An alternative pathway for the biosynthesis of isoprenoid compounds in bacteria

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The pattern of incorporation of radioactivity from [1-¹⁴C]acetate and [2-¹⁴C]acetate into the polyprenyl side-chain of ubiquinones in bacteria (*Azotobacter vinelandii*, *Pseudomonas sesami*, *Escherichia coli* and *Rhodopseudomonas capsulata*) was studied. For this purpose, a new degradation method involving a modified Barbier–Wieland reaction of laevulinic acid was developed, and used along with the iodoform reaction. Both C-1 and C-2 of acetate were incorporated exclusively into C-2 of laevulinic acid, suggesting that the well-known pathway through acetoacetyl-CoA ('acetoacetate pathway') was not operative in these bacteria. An alternative pathway ('acetolactate pathway'), starting with pyruvate and acetaldehyde as the distal precursors, and utilizing the reactions of leucine and valine metabolism, was postulated. It was also postulated that C-1 of acetate is incorporated not directly, but after oxidation to CO₂. The pattern of incorporation of radioactivity from [U-¹⁴C]valine, [U-¹⁴C]alanine and NaH¹⁴CO₃ into the side-chain of ubiquinone of *R. capsulata* was in agreement with the operation of the 'acetolactate pathway'.

It is well known that mevalonic acid is the precursor of the isoprene unit in a variety of compounds such as sterols and carotenoids (Folkers *et al.*, 1959). Isopentenyl pyrophosphate is a key intermediate in the metabolic pathway from mevalonic acid to polyprenoids. The carbon skeleton of mevalonic acid itself is derived entirely from the two carbon atoms of acetic acid (Folkers *et al.*, 1959; Clayton, 1965; Lynen *et al.*, 1959; Cornforth, 1969). Thus it would appear that acetate is the distal precursor of all the carbon atoms of the isoprene unit in fungi (Ottke *et al.*, 1950), higher plants (Park & Bonner, 1958) and animals (Bloch *et al.*, 1946; Little & Bloch, 1950; Cornforth & Popjak, 1954).

Acetate, mevalonic acid and isopentenyl pyrophosphate serve as precursors of a number of polyprenoid compounds in bacteria also (Thorne & Kodicek, 1962; Parson & Rudney, 1964; Durr & Habbal, 1972; Allen *et al.*, 1967). However, 'in bacterial systems, the intermediates between acetate and isopentenyl pyrophosphate, the immediate pre-

cursor of isoprenoid compounds, have not been clearly delineated' (Winrow & Rudney, 1971). We have now studied the biosynthesis of the polyisoprenoid side-chain of ubiquinones of bacteria and find that the pathway is different from that in the other organisms, and that only one of the five isoprene carbon atoms is derived from acetate.

Experimental

Materials

The common chemicals used were purchased mostly from BDH (India) or Sarabhai–Merck (India). Radioactive compounds (¹⁴C) were purchased from Bhabha Atomic Research Centre, Bombay, India.

Methods

Culture of micro-organisms. *Azotobacter vinelandii*, *Escherichia coli* and *Pseudomonas sesami* were grown as previously described (Raman *et al.*, 1965). *Rhodopseudomonas capsulata* was cultured according to the method suggested for *Rhodospirillum rubrum* (Ormerod *et al.*, 1961).

Isolation of ubiquinone. Ubiquinones were isolated from bacteria by the method described by Parson & Rudney (1964) and by Raman *et al.* (1965). The u.v.-absorption spectra of ubiquinone samples were obtained with a Hitachi Perkin–Elmer

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(model 124) spectrophotometer and ubiquinone was estimated from its absorbance at 275 nm (Crane & Dilley, 1963).

Degradation of ubiquinone. For ascertaining the radioactivity in each of the carbon atoms of the isoprene unit, the ubiquinone samples were degraded by a method which is outlined below.

(1). The purified ubiquinone samples were oxidized to laevulinic acid with a $\text{KMnO}_4/\text{NaIO}_4$ reagent (Lemieux & von Rudloff, 1955) in the presence of pyridine. The laevulinic acid was isolated after addition of carrier.

(2). A portion of the laevulinic acid was subjected to the iodoform reaction. The weight and radioactivity of iodoform were determined.

(3). Another portion of laevulinic acid was subjected to a modified Barbier–Wieland degradation (Theilheimer, 1950) as follows. The laevulinic acid was first converted to the methyl ester. The methyl laevulinate was condensed with 2-mercaptoethanol to form the hemithioketal of the keto group. This derivative was treated with phenyl magnesium bromide. The reaction product was isolated and converted, by treatment with acetic/perchloric acid mixture, to 1,1-diphenyl-4-oxo-1-pentene, $\text{CH}_3\text{—CO—CH}_2\text{—CH=C(C}_6\text{H}_5)_2$ which was oxidized with CrO_3 in H_2SO_4 to benzophenone (derived from C-1 of laevulinic acid), CO_2 (C-2 of laevulinic acid) and acetone (C-3, C-4 and C-5 of laevulinic acid). The CO_2 which was evolved during the oxidation was flushed out in a slow stream of N_2 and absorbed in 2% (w/v) NaOH. Radioactivity in the solution was measured. Acetone and benzophenone were obtained as a mixture by steam-distillation. Benzophenone was extracted from the mixture with light petroleum (b.p. $60^\circ\text{—}80^\circ\text{C}$) and estimated colorimetrically with 2,4-dinitrophenylhydrazine (Toren & Heinrich, 1955). Acetone, which remained in the aqueous layer, was treated with KOH and I_2 to give iodoform, the amount and radioactivity of which were measured. The radioactivity of benzophenone was also measured.

By this procedure, it was envisaged that radioactivity in C-1, C-2, C-3 and C-5 of laevulinic acid could be directly known and that in C-4 could be obtained by difference.

In preliminary experiments, on the oxidation of 1,1-diphenyl-4-oxo-1-pentene with chromic acid, the yield of benzophenone was nearly quantitative, and the yield of CO_2 (estimated gravimetrically as BaCO_3) was consistently about 95%. The yield of CO_2 was, however, not estimated in the experiments with radioactive material, but was assumed to be equal to that of benzophenone, since the two compounds are formed in what is virtually a single reaction. Therefore, the values for the radioactivity in C-2 of laevulinic acid could be too low by 5% and the values for C-4 could be correspondingly too

high. This possible error, however, did not turn out to be of serious consequence in interpreting the results.

Measurement of radioactivity. Radioactivity was measured in a Packard Tri-carb liquid-scintillation spectrometer. Non-aqueous samples were counted in a scintillation mixture consisting of 2,4-diphenyl-oxazole (5 g) and 1,4-bis-[2-(5-phenyloxazolyl)-benzene] (0.5 g) in sulphur-free toluene (1 litre). For aqueous samples, Bray's scintillation mixture (Bray, 1960) was used.

Results and discussion

Incorporation of radioactivity from [1- ^{14}C]acetate and [2- ^{14}C]acetate

Radioactivity from both [1- ^{14}C]acetate and [2- ^{14}C]acetate was incorporated into the ubiquinones of *A. vinelandii*, *E. coli*, *P. sesami* and *R. capsulata* (see Table 1), but, in all the bacteria except *R. capsulata*, the ratio of incorporation of C-1 to that of C-2 of acetate was much less than the ratio (2:3) that would be expected from the well-known pathway through acetoacetate and 3-hydroxy-3-methylglutarate (the 'acetoacetate pathway'). If the observed low ratio of incorporation of C-1 to C-2 of acetate were due to the operation of an alternative pathway, it is conceivable that the pattern of distribution of radioactivity in the isoprene unit would be different from the pattern established for the 'acetoacetate pathway' by Cornforth & Popjak (1954). In order to test this possibility, we degraded the side-chain of ubiquinone samples to laevulinic acid with $\text{KMnO}_4/\text{NaIO}_4$ reagent (Lemieux & von Rudloff, 1955) and further degraded the laevulinic acid to iodoform and succinic acid. As shown in Table 2, the iodoform samples from the ubiquinones of bacteria contained very little radioactivity even when [2- ^{14}C]acetate was the precursor. Thus, the degradation data indicate the absence of the 'acetoacetate pathway' in all the bacteria, including *R. capsulata*, in which the ratio (2:3) of incorporation of C-1 of acetate to that of C-2 would, at first sight, suggest the operation of the 'acetoacetate pathway'. On the other hand, the ubiquinone samples from the moulds *Aspergillus niger*, *Penicillium chrysogenum*, and *Phycomyces blakesleeanus* showed the pattern expected for the 'acetoacetate pathway' (see Table 2).

The finding that the bacteria do not synthesize isoprenoids through the 'acetoacetate pathway' prompted us to trace out the positions where carbon atoms of acetate are incorporated in the isoprenoid side chain of ubiquinones. Accordingly, we carried out a modified Barbier–Wieland degradation of the laevulinic acid obtained from the bacterial ubiquinones (Table 3). We found that C-1 and C-2 of acetate were both incorporated almost exclusively into C-2 of laevulinic acid. These results confirmed

Table 1. *Incorporation of radioactivity from [1-¹⁴C]acetate and [2-¹⁴C]acetate into ubiquinones of bacteria*

The bacterial cultures were grown as previously described (Raman *et al.*, 1965; Ormerod *et al.*, 1961). Radioactive precursors (50 μ Ci; 1.05 μ mol each) were added aseptically to the bacterial cultures. At the end of the growth period, the ubiquinones were isolated by the method described by Parson & Rudney (1964). Ubiquinone was estimated spectrophotometrically (Crane & Dille, 1963) and the radioactivity was measured in a liquid-scintillation spectrometer.

Bacterium and tracer used	Wet weight of cells (g)	Amount of ubiquinone (μ mol)	Specific radioactivity of ubiquinone (d.p.m./nmol)
<i>Azotobacter vinelandii</i>			
[1- ¹⁴ C]Acetate	5.7	1.24	3.4
[2- ¹⁴ C]Acetate	5.3	1.68	19.6
<i>Escherichia coli</i>			
[1- ¹⁴ C]Acetate	9.1	0.67	47
[2- ¹⁴ C]Acetate	8.8	0.73	284
<i>Pseudomonas sesami</i>			
[1- ¹⁴ C]Acetate	5.3	0.87	10
[2- ¹⁴ C]Acetate	5.9	0.95	87
<i>Rhodopseudomonas capsulata</i>			
[1- ¹⁴ C]Acetate	3.1	2.09	57
[2- ¹⁴ C]Acetate	3.5	1.90	79

Table 2. *Chemical degradation of radioactive ubiquinones synthesized from [1-¹⁴C]acetate and [2-¹⁴C]acetate: comparison of bacteria with fungi*

The radioactive samples of ubiquinone were degraded with KIO₄/KMnO₄ reagent to laevulinic acid, which was isolated with added carrier and degraded to iodoform and succinic acid.

Organism and precursor	Radioactivity (d.p.m.) in		
	Iodoform (A)	Succinic acid (B)	A/B
<i>A. vinelandii</i>			
[1- ¹⁴ C]Acetate	46	4200	0.01
[2- ¹⁴ C]Acetate	155	32 900	0.005
<i>E. coli</i>			
[1- ¹⁴ C]Acetate	195	31 500	0.006
[2- ¹⁴ C]Acetate	3650	207 000	0.018
<i>P. sesami</i>			
[1- ¹⁴ C]Acetate	140	8560	0.016
[2- ¹⁴ C]Acetate	420	84 500	0.005
<i>R. capsulata</i>			
[1- ¹⁴ C]Acetate	1700	117 400	0.014
[2- ¹⁴ C]Acetate	1580	150 000	0.011
<i>Penicillium chrysogenum</i>			
[1- ¹⁴ C]Acetate	156	18 900	0.008
[2- ¹⁴ C]Acetate	13 500	28 400	0.47
<i>Aspergillus niger</i>			
[1- ¹⁴ C]Acetate	256	46 200	0.005
[2- ¹⁴ C]Acetate	17 000	33 500	0.50
<i>Phycomyces blakesleeana</i>			
[1- ¹⁴ C]Acetate	40	4300	0.01
[2- ¹⁴ C]Acetate	4100	8000	0.5

the view that isoprenoids in these bacteria are not synthesized through the 'acetoacetate pathway'. The incorporation of C-2 of acetate in the observed position of the isoprene unit can be explained if the biosynthesis took place through the 'acetylactate

pathway', which is depicted in Fig. 1. The reactions of the pathway have been described as components of the pathways for biosynthesis and degradation of leucine (see Meister, 1965; Lehninger, 1975; Cunningham, 1978).

Table 3. Distribution of radioactivity in the carbon atoms of the laevulinic acid from ubiquinone synthesized from [^{14}C]acetate by bacteria.

An aliquot of ubiquinone, purified to constant specific radioactivity, was oxidized with a $\text{KMnO}_4/\text{NaIO}_4$ reagent to laevulinic acid. The latter was subjected to a modified Barbier–Wieland degradation (details given in the text) giving benzophenone, CO_2 and acetone. The radioactivity in each of these fragments was measured. This degradation method, in conjunction with the degradation of laevulinic acid to iodoform and succinic acid, provided the means for determining the radioactivity in each of the five carbon atoms of laevulinic acid.

Bacterium and tracer used	Radioactivity in the carbon atoms of laevulinic acid (% of the total five carbons)				
	C-1	C-2	C-3	C-4*	C-5
<i>A. vinelandii</i>					
[1- ^{14}C]Acetate	1.09	89.07	1.09	7.94	0.81
[2- ^{14}C]Acetate	0.49	95.41	0.39	3.34	0.39
<i>E. coli</i>					
[1- ^{14}C]Acetate	0.61	97.70	0.30	1.09	0.30
[2- ^{14}C]Acetate	0.18	90.43	0.11	9.16	0.12
<i>P. sesami</i>					
[1- ^{14}C]Acetate	1.61	84.40	1.61	11.31	1.07
[2- ^{14}C]Acetate	0.47	92.70	0.27	6.29	0.27
<i>R. capsulata</i>					
[1- ^{14}C]Acetate	1.44	93.67	1.44	2.73	0.72
[2- ^{14}C]Acetate	1.05	95.07	0.70	2.72	0.46

* The radioactivity in C-4 is a maximum limit (see the text).

Fig. 1. 'Acetolactate pathway'

The carbon atoms of acetate are marked to show their fate through the pathway.

Although the 'acetolactate pathway' explains the incorporation of C-2 of acetate in the observed position, it cannot, at first sight, explain the

incorporation of C-1 of acetate, which would be eliminated as CO_2 . However, a mechanism is known for the incorporation of CO_2 into isoprenoids, as

Fig. 2. Incorporation of CO_2 into isopentenyl pyrophosphate

depicted in Fig. 2 (see Goodwin, 1963; Braithwaite & Goodwin, 1960; Chichester *et al.*, 1959). If C-1 of acetate were converted first to CO_2 and then incorporated into isopentenyl pyrophosphate, as described in Fig. 2, it would ultimately be found at C-2 of laevulinic acid, and not at C-4, unlike in the 'acetoacetate pathway'. The pattern seen in the degradation studies (Table 3) is in agreement with the 'acetolactate pathway'.

Though the ratio of incorporation of C-1 of acetate to that of C-2 into the isoprene unit of the ubiquinone side-chain of *R. capsulata* was nearly 2:3, the pattern of radioactivity in the isoprene unit is in accordance with the 'acetolactate pathway' (Table 3). In view of the comparable effectiveness of incorporation of both $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]$ acetate in *R. capsulata*, we felt that this bacterium would manifest more clearly than the others any deviation from the well-known 'acetoacetate pathway' in the biosynthesis of ubiquinones. Accordingly, we chose this bacterium for further tests of the postulated 'acetolactate pathway'.

Incorporation of radioactivity from $[U\text{-}^{14}\text{C}]$ alanine, $[U\text{-}^{14}\text{C}]$ valine and $\text{NaH}^{14}\text{CO}_3$

Pyruvate and 2-oxoisovalerate (2-oxo-3-methylbutanoic acid) are two of the intermediates of the 'acetolactate pathway'. Therefore, further tests of the occurrence of the 'acetolactate pathway' were carried out using $[U\text{-}^{14}\text{C}]$ alanine and $[U\text{-}^{14}\text{C}]$ valine which would be converted metabolically to $[U\text{-}^{14}\text{C}]$ pyruvate and 2-oxo $[U\text{-}^{14}\text{C}]$ isovalerate respectively. The 'acetolactate pathway' (Fig. 1) along with the pathway depicted in Fig. 2, also provides a mechanism for the incorporation of carbon atoms of CO_2 . 3-Methylcrotonyl-CoA (see Fig. 1) with which CO_2 reacts in the 'acetolactate pathway' is produced from 2-oxo-4-methylpentanoic acid which in turn could rise from L-leucine. Therefore, addition of L-leucine to the bacterial cultures might cause increased incorporation of the carbon atom of CO_2 . The results of the experiments with $[U\text{-}^{14}\text{C}]$ alanine, $[U\text{-}^{14}\text{C}]$ valine and $\text{NaH}^{14}\text{CO}_3$ (with and without the addition of L-leucine) are shown in Table 4. Radioactivity from all the three precursors was

Table 4. Incorporation of radioactivity from [U-¹⁴C]alanine, [U-¹⁴C]valine and NaH¹⁴CO₃ into ubiquinone of *R. capsulata*

The experiments are described in the text and in the legend to Table 1.

Additions to the medium	Wet weight of cells (g)	Amount of ubiquinone (μmol)	Specific radioactivity of ubiquinone (d.p.m./nmol)
[U- ¹⁴ C]Alanine (20 μCi; 0.3 μmol)	3.3	2.40	59.6
[U- ¹⁴ C]Valine (20 μCi; 0.8 μmol)	3.3	2.54	36.0
NaH ¹⁴ CO ₃ (100 μCi; 2.07 μmol)	3.1	2.03	18.3
NaH ¹⁴ CO ₃ (100 μCi; 2.07 μmol) + L-leucine (100 mg/l)	3.2	1.97	25.0

Table 5. Distribution of radioactivity in the carbon atoms of laevulinic acid from ubiquinone synthesized from [U-¹⁴C]alanine, [U-¹⁴C]valine and NaH¹⁴CO₃ by *R. capsulata*

The experiments have been described in the text as well as in the legend to Table 3.

Additions to medium	Radioactivity in the carbon atoms of laevulinic acid (% of the total of five carbons)				
	C-1	C-2	C-3	C-4	C-5
[U- ¹⁴ C]Alanine (20 μCi)	24.35	0.61	23.73	27.58	23.73
[U- ¹⁴ C]Valine (20 μCi)	25.43	1.10	24.68	24.24	24.55
NaH ¹⁴ CO ₃ (100 μCi)	0.89	93.17	1.78	2.98	1.18
NaH ¹⁴ CO ₃ (100 μCi) + L-leucine	0.87	94.00	1.12	3.26	0.75

incorporated into ubiquinone; incorporation from NaH¹⁴CO₃ was increased when unlabelled L-leucine was concomitantly added.

The results of degradation of the ubiquinone samples synthesized from [U-¹⁴C]alanine, [U-¹⁴C]valine and NaH¹⁴CO₃ are shown in Table 5. When [U-¹⁴C]alanine or [U-¹⁴C]valine was the precursor, radioactivity was distributed equally among C-1, C-3, C-4 and C-5 of laevulinic acid, with negligible radioactivity in C-2. Radioactivity from NaH¹⁴CO₃ was incorporated almost exclusively into C-2 of laevulinic acid. These results are fully in accordance with the 'acetolactate pathway'. On the other hand, if the 'acetoacetate pathway' were operative, radioactivity from [U-¹⁴C]alanine could be incorporated only after its conversion to acetyl-CoA (through pyruvate) and therefore, all the carbon atoms of the isoprene unit would have been equally radioactive. The lack of radioactivity in C-2 of laevulinic acid when [U-¹⁴C]alanine or [U-¹⁴C]valine are used as the precursor suggests that the 'acetoacetate pathway' is not operative and that any [¹⁴C]acetyl-CoA that may have been produced from [U-¹⁴C]alanine through pyruvic acid has been diluted out. It was suggested above that C-1 of acetate could be incorporated after its conversion to CO₂ and would be found as C-2 of laevulinic acid. The observed incorporation of radioactivity from NaH¹⁴CO₃ into C-2 of laevulinic acid (Table 5) is in agreement with this suggestion.

The relatively high efficiency of *R. capsulata* in

incorporating C-1 of acetate, despite the absence of the 'acetoacetate pathway', could be due to the fact that culture conditions for this bacterium do not permit CO₂ to be lost freely to the atmosphere. In contrast, bacteria such as *A. vinelandii* are grown with vigorous aeration, leading to loss of CO₂, and hence, to lower incorporation of C-1 of acetate. An earlier finding of the lack of detectable incorporation of radioactivity from [1-¹⁴C]acetate into ubiquinone of some bacteria (Raman *et al.*, 1965) can be explained, among other things, through these considerations.

It is conceivable that part of the incorporation from C-2 of acetate is also through CO₂, since its position in the isoprene unit would be the same whether it be incorporated directly through the 'acetolactate pathway' or after conversion to CO₂. Some reactions of the 'acetolactate pathway' might be operative also in *P. blakesleeanus*, a fungus, which has been shown to incorporate radioactivity from ¹⁴CO₂ in the presence of leucine (Braithwaite & Goodwin, 1960; Chichester *et al.*, 1959).

The proposed mechanism for the incorporation of the carbon atom of CO₂ requires the formation of acetoacetyl-CoA as an intermediate. At first sight, therefore, the suggestion that acetoacetyl CoA is not formed directly from acetate in the pathway to isoprenoids is not compatible with the observation that the carbon atom of CO₂ is incorporated. The formation of acetoacetyl-CoA from two molecules of acetyl-CoA is, however, thermodynamically not

favoured (see Mahler & Cordes, 1966). The 'activated' acetoacetate utilized in fatty acid synthesis, on the other hand, is its thioester with acyl carrier protein, and is synthesized from acetyl- and malonyl-acyl carrier proteins. Although a similar pathway that utilizes malonate for the synthesis of mevalonate has been described (Brodie *et al.*, 1963), more recent work (Higgins & Kekwick, 1973) has shown that this 'malonyl pathway' is of no quantitative importance for mevalonate synthesis in animals. In bacteria too, it is plausible that the 'malonyl pathway' is not functional for mevalonate synthesis.

We also have preliminary evidence that the 'acetoacetate pathway' is not operative in *Lactobacillus casei* (a Gram-positive bacterium) for the biosynthesis of 'bactoprenol', a linear polyprenoid alcohol; its synthesis apparently takes place through the 'acetolactate pathway'. Since this bacterium utilizes mevalonate for isoprenoid synthesis (Thorne & Kodicek, 1966) it appears that the 'acetolactate pathway' also goes through mevalonate, although previous work (Raman *et al.*, 1965, 1969) has indicated that free mevalonate might not be an intermediate in Gram-negative bacteria.

In conclusion, it appears that the bacteria we have studied synthesize the isoprene unit through the 'acetolactate pathway'. However, De Rosa *et al.* (1977) have shown that the patterns of incorporation of ^{13}C from $[1-^{13}\text{C}]\text{acetate}$ and $[2-^{13}\text{C}]\text{-acetate}$ into the isoprenoid side-chain of caldariellaquinone of *Caldariella acidophila*, an extreme thermoacidophile bacterium, are consistent with the operation of the 'acetoacetate pathway'. Thus, it cannot be concluded that all bacteria synthesize isoprenoids through the 'acetolactate pathway'. It is, however, obvious that the postulated alternative pathway would have a bearing on the regulation of isoprenoid biosynthesis.

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