Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway in the green alga *Scenedesmus obliquus**

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Isoprenoid biosynthesis was investigated in the green alga *Scenedesmus obliquus* grown heterotrophically on ^{13}C-labelled glucose and acetate. Several isoprenoid compounds were isolated and investigated by ^{13}C-NMR spectroscopy. According to the ^{13}C-labelling pattern indicated by the ^{13}C-NMR spectra, the biosynthesis of all plastidic isoprenoids investigated (prenyl side-chains of chlorophylls and plastoquinone-9, and the carotenoids β-carotene and lutein), as well as of the non-plastidic cytoplasmic sterols, does not proceed via the classical acetate/mevalonate pathway (which leads from acetyl-CoA via mevalonate to isopentenyl diphosphate), but via the novel glyceraldehyde 3-phosphate/pyruvate route recently detected in eubacteria. Formation of isopentenyl diphosphate involves the condensation of a C_2 unit derived from pyruvate decarboxylation with glyceraldehyde 3-phosphate and a transposition yielding the branched C_5 skeleton of isoprenic units.

INTRODUCTION

Isopentenyl diphosphate (IPP) is the common precursor for all isoprenoids, including plant composite lipids possessing prenyl side-chains (for reviews see [1–3]). The biosynthesis of IPP was first investigated in yeast and mammalian liver tissue [4–7], but the pathways have not been fully resolved in higher plants and green alga [8,9]. The so-called acetate/mevalonate pathway includes the condensation of three units of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), reduction to mevalonate, two successive phosphorylation steps at C-5 of mevalonate and a decarboxylation/elimination step leading to IPP [1,9]. Labelling experiments with [^{14}C]acetate and [^{14}C]mevalonate had shown that in several non-green tissues of higher plants [10–13] and in the alga *Euglena gracilis* [14] the carotenoids were labelled in a pattern that seemed similar to the general isoprenoid labelling pattern found, for example, in squalene derivatives in other organisms, and this was interpreted on the basis of the classical acetate/mevalonate pathway. Thus there was no reason to believe that the biosynthesis of isoprenoids in higher plants or algae occurs other than via the acetate/mevalonate pathway (for review see [1]). However, we found that the antibiotic mevinolin, a highly specific inhibitor of mevalonate and sterol biosynthesis, efficiently blocked sterol biosynthesis in higher plants but did not affect the formation of chlorophylls and carotenoids in plastids [15–17]. This suggested that mevinolin cannot penetrate the chloroplast or, more likely, that chloroplasts possess a separate and different biosynthetic pathway for IPP formation which cannot be blocked by mevinolin.

A novel pathway leading to IPP formation was detected in several eubacteria by Rohmer and co-workers [18–20]. Incorporation of ^{13}C-labelled precursors into triterpenoids of the hopane series of *Zymomonas mobilis*, *Methylobacterium fujisawaense*, *M. organophilum*, *Alicyclobacillus acidoterrestris*, *Rhodopseudomonas palustris* and *R. acidophila* or into ubiquinone-8 in *Escherichia coli* showed that these species do not use the acetate/mevalonate pathway for the formation of isoprenoids, but instead use precursors derived from triose phosphate metabolism. The C_5 skeleton of isoprenic units, represented for instance by IPP, results from a sequence of two main steps: (1) condensation of a C_2 unit derived from pyruvate decarboxylation on the carbonyl group of glyceraldehyde 3-phosphate (GAP), and (2) a transposition step yielding the final branched carbon skeleton of IPP [20,20a].

More recently we detected that growth, multiplication and pigment formation in the green alga *Scenedesmus* were not influenced at all by the antibiotic mevinolin; this should have blocked at least sterol biosynthesis, which is strongly inhibited by mevinolin in higher plants. This prompted us to investigate whether *Scenedesmus* cells possess a different biosynthetic route for IPP formation. Here we describe several ^{13}C-labelling experiments with the unicellular green alga *Scenedesmus obliquus*. They demonstrate that the isoprenoid precursor IPP is also synthesized via the same novel GAP/pyruvate non-mevalonate pathway.

EXPERIMENTAL

Culture of *Scenedesmus*

The eukaryotic green alga *Scenedesmus obliquus* (strain 276-3d; Algensammlung, Göttingen, Germany) was grown heterotrophically on a liquid medium modified from that described by Bishop and Wong [21] containing D-glucose ($1\ g\cdot l^{-1}$) as sole carbon source plus K_2HPO_4 (4 mM), NaH_2PO_4 (4 mM), NH_4Cl (8 mM), $MgSO_4$ (1 mM), $CaCl_2$ (0.1 mM), $FeSO_4$-EDTA

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

* This paper is dedicated to Professor Trevor W. Goodwin, Liverpool, on the occasion of his 80th birthday.

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(36 μM), $(NH_4)_6Mo_7O_{24}$ (20 nM), H_3BO_3 (8 μM), $MnCl_2$ (2.5 μM), $ZnSO_4$ (1 μM) and $CuSO_4$ (0.5 μM). For each labelling experiment, glucose contained 10 % (w/w) D-[1-^{13}C]glucose, D-[6-^{13}C]glucose, D-[U-$^{13}C_6$]glucose (Sigma-Aldrich Chemie, Deisenhofen, Germany) or D-[4,5-$^{13}C_2$]glucose (Omicron Biochemical Inc., South Bends, IN, U.S.A.). For labelling studies with acetate, cells were grown on a medium similar to the glucose medium but containing K_2HPO_4 and NaH_2PO_4 at 5.33 mM and 10.66 mM respectively, plus sodium [1-^{13}C]acetate (1 g·l^{-1}; 10 % isotopic abundance).

The cells were cultured for 120 h at 25 °C under stimulatory light of 10 μmol of photon·m^{-2}·s^{-1} (Osram L 40 W fluorescent lamp) in 5 litre flasks aerated with air (20 l·h^{-1}). The very low light intensity was not enough for significant autotrophic growth, but promoted heterotrophic growth on glucose or acetate. In full darkness, *Scenedesmus* could only be cultured with the addition of yeast extract, which had to be avoided in this investigation, since it represented a source of carbon and could interfere with the incorporation of the labelled substrate. At the end of the exponential growth, the yield of *Scenedesmus* cells reached 0.5–0.6 g·l^{-1} (dry weight).

Extraction and isolation of isoprenoids

After 5 days of exponential growth, *Scenedesmus* cells from a culture (2–10 litres) were harvested by centrifugation (4000 ***g***, 4 °C, 5 min). Cells were broken by shaking with glass beads (1 mm diameter) in cold methanol (0 °C; 5 ml of methanol/ml cell volume) in a Vibrogen Zellmühle (Edmund Bühler GmbH, Tübingen, Germany) for 7 min. The suspension was filtered with a sintered glass filter. After addition of water to the methanol extract (0.5 vol.), the lipids were transferred to hexane and the hexane extract was dried over Na_2SO_4. The extraction of the cells was repeated three times using chloroform/methanol (2:1, v/v; 15 ml/ml of former cell volume). The whole chloroform/methanol extract and the hexane extract were combined and evaporated to dryness.

Isolation of isoprenoids and methyl palmitate

From the largest 10 litre *Scenedesmus* culture, sufficient amounts of phytol (15 mg), sterols (14 mg), lutein (3 mg), β-carotene (1.5 mg) and plastoquinone-9 (1 mg) were obtained to measure a ^{13}C-NMR spectrum for each compound. All other experiments were performed at a smaller scale (2 litre cultures) that yielded only phytol and sterols in sufficient amounts.

For the large-scale culture (10 litres), TLC separation (heptane/diethyl ether, 5:1, v/v) of the extracted lipids on silica gel (Merck No 5721) [3] yielded β-carotene (R_F 0.65), plastoquinone-9 (R_F 0.47) and a band containing chlorophylls, xanthophylls and sterols ($0 < R_F < 0.2$). β-Carotene was further purified by recrystallization in ethanol and a second TLC step (cyclohexane/peroxide-free diethyl ether, 40:1, v/v; R_F 0.70). Plastoquinone-9 was also purified by another TLC step using dichloromethane/cyclohexane (1:1, v/v; R_F 0.20) as eluent. The third most polar fraction was saponified overnight at room temperature with a 6 % (w/v) solution of KOH in methanol. In the case of the small-scale cultures (2 litres), the extracted lipids were saponified directly [6 % (w/v) KOH in methanol] without prior isolation of plastoquinone-9 and β-carotene.

After saponification, 2 vol. of water was added and unsaponifiable lipids were transferred to hexane and evaporated to dryness. The aqueous phase was acidified with H_2SO_4 (pH 2) and extracted again with hexane, yielding a fatty acid-containing fraction which was evaporated to dryness.

The unsaponifiable lipids were separated by TLC (dichloromethane; two migrations) yielding phytol (R_F 0.27), sterols (R_F 0.12) and xanthophylls on the baseline. Phytol and sterols were acetylated overnight at room temperature (acetic acid anhydride/pyridine/toluene, 1:1:2, by vol.). Phytyl acetate was purified by TLC (dichloromethane/cyclohexane, 1:1, v/v; R_F 0.30) on silver nitrate-impregnated silica gel [22]. The steryl acetates were also separated using the same method with absolute chloroform obtained by filtration on active alumina (Merck 1076, grade I) into a mixture of the acetates of ergost-7-enol and 22,23-dihydrochondrillasterol (R_F 0.40) and pure acetate of chondrillasterol (R_F 0.30). The two former sterols could be separated one from another by reverse-phase HPLC using a C_{18} Zorbax ODS column (4.6 mm × 25 cm) and methanol as eluent (1 ml·min^{-1}). TLC separation of the xanthophyll fraction (peroxide-free diethyl ether) gave pure lutein (R_F 0.50).

The fatty acid-containing fraction was methylated with a diazomethane solution in diethyl ether. The excess reagent was removed under a nitrogen stream. After TLC separation on silica gel with absolute chloroform, methyl palmitate (R_F 0.8) was obtained.

Polyterpenoid identification and evaluation of isotopic abundances

Isoprenoids from *S. obliquus* were identified by spectroscopic methods (^{1}H- and ^{13}C-NMR spectroscopy, GLC/MS or direct inlet MS) and comparison of the data obtained with those in the literature and reference compounds. β-Carotene was a gift from Hoffmann-La Roche (Basle, Switzerland). Methyl palmitate was obtained by treatment of palmitic acid with diazomethane, and glycerol triacetate by acetylation of glycerol. Phytol was isolated from spinach (*Spinacia oleracea* L.), plastoquinone-9 from beech leaves (*Fagus sylvatica* L.), and lutein and the sterol mixture from *S. obliquus* [chondrillasterol, 22,23-dihydrochondrillasterol and ergost-7-enol in the proportions 6:3:1 (by wt.)] [23] from a bulk culture (10 litres) of the alga grown autotrophically [100 mol of photon·m^{-2}·s^{-1}; Osram L 40 W fluorescent lamp; aeration with CO_2-enriched (3 %, v/v) air at 20 l·h^{-1}].

Assignment of the ^{13}C-NMR spectra was by comparison with data in the literature for carotenoids and phytol [24] and sterols [25–28]. The ^{13}C-NMR spectrum of plastoquinone-9 was assigned by comparison with that of ubiquinone Q-8 [20] obtained by the distortionless enhancement by polarization transfer (DEPT) technique. The ^{13}C-NMR spectrum of methyl palmitate was assigned by comparison with that of *N*-palmitoyl-*S*-[(2*R*,*S*)-2,3-dipalmitoyloxypropyl]-(*R*)-cysteine methyl ester [29].

Isotopic abundances were determined by ^{13}C-NMR spectroscopy in [^{2}H]chloroform on a Bruker AC 250 spectrometer as reported previously [20]. The spectra of the labelled compounds and the corresponding unlabelled reference compounds were recorded under identical experimental conditions corresponding to identical nuclear Overhauser effects for both measurements and taking the relaxation times into account. For the sake of clarity they are not indicated for each carbon atom, but only given as mean values of the isotopic abundances of all equivalent carbon atoms of all isoprenic units (see Table 1). For the estimation of isotopic abundances, the subsequent signals were normally utilized as internal references: the acetate methyl group of phytyl acetate, a carbonyl group of steryl acetate, and the ester methyl group of methyl palmitate. Otherwise the signal of a non-labelled carbon atom was chosen: C-4 of phytyl acetate in [6-^{13}C]glucose incorporation, C-2 of β-carotene, C-4 or C-18 of chondrillasteryl acetate in either [1-^{13}C]acetate or [4,5-$^{13}C_2$]glucose incorporations, and C-4 of 22,23-dihydrochondrillasteryl acetate in [1-^{13}C]glucose incorporation.

RESULTS

Labelling of isoprenic units after incorporation of ^{13}C-labelled glucose and acetate

In all labelling experiments a definite labelling pattern was found for all isoprenoid compounds. From cultures with [1-^{13}C]glucose, the labelling pattern of isoprenic units in phytol, chondrillasterol, 22,23-dihydrochondrillasterol, ergost-7-enol, lutein, β-carotene and the prenyl side-chain of plastoquinone-9 (Figure 1) were identical, indicating a common pathway for the biosynthesis of all these major plant isoprenoids (Table 1; Figure 2b). In labelling experiments with [6-^{13}C]glucose, [4,5-$^{13}C_2$]glucose, [U-$^{13}C_6$]glucose and [1-^{13}C]acetate performed on a smaller scale, only phytol and chondrillasterol were investigated. In each of these experiments, the C_5 isoprenic skeleton was labelled in the same way in both isoprenoids (Table 1; Figure 2b).

Occurrence of a non-mevalonate pathway

The results of all labelling experiments are shown in Tables 1 and 2, and Figures 1, 2(b) and 2(c). In *Scenedesmus*, [1-^{13}C]acetate gave [1-^{13}C]acetyl-CoA. This was confirmed by the label of palmitic acid, in which only odd-numbered carbon atoms were labelled (results not shown). If the classical pathway had been used, [1-^{13}C]acetyl-CoA should have been incorporated via HMG-CoA and mevalonate into IPP and would have labelled C-1 and C-3 of IPP (Figure 2a). In contrast, the label of C-1 of exogenously applied [1-^{13}C]acetate was only found in C-4 of IPP (Figure 2b).

From [1-^{13}C]glucose, [2-^{13}C]acetyl-CoA is formed via glycolysis and decarboxylation of pyruvate by pyruvate dehydrogenase. In palmitic acid synthesized from acetyl-CoA only the even-numbered carbon atoms were labelled, confirming that [1-^{13}C]glucose is metabolized to [2-^{13}C]acetyl-CoA in *Scenedesmus* (results not shown). According to the same scheme of glucose breakdown, addition of [4,5-$^{13}C_2$]glucose should yield [1-^{13}C]acetyl-CoA. In the biosynthesis of IPP via the acetate/mevalonate pathway [1-^{13}C]glucose should label carbon atoms C-2, C-4 and C-5 of IPP via [2-^{13}C]acetyl-CoA, and [4,5-$^{13}C_2$]glucose should label C-1 and C-3 of IPP via [1-^{13}C]acetyl-CoA (Figure 2a). In contrast, the ^{13}C label of [1-^{13}C]glucose was detected at C-1 and C-5 of IPP, and that of [4,5-$^{13}C_2$]glucose at C-2, C-3 and C-4 of IPP (Figure 2b). In conclusion, the isoprenic units in *Scenedesmus* are not labelled from acetyl-CoA via the classical mevalonate pathway. The labelling patterns are quite similar to those observed for bacterial isoprenoids formed by the GAP/pyruvate pathway for isoprenoid biosynthesis [20].

Labelling of isoprenic units from [4,5-$^{13}C_2$]glucose and [U-$^{13}C_6$]glucose

In incorporation experiments using [4,5-$^{13}C_2$]glucose and [U-$^{13}C_6$]glucose, splitting of ^{13}C resonance signals, as revealed by ^1J, ^2J and ^3J coupling constants, clearly showed that two intact units

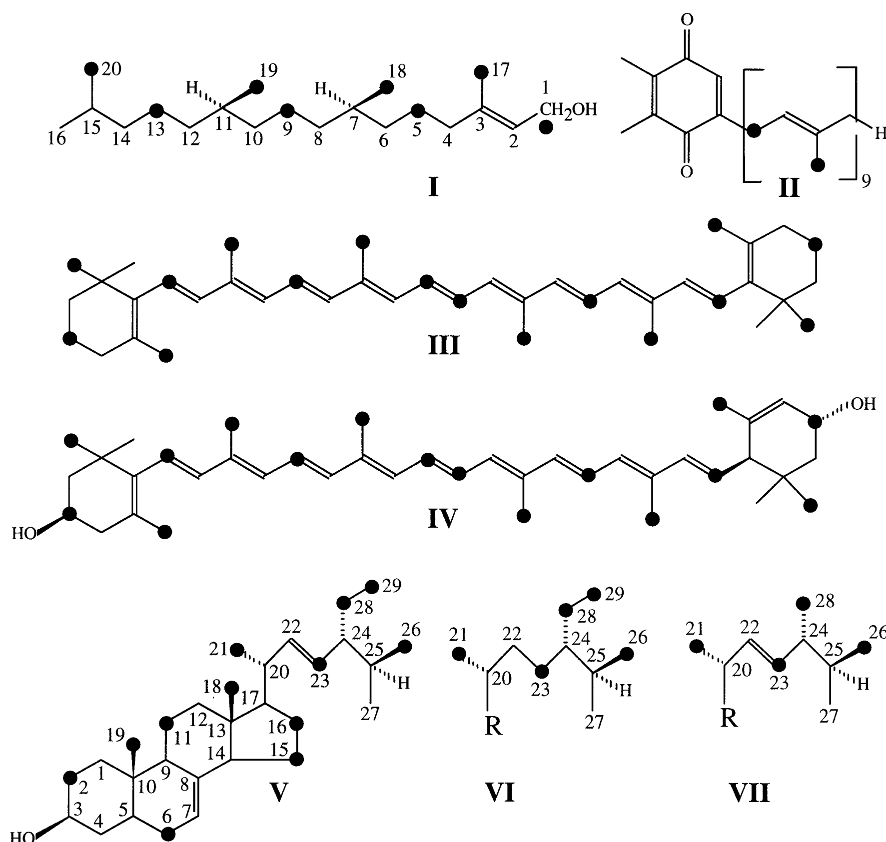

Figure 1 ^{13}C-labelling pattern in isoprenoids of *Scenedesmus obliquus* after growth on [1-^{13}C]glucose

^{13}C label is indicated by black circles. Compounds shown: phytol (**I**), plastoquinone-9 (**II**), β-carotene (**III**), lutein (**IV**), chondrillasterol (**V**), 22,23-dihydrochondrillasterol (**VI**) and ergost-7-enol (**VII**).

Table 1 Incorporation of ^{13}C-labelled glucose and acetate into the isoprenoids of *Scenedesmus obliquus*

In all experiments the isotopic abundance of [^{13}C]glucose or [^{13}C]acetate was 10%. All isoprenic units were identically labelled in each compound in all labelling experiments. For the sake of clarity, the values given represent the average percentage ^{13}C enrichment and are only indicated for the carbon atoms of IPP whose numbering is given in Figure 2(a). + indicates significant enrichment, which was not measured as absolute isotopic enrichment.

	^{13}C enrichment (%)					
Substrate	C-1	C-2	C-3	C-4	C-5	Calculated from:
[1-^{13}C]Glucose	+	–	–	–	+	Lutein, plastoquinone-9
	3.1	1.0	1.0	1.4	3.3	Phytol
	3.2	1.1	1.1	1.3	3.3	β-Carotene
	3.2	1.0	1.0	1.3	3.1	Chondrillasterol
	3.4	1.0	1.1	1.3	3.1	22,23-Dihydrochondrillasterol
	2.9	0.8	1.0	1.5	3.0	Ergost-7-enol
[6-^{13}C]Glucose	7.4	1.4	1.8	1.8	7.3	Phytol
	+	–	–	–	+	Chondrillasterol
[4,5-$^{13}C_2$]Glucose*	1.6	6.5	6.0	6.3	1.6	Phytol
	–	+	+	+	–	Chondrillasterol
[1-^{13}C]Acetate	1.4	1.5	1.1	7.1	1.4	Phytol
	1.2	1.3	1.0	5.9	1.1	Chondrillasterol

* ^{2}J ^{13}C/^{13}C coupling (2.7 Hz) between C-2 and C-4 of phytol, corresponding to C-2 and C-4 of IPP.

Table 2 ^{13}C/^{13}C coupling constants in the acetate groups of phytol and chondrillasterol of *Scenedesmus obliquus* after incubation with [U-$^{13}C_6$]glucose

For the numbering of the carbon atoms, see Figures 1 and 2(a). Not all possible coupling constants could be measured. Doublets of doublets appeared for the following carbon atoms of IPP: C-1 (^{1}J ^{13}C/^{13}C with ^{3}J ^{13}C/^{13}C), C-2 (^{1}J ^{13}C/^{13}C with ^{2}J ^{13}C/^{13}C) and C-4 (^{2}J ^{13}C/^{13}C with ^{3}J ^{13}C/^{13}C).

Isoprenic unit	Atoms of phytol (of IPP unit)	^{1}J (Hz)	^{2}J (Hz)	^{3}J (Hz)
1	C-1/C-2 (C-1/C-2)	50	–	–
	C-2/C-4 (C-2/C-4)	–	2.9	–
	C-1/C-4 (C-1/C-4)	–	–	5
	C-3/C-17 (C-3/C-5)	41.6	–	–
2	C-5/C-6 (C-1/C-2)	35	–	–
	C-5/C-8 (C-1/C-4)	–	–	2.8
	C-7/C-18 (C-3/C-5)	35.2	–	–
3	C-9/C-10 (C-1/C-2)	34.8	–	–
	C-9/C-12 (C-1/C-4)	–	–	2.8
	C-11/C-19 (C-3/C-5)	35.2	–	–
4	C-13/C-14 (C-1/C-2)	34.8	–	–
	C-13/C-16 (C-1/C-4)	–	–	2.7
	C-15/C-20 (C-3/C-5)	35.1	–	–
IPP unit	**Atoms of chondrillasterol (of IPP unit)**	**^{1}J (Hz)**	**^{2}J (Hz)**	**^{3}J (Hz)**
1	C-2/C-3 (C-1/C-2)	37.2	–	–
2	C-5/C-6 (C-1/C-2)	34.5	–	–
	C-1/C-6 (C-1/C-4)	–	–	2.5
	C-10/C-19 (C-3/C-5)	36.3	–	–
3	C-9/C-11 (C-1/C-2)	34.4	–	–
4	C-12/C-13 (C-1/C-2)	35.6	–	–
	C-12/C-15 (C-1/C-4)	–	–	3
	C-13/C-15 (C-2/C-4)	–	1.5	–
5	C-16/C-17 (C-1/C-2)	33.7	–	–
	C-16/C-22 (C-1/C-4)	–	–	2.5
	C-17/C-22 (C-2/C-4)	–	1.0	–
	C-20/C-21 (C-3/C-5)	34.3	–	–
6	C-23/C-24 (C-1/C-2)	43.6	–	–
	C-23/C-27 (C-1/C-4)	–	–	2.5
	C-25/C-26 (C-3/C-5)	35.7	–	–

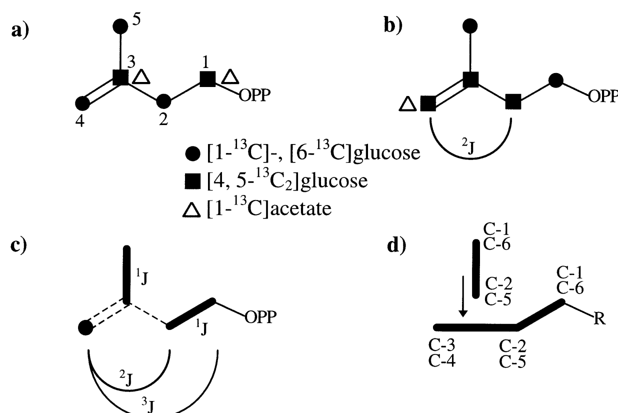

Figure 2 Labelling pattern of isoprenic units and ^{13}C/^{13}C couplings in *Scenedesmus* isoprenoids after incorporation of ^{13}C-labelled glucose and acetate

(**a**) Expected labelling pattern in IPP units from the classical acetate/mevalonate pathway. (**b**) Observed labelling pattern in IPP. ^{2}J ^{13}C/^{13}C coupling (2.7 Hz) is observed between C-2 and C-4 of IPP (C-2/C-4 of phytol). (**c**) Observed labelling pattern and ^{13}C/^{13}C couplings in IPP after incorporation of [U-$^{13}C_6$]glucose. Heavy lines indicate intact ^{13}C/^{13}C units from glucose molecules. (**d**) Origin of the carbon atoms of IPP from glucose, as derived from the data in (**b**) and (**c**). In agreement with (**c**), the insertion of a C_2 unit between two carbon atoms of a C_3 unit is illustrated.

were formed from glucose and incorporated into isoprenic units (Figure 2c). After addition of [4,5-$^{13}C_2$]glucose, the signals of carbon atoms C-2 and C-4 from phytol appeared as doublets with a ^{2}J ^{13}C/^{13}C coupling constant of 2.7 Hz (Figure 2b). No other coupling was detected in the ^{13}C-NMR spectra of the acetates of phytol and chondrillasterol, although all carbon atoms derived from C-2 and C-4 of IPP in all isoprenic units of both compounds were labelled. This probably means that all other ^{2}J ^{13}C/^{13}C coupling constants were too low to be measurable. However, for at least one isoprenic unit, C-2 and C-4 of IPP were shown to be introduced together from a precursor issued from a single glucose molecule. In the [U-$^{13}C_6$]glucose experiment, two other ^{2}J ^{13}C/^{13}C coupling constants could be observed in the spectrum of the acetate of chondrillasterol, indicating again that C-2 and C-4 of isoprene units arise from a single glucose molecule (Table 2). In addition, many ^{1}J and several ^{3}J ^{13}C/^{13}C coupling constants could be determined from the ^{13}C-NMR spectra of the acetates of phytol and chondrillasterol (Table 2). According to these data, a C_2 unit derived from glucose corresponds to C-3 and C-5 of IPP, and a C_3 unit to C-1, C-2 and C-4 (Figure 2c). In the biosynthesis of IPP, the C_2 precursor must be inserted between two carbon atoms of the C_3 unit corresponding to C-2 and C-4 of IPP. This is only possible if a transposition step yields the branched C_5 carbon skeleton of IPP after the addition of the C_2 unit to the C_3 unit and formation of a carbon bond. These labelling patterns are fully consistent with those observed after incorporation of [4,5-$^{13}C_2$]glucose into the hopanoids and ubiquinone of *Methylobacterium fujisawaense* and of [U-$^{13}C_6$]glucose into the hopanoids of *Zymomonas mobilis* [20a]. Again they indicate the existence of a non-mevalonate pathway in *Scenedesmus*.

In the experiment performed with [U-$^{13}C_6$]glucose [diluted 1:10 (w/w) with D-glucose], singlets were observed in addition to the doublets. The singlets of the carbon atoms derived from C-

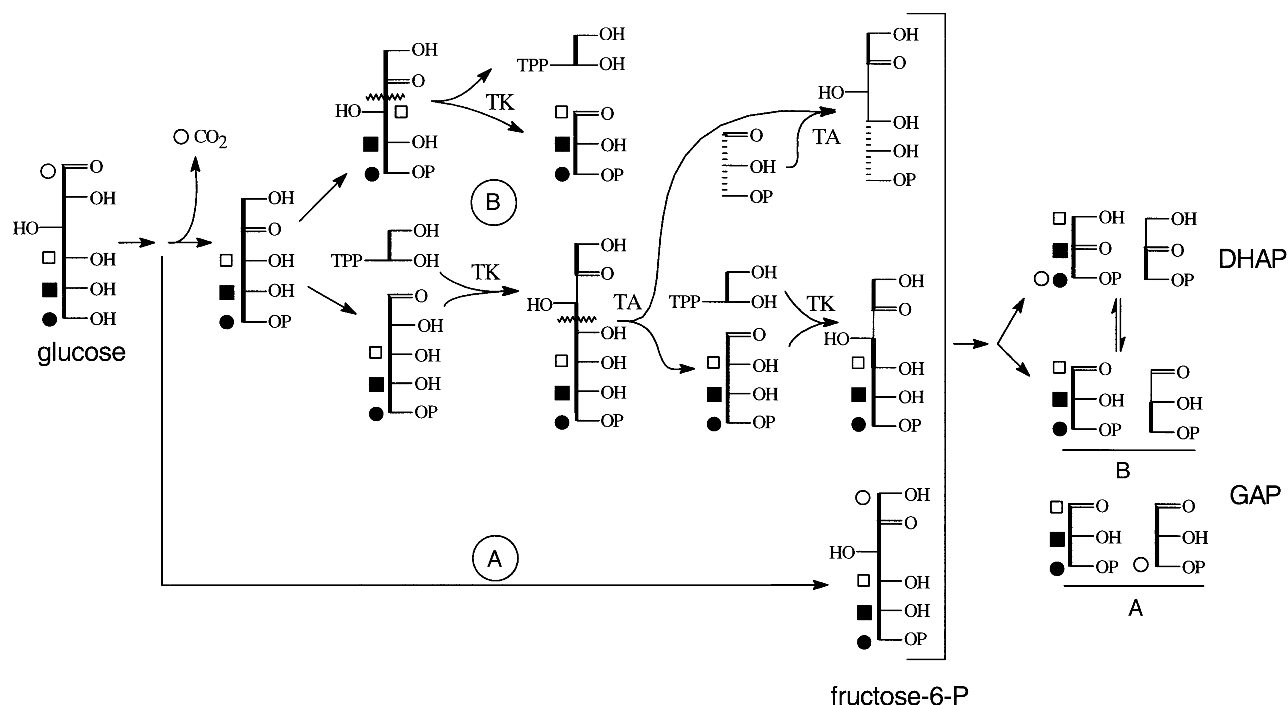

Figure 3 Involvement of the oxidative pentose phosphate cycle in glucose breakdown

The different carbon atoms of glucose appear in GAP as indicated by the symbols. GAP is produced directly via glycolysis (**A**) or via the oxidative pentose phosphate pathway (**B**). Heavy lines indicate intact C-C units from glucose molecules. DHAP, dihydroxyacetone phosphate; TA, transaldolase; TK, transketolase; TPP, thiamin diphosphate.

1, C-2, C-3 and C-5 of IPP showed an intensity corresponding to the natural abundance (results not shown). This means that nearly all of the carbon atoms that came from [U-$^{13}C_6$]glucose were introduced as a C_2 or C_3 unit. Only the singlets corresponding to carbon atoms derived from C-4 of IPP appeared with a much higher intensity. This feature will be discussed below in connection with the oxidative pentose phosphate cycle in glucose breakdown.

Pyruvate and triose phosphate as precursors of isoprenic units

The labelling patterns of isoprenic units derived from [6-^{13}C]glucose and [4,5-$^{13}C_2$]glucose (Figure 2b) make it plausible that the following units are incorporated together from glucose into IPP: C-6+C-5 from glucose is incorporated as the C_2 unit, and C-6+C-5+C-4 as the C_3 unit previously described. The C_3 unit, which is incorporated into IPP (Figure 2c), is labelled from C-1 and C-6 of glucose at the same terminal position (Figure 2b). A triose phosphate, derived via glycolysis from ^{13}C-labelled glucose, therefore appears to be the C_3 unit. Transformation of glucose into fructose bisphosphate via glucose 6-phosphate and fructose 6-phosphate and glycolytic cleavage of fructose bisphosphate by aldolase yields the two pools of dihydroxyacetone phosphate and GAP which are interchangeable by triose phosphate isomerase. GAP, and accordingly pyruvate, are labelled in the following manner: C-1 comes from C-3 and C-4 of glucose; C-2 from C-2 and C-5 of glucose; and C-3 from C-1 and C-6 of glucose. The results obtained from the glucose labelling experiments are in accordance with this labelling of triose phosphate (compare Figures 2b and 2d). The C_2 unit incorporated from glucose into C-5 and C-3 of IPP can be derived from a triose phosphate derivative, e.g. pyruvate, by loss of its C-1.

Possible implication of the glyoxylate shunt in labelling experiments with acetate

Assuming that the glyoxylate shunt operates when *Scenedesmus* grows on acetate, [1-^{13}C]acetate will be transformed via oxaloacetate, into [1-^{13}C]pyruvate and [1-^{13}C]GAP. If such triose phosphate derivatives are incorporated into IPP, as concluded from the glucose labelling experiments, the labelled C-1 of the triose phosphate will be incorporated into C-4 of IPP, and no labelling will be found in the carbon atoms of the pyruvate-derived C_2 subunit. Exactly this feature has been observed after incorporation of [1-^{13}C]acetate (Table 1; Figure 2b). Thus the labelling pattern of isoprenoids from [1-^{13}C]acetate supports the role of triose phosphate as direct precursor of IPP.

Role of the oxidative pentose phosphate pathway in labelling experiments with glucose

The cleavage of glucose via glycolysis into two interconvertible triose phosphates decreases the 10% ^{13}C abundance of the labelled precursor to 5% in the triose phosphates, and accordingly in the isoprenic units at the labelled positions. However, the ^{13}C abundance of phytol and chondrillasterol at labelled positions was about 3% in the case of labelling from [1-^{13}C]glucose and about 7% in the case of labelling from [6-^{13}C]glucose and [4,5-$^{13}C_2$]glucose (Table 1). This ratio of ^{13}C enrichments points to partial glucose metabolism through the oxidative pentose phosphate pathway, in addition to glycolysis, in heterotrophically grown *Scenedesmus* cultures: via phosphogluconate dehydrogenase, C-1 of phosphogluconate (i.e. of glucose) is lost as CO_2 (Figure 3). The oxidative pentose

phosphate cycle gives triose phosphate labelled from [6-^{13}C]glucose. In connection with glycolysis, the triose phosphate pool contains less label from [1-^{13}C]glucose and more label from [6-^{13}C]glucose, as expected from sole glycolytic cleavage. IPP was actually labelled from [1-^{13}C]glucose and [6-^{13}C]glucose with ^{13}C abundances of 3% and 7% respectively.

On addition of [U-$^{13}C_6$]glucose [diluted 1:10 (w/w) with non-labelled glucose], the ^{13}C-NMR spectra of the acetates of phytol and chondrillasterol showed, next to the doublet with a clear ^{2}J $^{13}C/^{13}C$ coupling, a singlet of rather high intensity for all carbon atoms that were derived from C-4 of IPP. Only these carbon atoms showed singlets that were definitely of greater intensity than the singlets expected from the natural ^{13}C abundance. Moreover, the carbon atoms that were derived from C-1 and C-2 of IPP showed ^{1}J $^{13}C/^{13}C$ coupling. The high singlet intensity and the ^{1}J $^{13}C/^{13}C$ coupling were observed in addition to the doublets of doublets (^{1}J $^{13}C/^{13}C$, ^{2}J $^{13}C/^{13}C$ and ^{3}J $^{13}C/^{13}C$ coupling; see Table 2) that indicated the incorporation of C_3 units into C-1, C-2 and C-4 of IPP. This feature is most probably related to the oxidative pentose phosphate pathway. By a transketolase reaction, an intact C_2 unit derived from C-2 and C-3 of glucose is added to erythrose 4-phosphate and ribose 5-phosphate in order to form fructose 6-phosphate and sedoheptulose 7-phosphate, yielding fructose bisphosphate through the catalysis of transaldolase and phosphofructokinase (Figure 3). After cleavage of the hexose bisphosphate by aldolase, GAP contains carbon atoms originating from two different glucose molecules: on the one hand C-2 and C-3 of GAP are provided by a first molecule, and on the other hand C-1 is from a second molecule (Figure 3). Incorporation into isoprenoids of such GAP molecules formed via this pathway from [U-$^{13}C_6$]glucose results in singlet signals for all carbon atoms derived from C-4 of IPP and doublets for those derived from C-1 and C-2. This was observed exactly in the ^{13}C-NMR spectra of phytol acetate and chondrillasteryl acetate after addition of [U-$^{13}C_6$]glucose.

DISCUSSION

Scenedesmus obliquus belongs to the eukaryotic green algae, which are similar to higher plants with respect to many physiological and biochemical aspects of photosynthesis. In their plastidic thylakoids they exhibit the same composition of pigments (chlorophylls *a* and *b*, carotenoids) and prenylquinones (plastoquinone, α-tocoquinone, phylloquinone) as higher plants [30–32]. For this reason, green alga species have been used intensively as model organisms for the investigation of photosynthesis in higher plants [33]. Their chloroplasts can be regarded as models for chloroplasts of higher plants. *Scenedesmus* is also able to grow heterotrophically on glucose, and its chloroplasts develop as green in darkness as well as in light. This species was therefore chosen to investigate the incorporation of ^{13}C-labelled substrates into the main chloroplast isoprenoids in order to verify the biosynthetic pathways involved in their formation and to compare these pathways with those utilized for sterol biosynthesis in the cytoplasm.

In a labelling experiment using [1-^{13}C]glucose, phytol from the chlorophyll side-chain, the polyprenyl side-chain of plastoquinone-9, the carotenoids β-carotene and lutein as well as the three main sterols (chondrillasterol, 22,23-dihydrochondrillasterol and ergost-7-enol) were not labelled in accordance with the classical acetate/mevalonate route, but in a pattern that pointed towards the existence of another pathway. Additional labelling experiments using other ^{13}C-labelled glucose samples and [1-^{13}C]acetate and determination of the labelling patterns in phytol and chondrillasterol were also in contradiction to the classical acetate/mevalonate pathway. In order to check whether acetyl-CoA was labelled as expected from the applied ^{13}C precursors, the labelling pattern of palmitic acid was determined in labelling experiments performed with [1-^{13}C]glucose and [1-^{13}C]acetate, and was in full agreement with acetyl-CoA formation via glycolysis from [1-^{13}C]glucose and with a direct transformation of the applied [1-^{13}C]acetate into acetyl-CoA. This confirmed that the unexpected ^{13}C-labelling pattern of the cytoplasmic and plastidic isoprenoid lipids observed did not arise from an unknown and unusual means of acetyl-CoA formation, but was due to a different pathway for IPP biosynthesis in *Scenedesmus*.

The $^{13}C/^{13}C$ couplings observed in the NMR spectra of the acetates of phytol and chondrillasterol after [U-$^{13}C_6$]glucose labelling showed clearly that one C_2 unit and one C_3 unit were incorporated intact from glucose into an isoprenic unit. The complex splitting of the ^{13}C-resonance signals of carbon atoms derived from C-1, C-2 and C-4 of IPP is due to the involvement of the oxidative pentose phosphate cycle in the formation of triose phosphate. The labelling pattern from glucose incorporation together with the $^{13}C/^{13}C$ coupling data indicate that triose phosphate is the most probable direct precursor of IPP in *Scenedesmus*.

The incorporation of [1-^{13}C]acetate via the glyoxylate shunt into IPP is also in accordance with triose phosphate as a direct precursor of IPP. The key enzymes of the glyoxylate shunt, i.e. isocitrate lyase and malate synthase, have been shown to be present in *Scenedesmus* when grown on acetate [34,35]. The transformation of ^{13}C-labelled succinate, produced by the glyoxylate shunt, via oxaloacetate and pyruvate into triose phosphate thus appears to occur in *Scenedesmus*. The label of the applied [1-^{13}C]acetate should show up at the C-1 position in the GAP produced. In fact, the labelling pattern found in isoprenic units was in agreement with such formation of [1-^{13}C]GAP from [1-^{13}C]acetate. This finding is further evidence for the proposal that triose phosphate is the direct precursor of the IPP formation in *Scenedesmus*.

The differential ^{13}C enrichment in the labelled positions of phytol and chondrillasterol using [1-^{13}C]glucose and [6-^{13}C]glucose, i.e. 3% and 7% respectively, can only be explained by the functioning of the oxidative pentose phosphate cycle in parallel with the glycolytic pathway. When glucose is metabolized via the oxidative pentose phosphate cycle, C-1 of [1-^{13}C]glucose is lost as $^{13}CO_2$. The triose phosphates formed are therefore not labelled. On using [6-^{13}C]glucose or [4,5-$^{13}C_2$]glucose, however, the resulting triose phosphates will be labelled with the same pattern as from glycolysis. Concerning the implication of the oxidative pentose phosphate pathway in the metabolic breakdown of glucose to triose phosphate, similar observations have been made in eubacteria. On applying [1-^{13}C]glucose or [6-^{13}C]glucose, different ^{13}C abundances in the same labelled carbon positions of the isoprenoids of *Alicyclobacillus acidoterrestris* and *E. coli* (3–3.5% and 6.5–7.0% respectively) were observed [20].

From the results described here a biosynthetic scheme for IPP biosynthesis in *Scenedesmus* can be proposed, which is identical to the scheme proposed by Rohmer et al. for the formation of hopanoids and ubiquinone from pyruvate and a triose phosphate derivative in several eubacteria [20,20a]. The triose phosphate derivative has been identified as GAP (or possibly, but less likely, free glyceraldehyde) by labelling experiments with *E. coli* mutants lacking enzymes involved in triose phosphate metabolism [20a]. The novel biosynthetic pathway of IPP formation occurring in *Scenedesmus* and also in some eubacteria involves the addition of a pyruvate-derived C_2 unit to GAP, most probably forming 1-deoxyxylulose 5-phosphate. A transposition yields the final

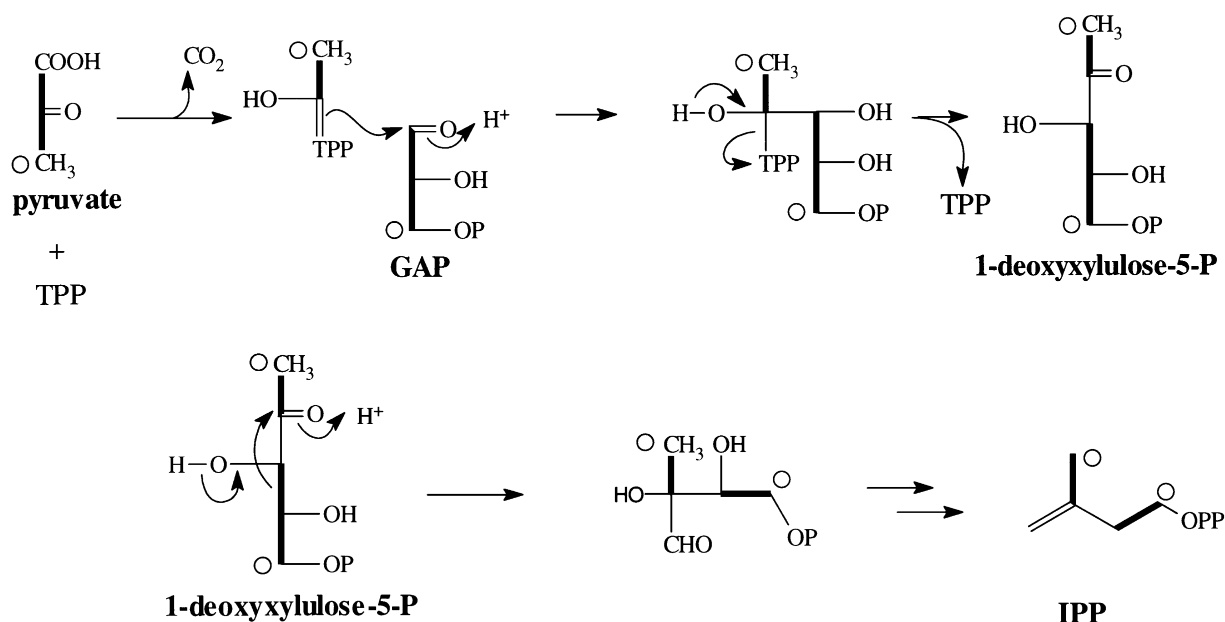

Figure 4 Hypothetical scheme for the biosynthesis of IPP from pyruvate and GAP in *Scenedesmus*

○, label from [1-^{13}C]glucose. TPP, thiamin diphosphate.

carbon skeleton of IPP (Figure 4). The successful incorporation of deuterium-labelled 1-deoxyxylulose into ubiquinone of *E. coli* is in accordance with the precursor role of such a carbohydrate in this non-mevalonate pathway [36].

In summary, the results of the present investigation show that a novel pathway for IPP biosynthesis exists not only in eubacteria for hopanoid and ubiquinone formation, but also in the eukaryotic alga *Scenedesmus*, in which the biosynthesis of all isoprenoid compounds so far investigated proceeds via this pathway. One indication of the occurrence of an IPP pathway in *Scenedesmus* differing from the classical acetate/mevalonate pathway was the inability of mevinolin, a specific inhibitor of HMG-CoA reductase in plants, animals and fungi [15,16,37], to inhibit the growth and multiplication of *Scenedesmus* even at concentrations of up to 1 mM [38]. Furthermore, the accumulation of photosynthetic pigments (chlorophylls and carotenoids) in growing *Scenedesmus* cultures was not influenced by mevinolin [38]. In addition, some authors had reported that, in green algae such as *Scenedesmus* [39] and *Chlorella* [40], ^{13}C- or ^{14}C-labelled mevalonate was not incorporated into carotenoids. These observations are now explained by the existence in *Scenedesmus* of a novel GAP/pyruvate pathway for IPP biosynthesis which cannot be blocked by mevinolin.

In higher plants, sterols are bound to cytoplasmic biomembranes [41,42] and their biosynthesis takes place in the cytoplasm/endoplasmic reticulum via the acetate/mevalonate pathway, which is blocked by mevinolin [8,9,15,16]. Plastidic isoprenoids such as carotenoids are synthesized via a plastidic IPP-biosynthetic pathway which is not influenced by mevinolin [15,16]. We expected that in the green alga *Scenedesmus*, in analogy to higher plants, the sterols would originate from the acetate/mevalonate pathway, which would be located in the cytoplasm, while the novel non-mevalonate IPP pathway would be bound to the plastids. This is, however, not the case. In contrast, in *Scenedesmus* the biosynthesis of the plastidic isoprenoids as well as that of the sterols proceeds via the novel non-mevalonate route. The reason for this is not clear and requires further investigation. That *Scenedesmus* chloroplasts would synthesize and contain sterols is not very likely, since chloroplasts of higher plants are free of sterols [41,42]. One might, however, speculate that in *Scenedesmus* the cytoplasmic acetate/mevalonate pathway was lost during the evolution of this green alga and is complemented by the presumed plastidic GAP/pyruvate IPP biosynthetic pathway, which would supply the IPP precursors for plastidic as well as cytoplasmic isoprenoid biosynthesis.

Since in higher plants the accumulation of chlorophylls containing isoprenic phytyl chains and carotenoids in plastids is not affected by mevinolin [16,17], one might assume that chloroplasts of higher plants also exhibit the novel triose phosphate biosynthetic pathway. Goodwin and co-workers found some indications that a separate plastidic site of IPP biosynthesis exists. In investigations with seedlings of maize [12,43–45], oat [45], barley [45], pea [45], lettuce [45], tobacco [46] and pine [47], plastidic isoprenoid compounds, such as the phytol side-chain of chlorophylls, β-carotene and plastoquinone-9, were preferentially labelled from $^{14}CO_2$. [^{14}C]Mevalonate, in turn, labelled only the cytosolic sterols at good rates. This was attributed to the impermeabilty of the chloroplast membrane to mevalonate [12,43–47]. In maize [48] and in *Euglena* [49] it had been shown in early investigations that ^{14}C-labelled pyruvate was efficiently incorporated into β-carotene, whereas acetate was not. The good incorporation of pyruvate [48,49] in comparison with that of acetate into plastidic isoprenoids, as well as the weak incorporation of mevalonate [43–47], can now be explained by the assumption of a plastidic GAP/pyruvate pathway for IPP biosynthesis in higher plants. A further indication of the existence of this alternative pathway in higher plants was recently given by Schwarz [50]. Incubation of *Ginkgo biloba* embryos in the presence of ^{13}C-labelled glucose or mevalonate revealed two distinct pathways of IPP formation in this plant. Sitosterol was labelled in agreement with the acetate/mevalonate pathway, but

ginkgolide A, a diterpene, was labelled from glucose in accordance with the GAP/pyruvate pathway demonstrated here for *Scenedesmus*. The present investigations, and many ambiguous or not readily interpretable results concerning isoprenoid biosynthesis in higher plants [8,51], could easily be rationalized by the occurrence of two different pathways for the formation of IPP in higher plants: (1) a cytoplasmic pathway which is involved in the biosynthesis of sterols and sesquiterpenes, and which can be inhibited by mevinolin; and (2) a mevalonate-independent plastidic IPP pathway responsible for carotenoid, phytol (chlorophyll) and plastoquinone-9 formation. The latter may be the novel IPP pathway established here for the green alga *Scenedesmus*, and this hypothesis is the subject of ongoing research.

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