Early steps of isoprenoid biosynthesis in *Escherichia coli*

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The incorporation of ^2H - and ^{13}C -labelled precursors into ubiquinone-8 (Uq-8) by strains of *Escherichia coli* was measured in order to define the pathway for the early steps in the biosynthesis of isoprenoids in these eubacteria. Cells grown with DL-[methyl- $^2\text{H}_6$]valine were found to label both the α -oxoisovaleric (' α -ketoisovaleric') acid α -oxoisohexanoic (' α -ketoisocaproic') acid, but not the Uq-8. Since these acids are required for the biosynthesis of isoprenoids by the acetolactate pathway, the operation of this pathway in the biosynthesis of Uq-8 is excluded. Cells grown with [1,2- $^{13}\text{C}_2$]acetate and non-labelled glucose readily incorporated $^{13}\text{C}_2$ units into fatty acids, but failed to incorporate any label into the Uq-8. Cells grown with [U- $^{13}\text{C}_6$]glucose and non-labelled acetate, however, were found to label both the fatty acids and the Uq-8. Oxidative cleavage with periodate/permanganate of the Uq-8 isolated from cells grown with U- $^{13}\text{C}_6$ -labelled glucose produced laevulinic acid, which was shown to be derived from two C_2 units and one C_1 unit of the labelled glucose by mass-spectral analysis of the 4,5-dihydro-6-methyl-2-phenylpyridazin-3(2*H*)-one derivative. The results of this work indicate that the C-2 and C-3 carbon unit of pyruvate, not acetyl-CoA, is the precursor to isopentenyl pyrophosphate (IPP) in these cells; however, the labelling pattern observed is consistent with the established acetoacetate pathway of isoprenoid biosynthesis. These data, coupled with the observed lack of inhibition of the growth of *E. coli* by mevinolin, a specific inhibitor of 3-hydroxy-3-methylglutaryl-CoA, can be best rationalized by the biosynthesis of IPP occurring in *E. coli* through a series of bound intermediates.

INTRODUCTION

The establishment of the pattern of incorporation of acetate via mevalonic acid and isopentenyl pyrophosphate (IPP) into isoprenoid compounds represents one of the cornerstones of metabolic biochemistry. The sequence of reactions involved in this important pathway were derived from work with animals and yeasts (Beytia & Porter, 1976) and, more recently, in archaeobacteria (Langworthy *et al.*, 1982; De Rosa *et al.*, 1986; Moldoveanu & Kates, 1988). The biosynthesis of isoprenoids has been less extensively studied in the eubacteria; however, current biosynthetic evidence indicates that the steps from IPP to isoprenoids in eubacteria are the same as those in eukaryotes (Fujisaki *et al.*, 1986*a,b*). In support of this idea, several mutants for many of the late steps in isoprenoid biosynthesis have recently been isolated and characterized in *Escherichia coli* by Sherman *et al.* (1989). In 1981, Pandian *et al.* proposed an alternative pathway for the biosynthesis of IPP which he termed the 'acetolactate pathway'. The early steps in this pathway lead to the production of α -oxoisohexanoic (' α -ketoisocaproic') acid by the same series of reactions as involved in the biosynthesis of leucine. The α -oxoisohexanoic acid, after oxidative decarboxylation to isovaleryl-CoA, is then dehydrogenated, hydrated and recarboxylated to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The HMG-CoA is then converted into IPP via mevalonate. Several observations in the literature, however, indicate that mevalonate may not be an intermediate in the biosynthesis of isoprenoids in some bacteria. These include the lack of incorporation of mevalonate into ubiquinone-10 (Uq-10) by growing cells of *Agrobacterium tumefaciens*, *Azotobacterium vinelandii*, *E. coli* and a *Pseudomonas* sp. (Raman *et al.*, 1965) and by permeabilized *E. coli* (Fujisaki *et al.*, 1986*a*). Mevalonate also failed to be incorporated into isoprenoid compounds by a series of bacteria isolated from soil which could use mevalonate as their sole carbon source (Takatsui *et al.*, 1983). Other observations include the inability of cell extracts of *Rhodo-*

spirillum rubrum to incorporate mevalonate into polyprenyl-phenol precursors of ubiquinones (Raman *et al.*, 1969), and the lack of incorporation of mevalonate into the modified bases of tRNA by *E. coli* (Peterkofsky, 1968). These observations prompted us to investigate the involvement of mevalonic acid in the early steps of isoprenoid biosynthesis in *E. coli*.

MATERIALS AND METHODS

Materials

[1,2- $^{13}\text{C}_2$]Sodium acetate (99 atom % ^{13}C), [U- $^{13}\text{C}_6$]glucose (98.5 atom % ^{13}C), and [1- ^2H]glucose (98 atom % ^2H) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), MSD Isotopes (Division of Merck Frosst Canada, Montreal, Canada), and Stohler Isotope Chemicals (Rutherford, NJ, U.S.A.) respectively. [U- $^2\text{H}_4$]Acetaldehyde (99 + atom % ^2H) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). DL-[methyl- $^2\text{H}_6$]Valine (> 98 atom % ^2H) was prepared by the Darapsky method as described by Gagnon *et al.* (1944). The 2-bromo[1,3- $^2\text{H}_6$]propane used in the synthesis was prepared as follows. [methyl- $^2\text{H}_6$]Acetone was reduced with NaBH_4 to [methyl- $^2\text{H}_6$]propan-2-ol, which was converted into the bromide by reaction with bromine in the presence of dimethylformamide and triphenylphosphine (Wiley *et al.*, 1964). Mevinolin was provided by Mr. Wan Ji of the Department of Plant Physiology and Weed Science at Virginia Polytechnic Institute and State University.

Preparation of [2,3,3,3- $^2\text{H}_4$]lactic acid

[2,3,3,3- $^2\text{H}_4$]Lactic acid was prepared by the condensation of NaCN with [U- $^2\text{H}_4$]acetaldehyde, followed by acid hydrolysis of the resulting cyanohydrin to lactic acid. Thus, to a reaction vial equipped with a rubber septum and containing an ice-cooled, stirred solution of 1 mmol of NaCN dissolved in 2.4 ml of water was added slowly 1 mmol of [U- $^2\text{H}_4$]acetaldehyde, followed by 1.27 ml of conc. H_2SO_4 in 3.5 ml of water. The solution was

Abbreviations used: Uq-8 etc., ubiquinone-8 etc.; IPP, isopentenyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMG, hydroxymethylglutarate.

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stirred at 0 °C for 1 h after the addition of the acid and then at room temperature for 30 min. The reaction product was extracted with diethyl ether (2 × 10 ml) and the ether extracts were combined and dried over anhydrous Na₂SO₄. After evaporation of the diethyl ether, the crude cyanohydrin obtained (1.72 g) was hydrolysed in 7 ml of 6 M-HCl at 100 °C for 1.5 h. Non-labelled DL-lactic acid (4 g) and water (approx. 10 ml) were added to the reaction mixture, which was then heated at 100 °C for 20 min in order to hydrolyse any [2,3,3,3-²H₄]lactic acid polymer which may have been formed during the acid hydrolysis of cyanohydrin. The lactic acid was extracted with methyl acetate, dried, and derivatized with bis(trimethylsilyl)trifluoroacetamide (Sigma). The analysis of the *OO*-bis(trimethylsilyl) derivative of the final lactic acid by g.c.-m.s. showed the sample to contain 18.3 % of the molecules as [²H₄]lactic acid.

Bacterial strains and growth

E. coli B was grown at 37 °C for ~ 12 h in a medium consisting of 9.9 g of K₂HPO₄, 0.5 g of NaCl, 0.8 mg of FeCl₃, 120 mg of MgSO₄, 5.3 g of NH₄Cl, 12.1 g of Tris/HCl, 2.9 g of sodium citrate, 0.44 mg of CaCl₂, 10 mg of thiamin and 4 g of either glucose, acetate, lactic acid or casamino acids/litre of medium. The carbon sources for the experiments (per litre) were 300 mg of DL-[methyl-²H₃]valine plus a mixture of amino acids (200 mg of arginine, 400 mg of aspartic acid, 25 mg of cysteine, 1.0 g of glutamic acid, 200 mg of glycine, 450 mg of lysine, 100 mg of methionine, 400 mg of proline, 300 mg of serine, 200 mg of threonine, 150 mg of histidine, 200 mg of phenylalanine, 400 mg of tyrosine and 100 mg of tryptophan) in the [²H₃]valine experiment; 0.4 g of [1,2-¹³C₂]acetate, 3.6 g of sodium acetate and 4 g of glucose or 5 g of casamino acid in Expts. 1 and 2; 0.4 g of [U-¹³C₆]glucose, 3.6 g of glucose and 4 g of sodium acetate in Expt. 3; 0.8 g of [1-²H]glucose, 3.2 g of glucose, 4 g of sodium acetate and 100 mg of L-methionine in Expt. 4; 4 g of lactic acid containing 18.3 % of [2,3,3,3-²H₄]lactic acid and 100 mg of L-methionine in Expt. 5.

Pyruvate dehydrogenase-defective *E. coli* mutant (CGSC# s5688, ΔaceE,F) was supplied by the *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, CT, U.S.A., and was grown in the *E. coli* B medium with acetate. In Expt. 6, the medium contained 0.1 g of methionine, 4 g of glucose, 3.6 g of sodium acetate, and 0.4 g of [1,2-¹³C₂]acetate per litre.

E. coli mutants YYC196 and MH6 (Chang & Cronan, 1983), both pyruvate dehydrogenase- and pyruvate oxidase-defective and both requiring acetate for growth, were supplied by Dr. Ying-Ying Chang, Department of Microbiology, University of Illinois, Urbana, IL, U.S.A. These cells, used in Expts. 7 and 8, were grown on a medium containing 4 g of glucose, 3.6 g of sodium acetate, and 0.4 g of [1,2-¹³C₂]acetate per litre.

In each experiment, 200 ml of medium was used. The labelled compounds were added to the medium at the concentrations indicated above, the pH was adjusted to 7 with HCl, and the medium was autoclaved. The glucose was autoclaved separately.

Effect of mevinolin on cell growth

E. coli B cells were grown in 5 ml of the medium containing both glucose and acetate as carbon sources (4 g/l) in the presence of mevinolin and mevinolin plus mevalonate (4 mM). Mevinolin concentrations of 1 μM and 68.3 μM were used. Cell growth was monitored by measuring cell density at 540 nm.

Isolation and derivatization of Uq-8

Uq-8 was isolated from the harvested cells (~ 200 mg) by extraction with 10 ml of dichloromethane/methanol (1:1, v/v) for 30 min at 50 °C. After centrifugation to remove insoluble

material, 0.5 vol. of water was added to the supernatant. After shaking and centrifugation, the dichloromethane layer was separated and dried over anhydrous Na₂SO₄, and the Uq-8 was purified by t.l.c. (silica-gel 60 F-254; E. Merck, AG, Darmstadt, Germany) using the solvent system hexane/diethyl ether (3:1, v/v). The yellow Uq-8 spot (*R_F* 0.5) was eluted with dichloromethane for m.s. analysis by direct probe insertion. Purified Uq-8 was derivatized by treatment with acetic anhydride and zinc dust as described by Campbell *et al.* (1971) to produce the diacetate derivative of dihydroubiquinone. The diacetate derivative of Uq-8 was then purified on t.l.c. with the same solvent system as that described above.

Cleavage of Uq-8 to laevulinic acid and the formation and analysis of the resulting 4,5-dihydro-6-methyl-2-phenylpyridazin-3(2*H*)-one derivative

Uq-8 was cleaved to laevulinic acid by the periodate/permanaganate oxidation procedure described by von Rudloff (1955). A t.l.c.-pure sample of Uq-8, isolated as described above from 2 g wet weight of cells, was dissolved in 50 μl of pyridine and 40 μl of 0.2 M-NaIO₄ and 40 μl of 4 mM-KMnO₄ were added. The resulting cloudy purple solution was allowed to react overnight in the dark at room temperature, was acidified with 3 M-HCl and was extracted three times with 0.5 ml of diethyl ether to extract the laevulinic acid. The combined ether layers were then shaken with 50 μl of 0.06 M-NaHCO₃ and the diethyl ether evaporated with a stream of N₂. (The addition of NaHCO₃ resulted in the formation of the sodium salt which was required to prevent the evaporation of the laevulinic acid.) To the resulting aqueous solution was added 120 μl of a solution 2.5 M in phenylhydrazine and 0.75 M in HCl in a mixture of methanol and water (3:1, v/v). After heating for 1 h at 70 °C, the solution was extracted with diethyl ether to recover the 4,5-dihydro-6-methyl-2-phenylpyridazin-3(2*H*)-one derivative of laevulinic acid. This material was readily assayed by g.c.-m.s. using the intense molecular ion at *m/z* 188 to measure the total incorporation of label into the laevulinic acid.

Mass spectrometry and determination of isotopic enrichments

Mass spectra were recorded using a VG 70-70EHF mass spectrometer operated at 70 eV. Samples of Uq-8 and its reduced diacetate derivative were introduced into the mass spectrometer by direct inlet, whereas g.c. was used for the introduction of the other samples. Isotopic abundances for both the labelled and unlabelled samples were obtained from the integration of the intensities of the ion + 1 *m/z*, ion + 2 *m/z*, ion + 3 *m/z* etc. and the percentage isotopic enrichment was calculated from these two sets of data as outlined by Biemann (1960).

M.s. of Uq-8 and dihydro-Uq-8 diacetate

The mass spectrum of Uq-8 shows an intense molecular ion at *m/z* 726 (structure **a**, Scheme 1), which allows for the measurement of the incorporation of stable isotopically labelled precursors into the entire molecule. The mass spectrum of Uq-8 also has fragment ions at *m/z* 235 and *m/z* 197 (structures **b** and **c**, Scheme 1). The *m/z*-235 ion allows for the measurement of label incorporated into the benzoquinone portion of the molecule and into the first three carbon atoms and the first methyl group of the attached isoprenoid unit. The *m/z*-197 ion allows for the measurement of label incorporated into the benzoquinone-ring portion and into the first carbon atom of the isoprenoid unit. The difference between the label incorporated into the entire molecule and that incorporated into the benzoquinone-containing *m/z*-197 fragment represents the label incorporated into the isoprenoid portion of the molecule. The incorporation of acetate or glucose into the methyl or methoxy group on the benzoquinone

Scheme 1. Structures and mass-spectral fragmentations of Uq-8 and the diacetate derivative of dihydroubiquinone

ring interferes with the measurement of m/z 235 and m/z 197. This incorporation could be greatly suppressed by growing the cells in the presence of methionine, since all methyl groups on the benzoquinone ring are derived from the methyl group of methionine (Jackman *et al.*, 1967). (The incorporation of a small amount of label into the methyl groups has little effect on the measurement of the total amount of label incorporated into the entire molecule from the M^+ m/z -726 ion, since the total incorporation into each of the eight isoprenoid units is much greater than the incorporation into the benzoquinone methyl groups.)

A serious problem in measuring the incorporation of more than on ^2H or ^{13}C into Uq-8 by using m.s. is the reduction (dismutation) of Uq-8 in the mass spectrometer, resulting in the generation of the hydroquinone of Uq-8, which has a molecular ion at m/z 728 (structure **e**, Scheme 1). This reaction, which has been studied in other quinones, leads to variations in the intensity of the $M^+ + 2$ ions (Aplin & Pike, 1966; Das *et al.*, 1965; Ukai *et al.*, 1967; Zhou & White, 1989). In our experience, the intensity

of the $M^+ + 2$ ion varied from sample to sample in a seemingly random fashion and caused an $\sim 20\%$ error in the measurement of the intensity of the $M^+ + 2$ ion of Uq-8. This problem was solved by converting Uq-8 into the hydroquinone diacetate derivative (structure **d**, Scheme 1). The mass spectrum of this diacetate showed a molecular ion at m/z 812, an intense fragment ion (**e**) at m/z 728, corresponding to the hydroquinone, and the same fragment ion (**c**) at m/z 197 as was observed in the mass spectrum of Uq-8 (Scheme 1). The intense m/z -728 ion allows for the accurate measurement of isotope incorporation into the entire molecule of Uq-8 without the interference caused by reduction in the mass spectrometer.

Analysis of isotope distribution of amino acids in cellular proteins

The isotopic distribution of ^2H or ^{13}C in the protein-bound amino acids in the cells was determined by g.c.-m.s. of their *N*-(trifluoroacetyl) and *NO*-bis(trifluoroacetyl) *n*-butyl esters as previously described (White, 1985).

Analysis of isotope distribution in fatty acids

The isotopic distribution of ^2H or ^{13}C in the cellular fatty acids was determined by g.c.-m.s. of their methyl esters in the following manner. After the t.l.c. purification of Uq-8, the polar phospholipids remaining at the origin were eluted with methanol. After evaporation of the methanol, the resulting phospholipids were heated at 50°C for 2–3 h with 3 M-HCl in anhydrous methanol. After evaporation of the HCl and methanol, the fatty acid methyl ester in the remaining residue was extracted with heptane and the heptane extract was washed with aq. NaHCO_3 . After the solution was dried over anhydrous Na_2SO_4 and concentrated, the resulting methyl fatty acids were analysed by g.c.-m.s. using a Hewlett-Packard HP5 capillary column (0.32 mm $\times$ 25 m) programmed from 100°C at $10^\circ/\text{min}$.

RESULTS

A critical series of reactions in the acetolactic acid pathway proposed by Pandian *et al.* (1981) for the biosynthesis of isoprenoid compounds in eubacteria is the conversion of α -oxoisovaleric acid to α -oxoisohexanoic acid. The role of these oxo acids in this pathway, and thus isoprenoid biosynthesis, can be readily tested by labelling these acids *in vivo* and measuring the incorporation of label into an isoprenoid-derived compound. (For the experiments reported here, Uq-8 was chosen because it was the most abundant isoprenoid formed in *E. coli*.) Since the conversion of α -oxoisovaleric acid into α -oxoisohexanoic acid is required for the conversion of valine into leucine (Rodwell, 1969), cells converting labelled valine into labelled leucine would have to contain these labelled α -oxo acids. Thus, by growing cells with [*methyl*- $^2\text{H}_6$]valine and measuring the extent of labelling in the valine, leucine and Uq-8 in the cells, the involvement of these α -oxo acids in isoprenoid biosynthesis can be firmly established. The results of this experiment showed the cellular valine and leucine to contain 75.9% and 80.8% of the molecules labelled with $^2\text{H}_6$, respectively, whereas no ^2H was found to be incorporated into the Uq-8. Since both the valine and leucine are labelled to about the same extent, this proves that both the α -oxoisovaleric acid and α -oxoisohexanoic acid present in the cells are also labelled and, since the Uq-8 contained no ^2H , these α -oxo acids cannot be involved in the biosynthesis of Uq-8. This result, therefore, eliminates the acetolactic acid pathway from being involved in isoprenoid biosynthesis in these cells.

Incorporation of labelled acetate and labelled glucose

With the elimination of the involvement of the acetolactic acid pathway in the early steps of IPP biosynthesis, the exact nature of the true pathway was explored by measuring the incorporation of several different labelled precursors into Uq-8 by *E. coli*. Cells grown with glucose and [$1,2\text{-}^{13}\text{C}_2$]acetate as their sole carbon sources showed no incorporation of label into the Uq-8; however, the labelled acetate was readily incorporated into fatty acids as a C_2 unit (Expt. 1, Table 1) where the m/z $M^+ + 2$ ion for the methyl palmitate isolated from these cells showed 28.6% incorporation but the m/z $M^+ + 2$ ion of Uq-8 showed no incorporation. [This observation is consistent with the results reported by Raman *et al.* (1965) for several different bacteria, including *E. coli*.] On the basis of the measured intensities of the ions associated with the molecular ion of the methyl palmitate and the fact that the fed acetate contained only 10% of the molecules with [$1,2\text{-}^{13}\text{C}_2$]acetate, 53.8% of the acetate used by these cells for the biosynthesis of fatty acids was derived from the fed acetate as an intact unit. Since acetyl-CoA is a key inter-

mediate in the biosynthesis of fatty acids, and since its acetate group must be labelled in these cells, these data also indicate that acetyl-CoA is not an intermediate in Uq-8 biosynthesis.

Growing *E. coli* B with [$1,2\text{-}^{13}\text{C}_2$]acetate plus casamino acids allowed for the incorporation of a small amount of a single carbon of the double-labelled acetate into the Uq-8 (Expt. 2, Table 1). This incorporation, however, was still much less than the 53.1% calculated to be incorporated from the methyl palmitate data. These data indicate that, under certain growth conditions, label from acetate could be incorporated into Uq-8, but only after being 'metabolically scrambled', e.g., via the glyoxylate cycle. The condensation of [$1,2\text{-}^{13}\text{C}_2$]acetate with glyoxylate forms malate, which is then converted into [$3\text{-}^{13}\text{C}$]pyruvate by 'malic' enzyme. The incorporation of the C-3 and C-2 unit of the resulting pyruvate into the IPP would explain the observed results.

Since glucose was the only carbon source other than acetate in Expt. 1, some glucose-derived intermediate must have served as the precursor to the Uq-8. In order to determine the number of carbon atoms in this precursor, cells were grown with glucose in which 10% of the molecules contained [$\text{U-}^{13}\text{C}_6$]glucose, and the incorporation of label into Uq-8 was measured. Molecules with from 1 to 11 ^{13}C atoms were observed with a smooth decrease in the abundance of molecules containing an increasing number of ^{13}C . Attempts to analyse the observed isotope pattern in order to determine the number of carbon atoms in the glucose-derived precursor were not successful. Specifically, we were unable to establish with certainty if the C_5 isoprenoid unit used in the biosynthesis was derived from two C_2 units and one C_1 unit or from one C_3 and one C_2 unit of the labelled glucose. In order to solve this problem, the Uq-8 was cleaved to laevulinic acid with periodate/permanganate, and the labelling pattern in the laevulinic acid was determined from the mass spectrum of its phenylhydrazine derivative (Scheme 2). The measured normalized ion intensities for this derivative showed 75.9% of the molecules to contain no ^{13}C ; 7.4%, one ^{13}C ; 14.7%, two ^{13}C ; 1.3%, three ^{13}C ; and 0.76%, four ^{13}C . From this simple labelling pattern it is clear that the laevulinic acid is derived from two C_2 units and one C_1 unit of the glucose, since the abundance of the $M^+ + 2$ ion is twice that of the $M^+ + 1$ ion. From the measured intensities of these ions and from the fact that the labelled glucose contained only 10% of the molecules with labelled glucose, it was calculated that 88% of the C_1 unit and 81% of the two C_2 units were derived from the glucose.

This labelling pattern could only have been obtained if the two $^{13}\text{C}_2$ units were at C-1–C-2 and C-3–C-4 and if the single ^{13}C was at the methylene of the isopentenyl pyrophosphate. This labelling pattern is consistent with glucose being metabolized to acetate, which is subsequently incorporated, via the established acetoacetate pathway, into isopentenyl pyrophosphate, which then supplies the carbon for the biosynthesis of the side chain of Uq-8. Also this labelling pattern is completely inconsistent with the acetolactate pathway for the biosynthesis of isopentenyl pyrophosphate.

The above experiments, however, did not allow for the determination of which of the glucose carbon atoms were incorporated into which positions of the IPP and thus into Uq-8. A partial remedy to this problem was effected by growing cells with [$1\text{-}^2\text{H}$]glucose (Expt. 4, Table 1) and measuring the position of label incorporation into the Uq-8 from the mass-spectral fragmentation of the isolated Uq-8. The results of this experiment showed the Uq-8 to be labelled, thus indicating that the C-1 and C-2 unit of the glucose is supplying at least one of the C_2 units in the biosynthesis. This is consistent with glucose being metabolized via glycolysis to pyruvate, whereupon C-2 and C-3 of the pyruvate supply the C_2 units for the biosynthesis.

Table 1. Incorporation of labelled compounds into Uq-8 and palmitic acid

Distribution of ² H or ¹³ C in: *																		
Expt.	Compounds fed	Uq-8				Uq-8 diacetate				Methyl palmitate								
		m/z 197				m/z 235				m/z 728				M ⁺ m/z 270†				
		+0	+1	+2	+3	+4	+0	+1	+2	+3	+4	+0	+1	+2				
1	[¹³ C] ₂ Acetate (10%) + glucose	96.4 (100)	3.6 (16.4)	0	0.6 (4.9)		99.4 (100)	0	0	98.9 (100)	0	0.4	0	0	63.0 (100)	2.6 (22.5)	28.6 (45.2)	
2	[¹³ C] ₂ Acetate (10%) + casamino acids	92.9 (100)	7.1 (20.3)	91.7 (100)	7.9 (6.1)		64.1 (100)	23.4 (93.7)	9.6 (62.0)	0	1.4 (12.9)	62.6 (100)	2.8 (22.8)	28.1 (45.7)				
3	[¹³ C] ₂ Glucose (10%) + acetate	(see the text)													65.9 (100)	6.2 (27.8)	22.5 (35.9)	
4	[1- ² H]Glucose (20%) + acetate + methonine	92.2 (100)	7.8 (21.1)	90.9 (100)	4.7 (19.8)	2.6 (8.0)	12.8† (36.1)	15.4 (43.4)	35.5 (100)	31.7 (89.2)	4.5 (12.7)	(Results not recorded)						
5	[² H] ₂ Lactic acid (18%)	86.8 (100)	9.0 (23.1)	84.1 (100)	7.9 (24.1)	3.8 (10.3)	28.7† (68.2)	8.1 (55.8)	21.2 (100)	13.5 (87.7)	6.1 (69.9)							
6	[¹³ C] ₂ Acetate (10%) + glucose§	98.1 (100)	1.9 (14.6)	90.5 (100)	2.5 (17.4)	7.0 (12.6)	93.7 (100)	4.0 (61.5)	2.2 (31.1)	0	0	47.8 (100)	1.9 (22.2)	36.6 (77.3)				
7	[¹³ C] ₂ Acetate (10%) + glucose§	97.5 (100)	2.5 (15.2)	98.5 (100)	0.4 (15.0)	1.1 (5.6)	94.7 (100)	3.7 (61.1)	1.6 (30.1)	12.0 (100)	5.0	45.5 (100)	1.7 (22.1)	36.9 (81.9)				
8	[¹³ C] ₂ Acetate (10%) + glucose§	98.9 (100)	1.1 (13.7)	96.6 (100)	0.8 (15.5)	2.6 (7.2)	96.5 (100)	0 (55.9)	3.5 (29.8)			46.2 (100)	1.5 (21.5)	37.7 (82.1)				
9	Unlabelled sample	(100)	12.6)	(100)	14.6	4.35	(100)	57.2	26.2	9.1	3.5)	(100)	18.3	0)				

* Values in parentheses are the observed normalized ion intensities.

† The intensities of $M^+ + 3$ and $M^+ + 4$ were used in the calculation, but not reported here.‡ The data were measured on $M^+ m/z$ 726 of non-derivatized Uq-8, which has a significant error in the measurement of the $M^+ + 2$ ion (see the text).§ *E. coli* mutant ΔaceE,F was used in Expt. 6, YYC196 in Expt. 7, and MH6 in Expt. 8; *E. coli* B was used in all other experiments.

Scheme 2. Cleavage of Uq-8 to laevulinic acid and the formation of 4,5-dihydro-6-methyl-2-phenylpyridazin-3(2H)-one

Incorporation of labelled lactic acid

That pyruvate can supply at least the C-3 and C-4 C_2 unit of the IPP was confirmed by establishing that pyruvate containing three 2H atoms in the methyl group was incorporated into the methyl group of the isoprenoid with retention of up to three 2H atoms. Since IPP has only one methyl group, the incorporation of three 2H atoms also proves that C-3 of pyruvate becomes the C-4 position of the IPP. More importantly, the incorporation of three 2H atoms from labelled pyruvate would indicate that no intermediate derived from phosphoenolpyruvate, which would have retained only two 2H atoms, could be a source of the C_2 unit. Due to the extensive exchange of the 2H of [methyl- 2H_3]pyruvate with the H^+ of water (White, 1978), this experiment was conducted by feeding cells with [methyl- 2H_3]lactate, which would be converted, *in vivo*, into the labelled pyruvate. As Table 1 (Expt. 5) shows, the m/z -726 molecular ion of the Uq-8 isolated from cells grown with [methyl- 2H_3]lactate contained a small number of molecules with three 2H atoms. It is also clear from the data that molecules with one and two 2H atoms were also present. (The number of molecules with two 2H atoms is uncertain, because of variations in the $M^+ + 2$ ion of Uq-8.) This shift in the expected pattern from completely 2H -labelled to partially 2H -labelled results from the exchange of pyruvate discussed above. The presence of this exchange can be independently confirmed by the measurement of similar 2H distribution patterns in the pyruvate-derived amino acids alanine, valine, isoleucine and leucine (Table 2).

Incorporation of labelled acetate in *E. coli* mutants

The data obtained from the above experiments indicated that the isoprenoid units of ubiquinone in *E. coli* appear to be

Table 2. Incorporation of [2H_4]lactic acid into cellular protein

Amino acid†	Distribution of $^2H^*$				Ions used for measurement
	+0	+1	+2	+3	
Alanine	90.5 (100)	1.4 33.0	4.9 7.3	3.2 5.2	m/z 140 ($M^+ - COOC_4H_9$)
Valine	79.7 (100)	5.9 21.0	7.3 11.9	6.9 17.6	m/z 168 ($M^+ - COOC_4H_9$)
Leucine	80.4 (100)	2.4 23.0	9.2 12.0	8.0 12.1	m/z 182 ($M^+ - COOC_4H_9$)
Isoleucine	94.2 (100)	2.8 17.2	3.7 4.9	2.2 2.9	m/z 182 ($M^+ - COOC_4H_9$)

* Values in parentheses are the observed normalized ion intensities.

† The intensity data were obtained from the *N*-trifluoroacetyl *n*-butyl ester derivatives of the amino acids listed. The measured intensities for unlabelled derivatized samples were: 100, 31.4, 1.4 and 0% for the m/z -140, -141, -142 and -143 ions in alanine; 100, 13.6, 1.8 and 7.5% for the m/z -168, -169, -170 and -171 ions in valine; 100, 20.0, 0 and 0% for the m/z -182, -183, -184 and -185 ions in leucine; and 100, 14.2, 0.6 and 0% for the m/z -182, -183, -184, and -185 ions in isoleucine respectively. The amino acids came from the cell pellet of Expt. 5, Table 1.

biosynthesized from C_2 units derived from glucose via pyruvate, which do not mix with the acetyl-CoA pool. This could occur if the acetyl groups generated by the cleavage of the pyruvate were directly channelled from their site of synthesis to their site of incorporation into the IPP without exchange with the cellular acetyl-CoA pool. Since a covalently bound acetyl group is a key intermediate in reactions catalysed by the pyruvate dehydrogenase complex, it was decided to test for the possible involvement of this enzyme in the biosynthesis of IPP by measuring the incorporation of labelled acetate into Uq-8 by mutants lacking this enzyme. It was rationalized that, since these mutants grow without the addition of Uq-8, they must be able to use an alternative pathway, one perhaps originating from acetate. The utilization of this alternative pathway could be confirmed by observing the incorporation of labelled acetate into Uq-8. Growing the mutant with [1,2- $^{13}C_2$]acetate and glucose, however, showed no incorporation of label into Uq-8 (Expt. 6, Table 1). The incorporation of labelled acetate into the fatty acids (90%) by these mutant cells was higher than that recorded in wild-type cells (53.8%), since a larger proportion of the acetyl-CoA used for the fatty acid biosynthesis must be derived from the fed acetate.

Pyruvate can also be metabolized to free acetate in *E. coli* by pyruvate oxidase (Hamilton *et al.*, 1986; Grabau & Cronan, 1984; Chang & Cronan, 1988), which could also be supplying the acetate for Uq-8 biosynthesis. Pyruvate oxidase is a membrane-bound enzyme that requires thiamin pyrophosphate, as does pyruvate dehydrogenase, but not lipoic acid, for activity. The physiological role of this enzyme is not clear (Grabau & Cronan, 1984; Chang & Cronan, 1988). The involvement of this enzyme in isoprenoid biosynthesis in *E. coli* was studied by using *E. coli* mutants (YYC196 and MH6) that do not produce either pyruvate dehydrogenase or pyruvate oxidase. Both mutants were grown with [1,2- $^{13}C_2$]acetate in the presence of glucose, and as in the ($\Delta aceE, F$) mutant and wild-type, labelled acetate was not incorporated into ubiquinone (Expts. 7 and 8, Table 1) in either mutant, and [1,2- $^{13}C_2$]acetate was incorporated into fatty acids to the extent of ~95% in both mutants. Also, as in the wild-type cells, [U - $^{13}C_6$]glucose was readily incorporated into the Uq-8. These results indicate that glucose serves as a carbon source for the isoprenoid even when both pyruvate dehydrogenase and pyruvate oxidase are absent, and it suggests that a unique 'pyruvate dehydrogenase' is specifically involved in this pathway.

Effect of mevinolin on cell growth

A central question with regard to the early steps of isoprenoid biosynthesis in *E. coli* is whether or not mevalonate is an intermediate. Several studies (Raman *et al.*, 1965, 1969; Fujisaki *et al.*, 1986a; Takatsuji *et al.*, 1983; Peterkofsky, 1968) have indicated that free mevalonate does not appear to be involved in the biosynthesis of isoprenoids in several different eubacteria. However, there is no definitive evidence that mevalonate is not involved in the pathway. Mevinolin, a fungal product, has been found to be an effective inhibitor of HMG-CoA reductase, which catalyses the formation of mevalonate for isoprenoid biosynthesis in eukaryotes and archaeobacteria (Endo, 1981; Cabrera *et al.*, 1986; Bach & Lichtenthaler, 1987). It has been demonstrated that mevinolin completely stops the growth of *Halobacterium halobium* at a concentration of 1 μM (Cabrera *et al.*, 1986), *Thermoproteus tenax* at 12.5 μM (Huser *et al.*, 1986), and many strains of yeast at 10–50 μM (Ikeura *et al.*, 1988). In each case, cell growth could be recovered by the addition of mevalonate. The effect of mevinolin on eubacteria has been studied in two thermophilic eubacteria, *Bacillus stearothermophilus* and strain FJSS3-B.1, a newly isolated thermophilic anaerobic eubacterium (Huser *et al.*, 1986). It was found that mevinolin did not inhibit the growth of either strain at concentrations up to 125 μM . To test the involvement of mevalonate in *E. coli*, similar experiments were performed with *E. coli* B. It was found that mevinolin did not affect the growth of *E. coli* at 1 μM or 68.3 μM , concentrations which completely inhibited the growth of *H. halobium*. Although this negative result does not prove the lack of involvement of mevalonate and HMG-CoA reductase in the biosynthesis of isoprenoid in eubacteria, it does indicate that there may be some fundamental difference in isoprenoid biosynthesis between eubacteria and the eukaryotes and archaeobacteria.

DISCUSSION

That IPP can serve as the precursor for the biosynthesis of isoprenoids in *E. coli* is now well established (Fujisaki *et al.*, 1986a,b). This information, combined with the observations presented here, made it necessary to consider just how IPP can be generated by *E. coli* without the involvement of acetyl-CoA. This analysis must take into consideration the possibility that mevalonic acid is not an intermediate or, at least, not a free intermediate, in the biosynthesis of isoprenoids in *E. coli* and a large number of other eubacteria. The simplest mechanism which would account for these observations is the cleavage of pyruvate by the cells to generate an acetyl group that is bound to a protein, most likely through a thiol-ester linkage. This acetylated protein could be analogous to the acyl-carrier protein in fatty acid biosynthesis (Wakil, 1989). This acetylated protein would supply the acetate units for the biosynthesis of IPP, which would proceed very much like that established for the acetoacetate biosynthetic pathway to IPP. This idea, however, does not explain the apparent lack of involvement of mevalonate in the biosynthesis of isoprenoids in bacteria. There are two possible explanations for lack of involvement of mevalonate. One is that all the intermediates from the acetate-derived pyruvate to the IPP are bound to proteins and do not exchange with cellular parts of free intermediates. Acetyl groups bound to proteins by thiol-ester linkages are well-documented, with pyruvate dehydrogenase and pyruvate formate-lyase (Knappe *et al.*, 1974) being specific examples. As our work demonstrates, however, the bound acetates of these enzymes do not appear to be involved in the biosynthesis of isoprenoids.

A second explanation would be that all of the steps, up to the formation of 3-hydroxy-3-methylglutarate (HMG), proceed

via bound intermediates and that the conversion of HMG into IPP does not proceed via free mevalonate. One can envision two routes whereby this could occur. In the first, the protein-bound HMG is reduced to bound mevalonate, which is then converted into the pyrophosphate ester before it is released from the enzyme. Subsequent decarboxylation and dehydration would then lead to IPP by the normal series of reactions. In the second route, the HMG is released from its bound state and, after decarboxylation and dehydration, is converted into either 3-methylbut-2-enoic acid or 3-methylbut-3-enoic acid (Martin & Simpson, 1989). These acids are then converted into either IPP or dimethylallyl pyrophosphate by reduction of the acid group to an alcohol and by the subsequent formation of the pyrophosphate ester. If dimethylallyl pyrophosphate were the major reaction product formed, it could be readily converted into IPP by the IPP isomerase present in *E. coli* (Fujisaki *et al.*, 1986a,b). Any of these explanations would be consistent with the absence of free mevalonate in *E. coli* and would explain why mevalonate is not used by these and other eubacteria for isoprenoid biosynthesis.

An attempt to measure the incorporation of [*methyl-²H]₃-methylbut-2-enoic acid into Uq-8 by *E. coli* showed no detectable incorporation, indicating either that this compound is not an intermediate in the pathway or that the compound is not permeable to the cells. Thus further work to confirm any of the proposed intermediates will require the use of permeabilized cells.*

The work reported here, which shows that free acetyl-CoA is not involved in isoprenoid biosynthesis in the eubacterium *E. coli*, is in sharp contrast with what has been observed in both eukaryotes (Beytia & Porter, 1976) and in archaeobacteria (Langworthy *et al.*, 1982; De Rosa *et al.*, 1986; Moldoveanu & Kates, 1988), which use acetyl-CoA in the biosynthesis of isoprenoids. If this pathway for the biosynthesis of isoprenoids is a general characteristic of all eubacteria, then it may represent a clear biochemical marker that separates eubacteria from archaeobacteria and eucaryotes.

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