.8**	1.3	**2.1**	–	–	–	1.0	**2.0**	1.2	**2.1**	1.1
Synechocystis PCC 6714 [1-^{13}C]Glucose (15%)	Methyl palmitate	1.8	**2.2**	1.2	**2.1**	1.4	**2.2**	–	–	–	1.3	**2.3**	1.4	**2.3**	1.1
Chlam. reinhardtii [1-^{13}C]Glucose (10%)	Methyl palmitate	1.4	**3.1**	1.0	**3.0**	1.3	**3.1**	–	–	–	1.1	**3.2**	1.1	**3.3**	1.1
Chlor. fusca [1-^{13}C]Glucose (10%)	Methyl palmitate	1.4	**3.4**	0.9	**3.2**	1.2	**3.4**	–	–	–	1.1	**3.5**	1.1	**3.6**	1.1
S. obliquus [3-^{13}C]Pyruvate (99%) + Glucose	Methyl palmitate	–	**6.6**	1.8	**6.0**	–	**5.6**	–	–	–		**6.1**	1.8	**5.2**	1.1
Eu. gracilis [1-^{13}C]Glucose (10%)	Methyl palmitate	1.9	**4.7**	1.1	**4.4**	1.4	**4.6**	–	–	–	1.1	**4.8**	1.5	**5.5**	1.1
Cy. caldarium [1-^{13}C]Glucose (10%)	Methyl palmitate	1.0	**4.4**	0.8	**3.9**	1.1	**4.1**	–	–	–	1.0	**4.3**	1.0	**4.5**	1.1
O. danica [1-^{13}C]Glucose (10%)	Methyl myristate	2.9	**4.5**	2.9	**4.4**	2.9	**4.4**	–	2.8	**4.5**	2.8	**4.7**			1.1

* The signal of the ester methyl group (OMe; isotopic abundance 1.1%) was utilized as reference for the evaluation of isotopic abundances.

Table 3 Isotopic abundances of docosanyl acetate from *Chlam. reinhardtii* and *Chlor. fusca* after incorporation of [1-^{13}C]glucose

Docosanol was always the major compound of the aliphatic long-chain alcohol fractions in *Chlam. reinhardtii* [C_{20} (5%), C_{22} (76%), C_{24} (12%), C_{26} (< 1%), C_{28} (7%) and C_{30} (< 1%)] and in *Chlor. fusca* [C_{20} (13%), C_{22} (75%), C_{24} (7%), C_{26} (< 1%), C_{28} (5%) and C_{30} (< 1%)]. Values in bold highlight the strong labelling.

Carbon atoms	Docosanyl acetate	
	Chlam. reinhardtii [1-^{13}C]Glucose	*Chlor. fusca* [1-^{13}C]Glucose
C-1	1.2	1.2
C-2	**3.3**	**3.8**
C-3	1.0	1.1
C-4	**3.6**	**3.6**
C-5	1.5	1.4
C-6	**2.9**	**3.4**
C-7 to C-18	–	–
C-19	1.2	1.3
C-20	**3.4**	**4.2**
C-21	1.2	1.1
C-22	**3.5**	**4.0**
MeCO–*	1.1	1.1
Me**CO**–	1.1	1.0

* The signal of the carbonyl of the acetoxy group (MeCO–) was utilized as reference for the evaluation of isotopic abundances.

Occurrence of the GAP/pyruvate pathway in the cyanobacterium *Synechocystis*

In *Synechocystis* PCC 6714, only phytol and β-carotene biosynthesis were investigated. Cyanobacteria do not synthesize sterols [45,46], and the amount of cells obtained from the labelling experiments was insufficient for the analysis of triterpenes of the hopane series [26]. The biosynthesis of the two isoprenoids from the photosynthetic apparatus only occurred via the GAP/pyruvate pathway from labelled glucose (Table 1). Isotopic abundances resulting from incorporation of [1-^{13}C]glucose were much lower than those found for other phototrophic organisms. Significant glucose catabolism via the oxidative pathway was most probably the main reason for this rather low yield [47]. Confirmation of this hypothesis was obtained by the excellent incorporation of [6-^{13}C]glucose. Indeed, owing to the reversibility of the reactions involved in glycolysis, C-1 and C-6 of glucose are metabolically equivalent, but C-6 is not lost as CO_2 in the oxidative pentose phosphate pathway. Such a competition between these two glucose catabolic pathways was already observed in other prokaryotes (*E. coli* and *Alicyclobacillus acidoterrestris*) [6].

MVA versus GAP/pyruvate pathway

Incorporation of a ^{13}C-labelled precursor is, at present, the only reliable method for the identification of the route utilized for isoprenoid biosynthesis. The labelling pattern resulting from either the MVA or the GAP/pyruvate route can be clearly differentiated, whatever carbon source is utilized [4,6]. This labelling method is, however, time consuming and must be performed on a sufficiently large scale to obtain the amounts of isoprenoids required for ^{13}C-NMR spectroscopy. It is therefore not very sensitive, and does not usually allow the detection of a minor contribution (e.g. below approx. 10%, depending on the size of the analysed isoprenoid sample) of a second pathway occurring simultaneously. Incorporation of labelled MVA or D-

1-deoxyxylulose determined by analysis of the isoprenoids using MS is much simpler and well suited when positive results are obtained [15,17], but the method is apparently not suitable for general use, as shown by the failure to incorporate D-1-deoxyxylulose by organisms such as *Methylobacterium organophilum* and *Corynebacterium ammoniagenes* (T. Duvold, P. Cali and M. Rohmer, unpublished work) or *O. danica* (C. Müller and H. K. Lichtenthaler, unpublished work), which were shown in glucose-labelling experiments to synthesize their isoprenoids via the GAP/pyruvate pathway [4,48]. Furthermore, addition of a precursor to a culture medium may modify the regulation of metabolic pathways occurring in normal growth conditions, and lead to a possible overestimation of their contribution to the formation of a metabolite [43]. Negative results do not imply the absence of a metabolic pathway, but simply that it was not utilized in a significant manner in the growth conditions utilized for the labelling experiments. Furthermore, all data acquired recently on isoprenoid biosynthesis in phototrophic eukaryotes were obtained using feeding experiments with an exogenous carbon source. These growth conditions do not reflect the normal growth conditions for a phototrophic organism utilizing only CO_2 as carbon source. These features point out the difficulties that are encountered when the contribution of each pathway involved in isoprenoid biosynthesis is evaluated under normal physiological conditions. Exchanges of intermediates (IPP and FPP) between cytoplasm and chloroplasts were detected. Mixed labelling patterns were found in ginkgolides from *Ginkgo biloba* [13], in phytol from the liverwort *Heteroscyphus planus* [42] after feeding of ^{13}C-labelled MVA, in both ferrugineol and sitosterol after addition of ^{2}H-labelled D-1-deoxyxylulose to tissue cultures of *Salvia miltiorrhiza* [18], and in the sterols (as well as in phytol) of *Cy. caldarium* during phototrophic growth after feeding of either MVA or methyl D-1-deoxyxyluloside [17].

The GAP/pyruvate pathway was detected in three species of green algae for the formation of all isoprenoids investigated. Its significance in being the only biosynthetic pathway in green algae, and that of its subcellular localization, are the subject of further experimentation. This route is also involved in the formation of the chloroplast isoprenoids of the Rhodophyte *Cy. caldarium* and the Chrysophyte *O. danica*, whereas the sterols found in the cytoplasm of these organisms arose from the MVA pathway. The same feature was found in higher plants: plastidic isoprenoids (carotenoids, the phytyl chain of chlorophylls and the prenyl side-chain of plastoquinone) were derived from the GAP/pyruvate pathway, whereas the cytoplasmic sterols, as well as the prenyl chain of ubiquinone found in mitochondria, were synthesized via the acetate/MVA pathway [49]. The emission of isoprene [11] (which is directly linked to photosynthesis) and the biosynthesis of monoterpenes [12] and diterpenes [13–15] (which are believed to be synthesized in the plastids of higher plants) were also found to proceed according to the novel IPP pathway. Finally, the CLA1 gene product in *Arabidopsis thaliana* is a thiamin-binding protein catalysing chemical reactions similar to those catalysed by transketolases [50]. It is most probably identical with the deoxyxylulose 5-phosphate synthase. Analyses of the predicted amino acid sequence suggested that this protein may be transported to the chloroplasts. All these data strongly support the hypothesis that the GAP/pyruvate pathway is linked to the plastidic compartment, and is closely related to photosynthesis. They are also in accordance with the prokaryotic origin of the chloroplasts, which are presumed to be derived from a cyanobacteria-like endosymbiont, i.e. the bacterial GAP/pyruvate pathway for isoprenoid biosynthesis is conserved, which was shown here to be present in the cyanobacterium *Synechocystis* PCC 6714.

The diversity of the labelling patterns observed in the isoprenoids of selected species from different phyla reflects the polyphyletic origin of unicellular algae. As emphasized by the analysis of the composition of the pigments in the photosynthetic apparatus, the origin of photosynthesis is diverse [51]. The case of *Eu. gracilis* is particularly striking. It is the only phototrophic eukaryote in which the GAP/pyruvate pathway was not detected. Euglenophytes occupy a seemingly peculiar position among phototrophic eukaryotes. In contrast with the chloroplasts from plants, green algae and red algae, which possess two membranes, their chloroplasts are surrounded by three membranes and are therefore believed to represent remnants from a symbiotic alga [52,53]. Analysis of mitochondrial DNA sequences suggested that Euglenophytes are related to heterotrophic protists, rather than to green or yellow algae [54]. This does not allow a satisfactory explanation for the absence of the GAP/pyruvate route in Euglenophytes, because these micro-organisms, like some heterotrophic soil amoebae (*Acanthamoeba polyphaga* and *Naegleria gruberi*) [55,56], share an important common feature with all other phototrophic organisms: *Eu. gracilis* [57] and the heterotrophic apoplastidic Euglenophyte *Astasia longa* [58] synthesize their sterols via the cycloartenol route like all plants and algae.

Finally, labelling experiments performed with glucose would also provide an insight into the stereochemistry of the cyclization step in the biosynthesis of carotenoids that are formed via the GAP/pyruvate pathway, where the methyl groups on the cyclohexene ring can be differentiated by NMR. This was only elucidated for carotenoids derived from the MVA route [59,60]. Each methyl group of the *gem*-dimethyl groups on the two cyclohexene rings of lutein from *Chlam. reinhardtii* preserved its integrity. Only the pseudo-axial methyl group was labelled from [1-^{13}C]glucose. With additional data on the configuration of the polyene precursors (*E* or *Z*) and the selectivity of the protonation of the terminal double-bond, this would allow one to determine the stereochemistry of the cyclization [58].

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