In the experiments described above, signals from extracellular metabolites which have diffused through the cell membrane are being observed. Therefore, the rate of formation of each metabolite observed by the present method may also be affected, at least to some extent, by the rate of diffusion of the metabolites through the cell membrane. With a more concentrated cell suspension and/or a large bore NMR tube, it may be possible to observe intracellular metabolites separately, and to determine the intracellular pH by using the chemical shifts of these metabolites (Navon et al., 1977).

It has been noticed that several small signals exist in the low-field NH and aromatic proton region. These signals may also give further information about the anaerobic metabolism of *E. coli* cells under investigation.

References

Arata, Y., & Ozawa, H. (1976) *J. Magn. Reson. 21,* 67.

Arata, Y., Ozawa, H., Ogino, T., & Fujiwara, S. (1978) *Pure Appl. Chem.* (in press).

Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13,* 243.

Daniels, A., Korda, A., Tanswell, P., Williams, A., & Williams, R. J. P. (1974) *Proc. R. Soc. London, Ser. B 187,* 353.

Daniels, A., Williams, R. J. P., & Wright, C. F. (1976) *Nature (London) 261,* 321.

Dwek, R. A., Campbell, I. D., Richards, R. E., & Williams, R. J. P., Eds. (1977) *NMR in Biology,* Academic Press, New York, N.Y.

Eakin, R. T., Morgan, L. O., Gregg, C. T., & Matwiyoff, N. A. (1972) *FEBS Lett. 28,* 259.

Gupta, R. K., Ferretti, J. A., & Becker, E. D. (1974) *J. Magn. Reson. 13,* 275.

Kainosho, M., Ajisaka, K., & Nakazawa, H. (1977) *FEBS Lett. 80,* 385.

Metzler, D. E. (1977) *Biochemistry: The Chemical Reactions of Living Cells,* Academic Press, New York, N.Y.

Moon, R. B., & Richards, J. H. (1973) *J. Biol. Chem. 248,* 7276.

Navon, G., Ogawa, S., Shulman, R. G., & Yamane, T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 888.

Ogino, T., Arata, Y., Fujiwara, S., Shoun, H., & Beppu, T. (1978) *J. Magn. Reson.* (in press).

Stokes, J. L. (1949) *J. Bacteriol. 57,* 147.

Wood, W. A. (1952) *Bacteria 2,* 59.

Three Hydroxylations Incorporating Molecular Oxygen in the Aerobic Biosynthesis of Ubiquinone in *Escherichia coli*†

Ken Alexander and Ian G. Young*

ABSTRACT: The biosynthetic origin of the oxygen atoms of ubiquinone 8 from aerobically grown *Escherichia coli* was studied by ^{18}O labeling. An apparatus was developed which allowed the growth of cells under a defined atmosphere. Mass spectral analysis of ubiquinone 8 from cells grown under highly enriched $^{18}O_2$ showed that three oxygen atoms of the quinone are derived from molecular oxygen. It was established that the molecular oxygen is incorporated into the two methoxyl groups (at C-5 and C-6) and one of the carbonyl positions of the ubiquinone molecule by demonstrating that only one of the incorporated oxygens will exchange with water under acidic conditions that specifically catalyze the exchange of carbonyl, but not methoxyl, oxygens. That the C-4 carbonyl oxygen is derived from molecular oxygen was shown by the incorporation of three atoms of $^{18}O_2$ into ubiquinone 8 biosynthesized from added 4-hydroxybenzoic acid. Comparison of ubiquinone 8 and menaquinone 8 from *E. coli* grown under $^{18}O_2$ confirmed that the labeled carbonyl oxygen of the [$^{18}O_2$]ubiquinone 8 is incorporated biosynthetically and not by chemical exchange in the cell. It is concluded that the three hydroxylation reactions involved in the pathway for the aerobic biosynthesis of ubiquinone are all catalyzed by monooxygenases. The implications of this study for the anaerobic biosynthesis of ubiquinone 8 in *E. coli* are discussed.

Ubiquinone is well established as a component of the respiratory chain of mitochondria (Crane & Sun, 1972) and also functions in respiration in many microorganisms (Haddock & Jones, 1977; Jones, 1977). The biosynthetic pathway to ubiquinone (IX) from 4-hydroxybenzoate (I) involves three hydroxylation reactions (Figure 1) (Young et al., 1973). Two of the hydroxyl groups are subsequently methylated and become the C-5 and C-6 ring methoxyl groups of ubiquinone, and the third hydroxyl forms the C-4 carbonyl of the quinone ring. The origin of these three oxygens has received comparatively little attention. It has been shown using ^{18}O labeling that molecular oxygen is incorporated into the two-ring methoxyl groups in the case of the aerobe *Pseudomonas desmolytica* (Uchida & Aida, 1972). However, no labeling of the quinone carbonyl was detected, suggesting that this oxygen might arise from water.

In the case of *E. coli,* which is a facultative anaerobe, the origin of the three hydroxyl groups has not previously been studied. We have recently found that strains of *E. coli* K12 produce up to 70% of the aerobic levels of ubiquinone when grown anaerobically with fumarate as electron acceptor (Alexander & Young, 1978). This finding suggested that *E. coli* is able to synthesize ubiquinone by an anaerobic mechanism and raised the question as to the origin of the three oxygen atoms under aerobic conditions. In the present paper, we show that under aerobic conditions each of the three oxygen atoms is derived from molecular oxygen.

† From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia. *Received June 16, 1978.*

0006-2960/78/0417-4745$01.00/0

Experimental Section

Bacterial Strains. Two isogenic derivatives of *E. coli* K12, AB3311 (Hfr, *metB*) and AN164 (Hfr, *metB, aroB, rpsL*) were used.

Media. The mineral salts medium used has been described previously (Stroobant et al., 1972). For the growth of AB3311, the basal medium was supplemented with sodium succinate (50 mM), potassium bicarbonate (20 mM), thiamin hydrochloride (3 μM), L-methionine (0.15 mM), and casamino acids (0.1%). The medium used for the growth of AN164 was further supplemented with 4-hydroxybenzoic acid (0.1 mM), L-phenylalanine (0.2 mM), L-tyrosine (0.2 mM), L-tryptophan (0.2 mM), 4-aminobenzoic acid (1 μM), and 2,3-dihydroxybenzoic acid (10 μM).

Growth of Cells. Cells were grown in 1-L quantities in a baffled 2-L conical flask designed to permit growth on sterile media under a defined atmosphere. Access to the inverted flask for the evacuation and introduction of gases and liquids was provided through two narrow bored glass tubes with vacuum taps and one tube fitted with a rubber septum. The tubes were attached to the top of the flask and reached within 3 cm of the base such that when the flask was turned upright for incubation the ends of the tubes were below the level of the medium, preventing any possible leakage of the defined atmosphere through the taps. The minimal medium was autoclaved in a separate vessel and cooled under nitrogen–carbon dioxide (95:5), and the sterile supplements were added. The medium was then pumped into the sterile inverted culture vessel followed by the inoculum, which consisted of cells grown aerobically on succinate minimal medium. Throughout this operation the culture flask was continuously gassed with nitrogen–carbon dioxide (95:5). The vessel was then partially evacuated and placed upright, and 300 mL of gas (either $^{16}O_2$ or $^{18}O_2$) was introduced. The culture flask was then shaken at 37 °C until growth had ceased. The yield of cells obtained was 1.25 g wet weight. Cultures were checked for purity to ensure that no contamination or reversion had occurred.

Nitrogen Gas. Oxygen-free gas containing 95% nitrogen and 5% carbon dioxide was obtained from Commonwealth Industrial Gases Ltd. and was purified of any residual traces of oxygen by bubbling through Fieser's solution (Fieser, 1924).

$^{18}O_2$ and $H_2{}^{18}O$. The $^{18}O_2$ (^{18}O enrichment = 99%) used for the biosynthetic labeling studies and the $H_2{}^{18}O$ (^{18}O enrichment = 50%) employed in the acid-catalyzed exchange reaction with [^{16}O]ubiquinone 8 were purchased from Norsk Hydro. The $H_2{}^{18}O$ was diluted with aqueous 2 N HCl for the exchange reaction to give a final ^{18}O enrichment of 46.3% in the reaction mixture.

Isolation of Biosynthetic Octaprenylquinones. Cells were harvested at 0–4 °C and extracted under nitrogen by the Soxhlet procedure as described previously (Young et al., 1973), and the mass spectrum of a portion of the lipid extract, containing the quinones in their reduced form as quinols, was measured. The remainder of the extract was chromatographed on silica gel thin-layer plates using chloroform–light petroleum (60–80 °C) (7:3, v/v) as solvent. The dark-yellow band containing ubiquinone 8 (R_f 0.4) and the pale-yellow menaquinone 8 band (R_f 0.7) were each eluted with ethanol and their mass spectra determined. Concentrations of the isolated quinones were estimated spectrophotometrically (Crane & Barr, 1971; Dunphy & Brodie, 1971; Baum & Dolin, 1965).

Acid-Catalyzed Exchange. The reaction was performed essentially according to the method of Uchida & Aida (1972). The reaction mixture consisted of 20 μL of $H_2{}^{18}O$ (or $H_2{}^{16}O$) and 50 μg of [^{16}O]ubiquinone 8 (or [^{18}O]ubiquinone 8) in 200 μL of ethanol containing 2 μL of 2 N HCl (aqueous). After 5 h at 85 °C under vacuum in a sealed ampule, the mixture was extracted with light petroleum and the mass spectrum of the recovered ubiquinone 8 was measured.

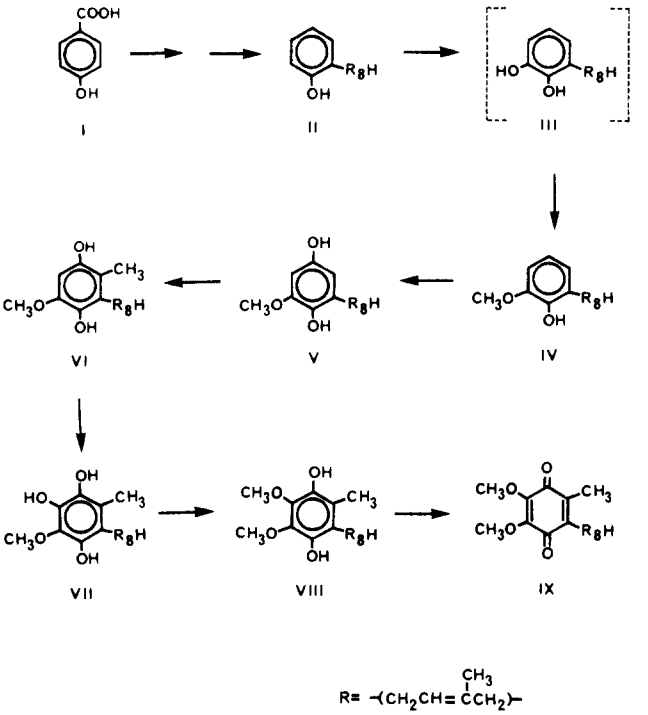

FIGURE 1: Scheme for ubiquinone biosynthesis in *E. coli* showing the three hydroxylation reactions. I, 4-hydroxybenzoic acid; II, 2-octaprenylphenol; III, 2-octaprenyl-6-hydroxyphenol (hypothetical intermediate) (Young et al., 1973); IV, 2-octaprenyl-6-methoxyphenol; V, 2-octaprenyl-6-methoxy-1,4-benzoquinol; VI, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol; VII, 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol; VIII, ubiquinol 8; IX, ubiquinone 8.

Isotope Exchange during Chromatography. To monitor the effects of chromatography on labeled oxygen atoms of ubiquinone, samples of purified biosynthetic [^{18}O]ubiquinone 8 or of ubiquinone 8 labeled exclusively in either the carbonyl or methoxy oxygens were chromatographed on silica gel thin-layer plates. Chloroform–light petroleum (7:3, v/v) was used as solvent and the ubiquinone 8 was eluted with ethanol. Mass spectra of samples were measured before and after chromatography. Ubiquinone 8 was labeled in the two carbonyl oxygens at C-1 and C-4 by acid-catalyzed exchange with $H_2{}^{18}O$. Ubiquinone 8 was labeled exclusively in the two methoxyl oxygens at C-5 and C-6 by aerobic biosynthesis under $^{18}O_2$, followed by acid-catalyzed exchange of the biosynthetic [^{18}O]ubiquinone 8 with $H_2{}^{16}O$.

Spectroscopy. Mass spectra were measured using an AEI MS9 double-focus mass spectrometer with a direct insertion probe. The spectrometer was operated at 70 eV with an electron current of 100 μA, an accelerating voltage of 8 kV and an ion source temperature of 210 °C. Ultraviolet spectra were measured on a Cary 14 spectrophotometer.

Results

Incorporation of Three Atoms of $^{18}O_2$ into Ubiquinone. ^{18}O labeling experiments were carried out to determine how many of the three oxygen atoms incorporated into ubiquinone (IX) during its biosynthesis from 4-hydroxybenzoate (I) are derived from molecular oxygen. A special culture flask was used which permitted the growth of *E. coli* under a defined atmosphere (see Experimental Section). Aerobic growth under these conditions was limited by the amount of oxygen available in

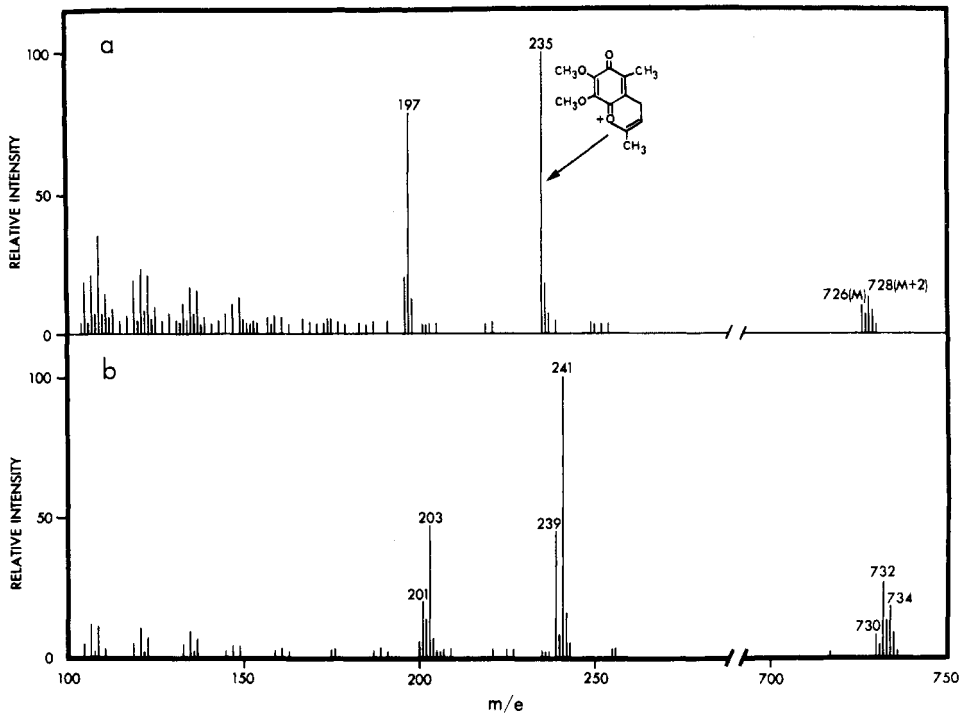

FIGURE 2: Mass spectra of ubiquinone from *E. coli* (strain AB3311). Cells were grown on succinate in the presence of: (a) limiting $^{16}O_2$; (b) limiting $^{18}O_2$ (99% enriched). Peaks with relative intensities less than 2% are not shown.

TABLE I: Octaprenylquinones Synthesized by *E. coli* (Strain AB3311) in the Presence of Limiting $^{18}O_2$.

	ubiquinone 8	menaquinone 8
level formed[a]	298	34
measured mass of molecular ion	732.5711	716.5521
assignment	$C_{49}H_{74}{}^{16}O^{18}O_3$	$C_{51}H_{72}{}^{16}O_2$
required mass	732.5714	716.5532

[a] Nanomoles per gram wet weight of cells.

the closed system. Further growth under anaerobic conditions was prevented by using succinate as sole carbon source. With succinate as carbon source, the growth is strictly proportional to the amount of oxygen present (data not shown). Experiments were carried out on a small scale using highly enriched $^{18}O_2$ to improve the sensitivity of ^{18}O labeling in case any exchange occurred during isolation and purification of the quinone (Samuel, 1962).

The incorporation of $^{18}O_2$ into ubiquinone was assessed by isolating the quinone from cells of strain AB 3311 grown under an $^{18}O_2$ atmosphere and examining it by mass spectrometry. The mass spectra of ubiquinones (Muraca et al., 1967; Morimoto et al., 1967) have a small parent molecular ion peak (M) and an intense peak at *m/e* 235, due to the formation of a highly stabilized pyrylium ion. This fragment, the 2,5-dimethyl-7,8-dimethoxy-6-cyclohexadienone-1-pyrylium ion, contains the four nuclear oxygen atoms of the parent ubiquinone molecule. Additional features of the spectrum include an M + 2 peak assigned to the formation of the hydroquinone species and a peak at *m/e* 197 due to a benzyl ion fragment. The extent of hydroquinone formation and the ratio of intensities of the M/M + 2 peaks are variable functions of residual moisture in the spectrometer (Heiss et al., 1969), and quantitative estimations of ^{18}O incorporation into ubiquinone are most conveniently determined by analysis of the pyrylium ion region of spectra.

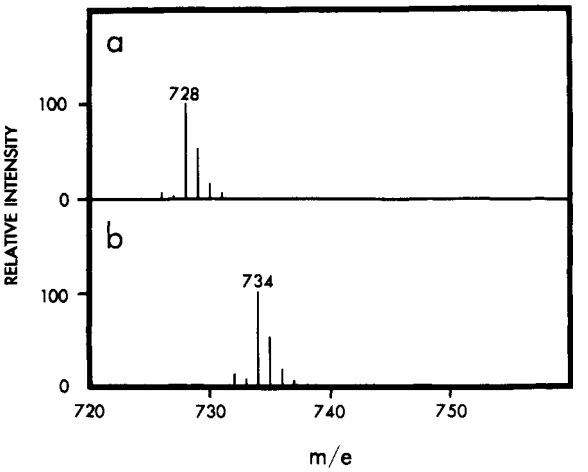

FIGURE 3: Molecular ion regions of mass spectra of ubiquinol from cells grown in the presence of: (a) limiting $^{16}O_2$; (b) limiting $^{18}O_2$ (99% enriched). Mass spectra were measured directly on crude extracts from *E. coli* (strain AB3311), without chromatographic purification of the extracted ubiquinol.

The mass spectrum of ubiquinone biosynthesized aerobically by *E. coli* was characteristic of ubiquinone 8 (Muraca et al., 1967; Morimoto et al., 1967) having a parent molecular ion peak at *m/e* 726 (M) and a base peak at *m/e* 235 (Figure 2a). The original peaks of [^{16}O]ubiquinone 8 at *m/e* 197, 235, 726 (M), and 728 (M + 2) (Figure 2a) were each replaced in the mass spectrum of ubiquinone 8 biosynthesized in the presence of $^{18}O_2$ by prominent new peaks of 6 additional mass units, and by minor contributions of 4 additional mass units (Figure 2b). This increase in molecular weight of the parent ion and its principal fragments is due to the incorporation of $^{18}O_2$ into the biosynthesized ubiquinone 8. Analysis of the peak at *m/e* 732 under high resolution established the presence of the parent ion (M) of a molecule of ubiquinone 8 containing three atoms of ^{18}O, with an elemental composition of $C_{49}H_{74}{}^{16}O^{18}O_3$ (Table I). The minor peak at *m/e* 730 (Figure 2b) implied the

TABLE II: Comparison of Pyrylium Ions from Mass Spectra of Ubiquinone 8 Synthesized by *E. coli* (Strain AB3311) in the Presence of either $^{16}O_2$ or $^{18}O_2$.[a]

	rel intensities of peaks[b]			Theor values for incorp of ^{18}O atoms	
		ubiquinone synth under $^{18}O_2$			
m/e	[^{16}O]ubiquinone	chromatogr once	chromatogr twice	2 ^{18}O atoms[c]	3 ^{18}O atoms[d]
235	100	2.5	2.5	0	0
+2	0	2.0	4.0	2.0	0
+4	4.5	46.0	66.0	100	3.0
+6	2.0	100	100	0	100
+8	1.0	0	2.0	0	0

[a] ^{18}O enrichment = 99%. [b] To enable comparison of the observed peaks with theoretical values, it is necessary to remove the contributions at peaks 2 mass units above the pyrylium ions due to natural isotopic abundance and unrelated fragments. The data have been corrected for these minor contributions on the basis of the intensities observed with [^{16}O]ubiquinone (*m/e* 235, 100; *m/e* 237, 7.0). [c] M; M + 2; M + 4 = 1^2; $1 \times 99 \times 2$; 99^2. [d] M; M + 2; M + 4; M + 6 = 1^3; $1^2 \times 99 \times 3$; $1 \times 99^2 \times 3$; 99^3.

TABLE III: Chemical Exchange of Carbonyl Oxygens of Ubiquinone 8. Comparison of Pyrylium Ions from Mass Spectra of [^{16}O]Ubiquinone, before and after Acid-Catalyzed Exchange with $H_2{}^{18}O$.[a]

	rel intensities of peaks[b]		theor values for incorp of 2 ^{18}O atoms[c]
m/e	before react.	after react.	
235	100	58.5	58.0
+2	0	100	100
+4	4.5	37.5	43.0
+6	2.0	4.5	0
+8	1.0	2.5	0

[a] ^{18}O enrichment = 46.3%. [b] Corrected (see Table II). [c] M; M + 2; M + 4 = 53.7^2; $53.7 \times 46.3 \times 2$; 46.3^2.

TABLE IV: Acid-Catalyzed Exchange of Biosynthetic [^{18}O]Ubiquinone 8[a] with $H_2{}^{16}O$.

	rel intensities of pyrylium ion pks[b]		theor values for presence of 2 ^{18}O atoms[c]
m/e	before react.	after react.	
235	2.5	2.0	0
+2	2.0	2.5	2.0
+4	46.0	100	100
+6	100	0.5	0
+8	0	1.0	0

[a] Biosynthesized in the presence of 99% enriched $^{18}O_2$ (cf. Table II). [b] Corrected (see Table II). [c] M; M + 2; M + 4 = 1^2; $1 \times 99 \times 2$; 99^2.

additional presence of a small quantity of ubiquinone 8 containing two atoms of ^{18}O. This was clarified by an analysis of the region of the spectrum corresponding to the pyrylium ion fragment of ubiquinone 8. Comparison of the relative intensities of the peaks associated with this fragment revealed that 70% of the biosynthesized ubiquinone 8 had incorporated three atoms of ^{18}O, while the remainder contained two labeled oxygens (Table II). This was evidently the result of a partial loss of the third ^{18}O atom by chemical exchange during the chromatographic purification of the biosynthesized [^{18}O]-ubiquinone 8, since a second chromatography of the isolated quinone led to a further loss of the third ^{18}O atom (Table II). That three atoms of ^{18}O per molecule were present prior to any purification was confirmed by comparing the molecular ion regions of the mass spectra of crude extracts from cells grown in the presence of either $^{16}O_2$ or $^{18}O_2$ (Figure 3). The peak at *m/e* 728 assigned to the molecular ion of [^{16}O]ubiquinol 8 is replaced by a single new peak at *m/e* 734 due to the incorporation of three atoms of ^{18}O.

It is clear from these experiments that chromatography of the crude extract on silica gel results in oxidation of the ubiquinol 8 to ubiquinone 8 and is accompanied by a partial exchange of one of the three incorporated ^{18}O atoms.

Location of the Three Atoms Derived from Oxygen. The nuclear locations of the three oxygen atoms incorporated during the biosynthesis of ubiquinone 8 were investigated by their capacity to exchange with water. Under acidic conditions the carbonyl oxygen atoms of ubiquinone exchange readily with water, whereas the methoxyl oxygens do not (Uchida & Aida, 1972). Thus, treatment of [^{16}O]ubiquinone 8 in ethanol with $H_2{}^{18}O$ in acid solution led to an almost complete exchange of the two carbonyl oxygens (Table III). A sample of the [^{18}O]ubiquinone that had been biosynthesized in the presence of molecular $^{18}O_2$ (Figure 2b) was allowed to undergo chemical exchange with $H_2{}^{16}O$ under the conditions used by Uchida & Aida (1972). The mass spectrum of the product was characteristic of a ubiquinone 8 molecule containing only two atoms of $^{18}O_2$, with a parent molecular ion peak at *m/e* 730 (M) (Figure 4). Detailed examination of the pyrylium ion region of this spectrum showed the disappearance of any contribution from a third ^{18}O atom at *m/e* 241 (Table IV). There was a corresponding increase in intensity of the peak at *m/e* 239 due to the presence of two atoms of ^{18}O in the exchanged ubiquinone 8 product. The loss of only one atom of ^{18}O during the acid-catalyzed exchange corresponds to the loss of one carbonyl ^{18}O atom and the retention of two labeled methoxyl oxygens. It is concluded that both of the methoxyl oxygens at C-5 and C-6 and one of the carbonyl oxygen atoms of ubiquinone 8 are derived from molecular oxygen, under conditions of aerobic biosynthesis.

In a separate comparative study using [^{18}O]ubiquinone 8, labeled exclusively in either the methoxyl or carbonyl positions, it was established that oxygens in the carbonyl but not the methoxyl groups are also partially exchanged during chromatography (Table V). Thus, the loss of a proportion of the label from one of the ^{18}O atoms that was observed during the chromatography of the biosynthesized [^{18}O]ubiquinone 8 (Table II) can be attributed to a partial exchange of the labeled carbonyl oxygen.

Identification of the Labeled Carbonyl Oxygen. In order to define which of the quinone carbonyls carried the ^{18}O label an *aroB* derivative of AB3311 (strain AN164) was used. This strain is completely blocked in the common pathway of aromatic biosynthesis and is therefore unable to synthesize any of the aromatic end products, including ubiquinone. It was grown under $^{18}O_2$ in the presence of L-phenylalanine, L-tyro-

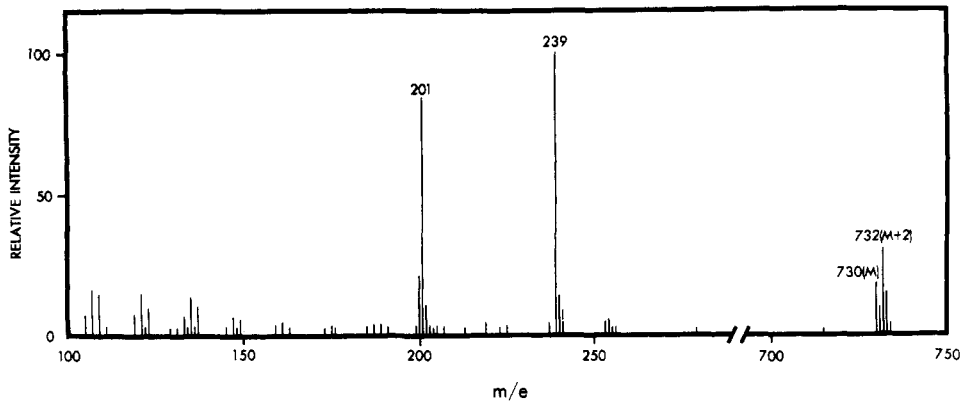

FIGURE 4: Mass spectrum of biosynthetic [^{18}O]ubiquinone after acid-catalyzed exchange with $H_2{}^{16}O$. Peaks with relative intensities less than 2% are not shown.

sine, L-tryptophan, 4-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and an excess of 4-hydroxybenzoic acid (I). Under these conditions, no ^{18}O labeling of the C-1 carbonyl oxygen of ubiquinone 8 (IX) can occur. Examination of the molecular ion region of the mass spectrum of a crude extract from cells grown in the presence of $^{18}O_2$ indicated that three atoms of molecular oxygen had been incorporated. This was confirmed by the mass spectrum of a chromatographically purified sample of the synthesized [^{18}O]ubiquinone 8. High-resolution mass measurement showed the presence of a molecular ion (M) with a measured mass of 732.5711 ($C_{49}H_{74}{}^{16}O^{18}O_3$ requires 732.5714). This, taken together with the exchange data described above, establishes that molecular oxygen is incorporated into the C-4 carbonyl of the quinone ring.

Biosynthesis of Menaquinone in the Presence of $^{18}O_2$. Under aerobic conditions, menaquinone 8 is a minor biosynthetic product of *E. coli* (Wallace & Young, 1977; Whistance et al., 1969). Unlike the case for ubiquinone 8, the pathway for the biosynthesis of menaquinone 8 does not involve any hydroxylation reactions (Young, 1975). The latter quinone was therefore employed as an internal control to confirm that carbonyl oxygens of biosynthetic octaprenylquinones do not incorporate any label by chemical exchange in vivo during the growth of strain AB 3311 under $^{18}O_2$. In agreement with other workers (Snyder and Rapoport, 1970), we found no incorporation of $^{18}O_2$ into the two carbonyl groups of menaquinone 8. Accurate mass measurement of the molecular ion peak of the biosynthesized naphthoquinone established that both of its carbonyl groups contained only ^{16}O (Table I). This, taken in conjunction with our finding that one of the carbonyl oxygens of ubiquinone 8 also remains unlabeled, provides strong evidence that the in vivo incorporation of an atom of $^{18}O_2$ into the remaining carbonyl oxygen of ubiquinone 8 occurs biosynthetically and not by chemical exchange in the cell.

TABLE V: Effect of Chromatography on Isotopically Labeled Ubiquinone 8.

	rel intensities of pyrylium ion peaks[a]			
	[1,4-^{18}O]carbonyl-labeled ubiquinone[b]		[5,6-^{18}O]methoxy-labeled ubiquinone[c]	
m/e	before chromatogr	after chromatogr	before chromatogr	after chromatogr
235	58.5	100	2.0	2.0
+2	100	90.0	2.5	4.5
+4	37.5	22.5	100	100
+6	4.5	2.0	0.5	2.0
+8	2.5	2.0	1.0	1.0

[a] Corrected (see Table II). [b] Both carbonyl oxygens labeled by acid-catalyzed exchange with 46.3% enriched $H_2{}^{18}O$ (cf. Table III). [c] Labeled exclusively in the two methoxyl oxygens by a combination of biosynthetic and chemical methods (see Experimental Section and Table IV; ^{18}O enrichment of methoxyl oxygens = 99%).

Discussion

The results presented in this paper clearly demonstrate that under aerobic conditions the oxygens of the two methoxyl groups and the C-4 carbonyl group of ubiquinone 8 are derived from molecular oxygen. It is concluded that the three hydroxylation reactions involved in the pathway for the aerobic biosynthesis of ubiquinone (Figure 1) are all catalyzed by monooxygenases.

A previous study of ubiquinone biosynthesis in *Pseudomonas desmolytica* reported the incorporation of $^{18}O_2$ of relatively low enrichment into ubiquinone 9, but only into the two methoxyl positions (Uchida and Aida, 1972). The failure to detect any ^{18}O label in a carbonyl oxygen of the chromatographically purified ubiquinone 9 is likely to have been due to the susceptibility of such atoms to chemical exchange during chromatography. We have found that the carbonyl oxygen atoms of ubiquinone 8 are partially labile during chromatography, and only the methoxyl oxygens do not undergo any exchange. Ubiquinone is widely distributed in nature and is synthesized in animals, fungi, plants, and many bacteria (Ramasarma, 1968; Bentley, 1975). It seems likely that the incorporation of molecular oxygen into ubiquinone at positions C-4, C-5, and C-6 is not unique to *E. coli* but is common to other organisms which synthesize ubiquinone aerobically.

E. coli can also synthesize ubiquinone when growing under strictly anaerobic conditions (Alexander and Young, 1978). The involvement of monooxygenases in the three hydroxylation reactions of the aerobic pathway (Figure 1) implies that these reactions must be catalyzed by alternative enzymes under anaerobic conditions. In the following paper of this issue (Alexander and Young, 1978) an examination of mutants blocked in the various reactions of the aerobic pathway has shown that the three hydroxylation reactions described above are duplicated under anaerobic conditions, allowing ubiquinone synthesis to occur in the absence of molecular oxygen.

Acknowledgments

Mrs. D. Marsden is thanked for excellent technical assistance. We are grateful to Mr. T. Davies of the Research School of Chemistry, Australian National University, for the measurement of mass spectra. Dr. J. K. MacLeod of the Research School of Chemistry, Australian National University, and Dr.

C. B. Osmond of the Research School of Biological Sciences are thanked for helpful discussions.

References

Alexander, K., & Young, I. G. (1978) *Biochemistry 17* (following paper in this issue).
Baum, R. H., & Dolin, M. I. (1965) *J. Biol. Chem. 240*, 3425.
Bentley, R. (1975) *Biosynthesis 3*, 181.
Crane, F. L., & Barr, R. (1971) *Methods Enzymol. 18C*, 137.
Crane, F. L., & Sun, F. F. (1972) in Electron and Coupled Energy Transfer in Biological Systems, Vol. 1B, King, T. E., and Klingenberg, M., Ed., New York, N.Y., Marcel Dekker, p 477.
Dunphy, P. J., & Brodie, A. F. (1971) *Methods Enzymol. 18C*, 407.
Fieser, L. F. (1924) *J. Am. Chem. Soc. 46*, 2639.
Haddock, B. A., & Jones, C. W. (1977) *Bacteriol. Rev. 41*, 47.
Heiss, J., Zeller, K. P., & Rieker, A. (1969) *Org. Mass Spectrom. 2*, 1325.
Jones, C. W. (1977) *Symp. Soc. Gen. Microbiol. 27*, 23.
Morimoto, H., Shima, T., Imada, I., Sasaki, M., & Ouchida, A. (1967) *Justus Liebigs Ann. Chem. 702*, 137.
Muraca, R. F., Whittick, J. S., Daves, G. D., Jr., Friis, P., & Folkers, K. (1967) *J. Am. Chem. Soc. 89*, 1505.
Ramasarma, T. (1968) *Adv. Lipid Res. 6*, 107.
Samuel, D. (1962) in Oxygenases, Hayaishi, O., Ed., New York, N.Y., Academic Press, p 31.
Snyder, C. D., & Rapoport, H. (1970) *Biochemistry 9*, 2033.
Stroobant, P., Young, I. G., & Gibson, F. (1972) *J. Bacteriol. 109*, 134.
Uchida, K., & Aida, K. (1972) *Biochem. Biophys. Res. Commun. 46*, 130.
Wallace, B. J., & Young, I. G., (1977) *Biochim. Biophys. Acta 461*, 84.
Whistance, G. R., Dillon, J. F., & Threlfall, D. R. (1969) *Biochem. J. 111*, 461.
Young, I. G. (1975) *Biochemistry* 14, 399.
Young, I. G., Stroobant, P., MacDonald, C. G., & Gibson, F. (1973) *J. Bacteriol. 114*, 42.

Alternative Hydroxylases for the Aerobic and Anaerobic Biosynthesis of Ubiquinone in *Escherichia coli*†

Ken Alexander and I. G. Young*

ABSTRACT: The synthesis of ubiquinone under anaerobic conditions was examined in a variety of strains of *Escherichia coli* K12. All were shown to synthesize appreciable quantities of ubiquinone 8 when grown anaerobically on glycerol in the presence of fumarate. Under these conditions, ubiquinone 8 was in most cases the principal quinone formed, and levels in the range 50–70% of those obtained aerobically were observed. Studies with mutants blocked in the various reactions of the aerobic pathway for ubiquinone 8 synthesis established that under anaerobic conditions three alternative hydroxylation reactions not involving molecular oxygen are used to derive the C-4, -5, and -6 oxygens of ubiquinone 8. Thus, mutants blocked in either of the three hydroxylation reactions of the aerobic pathway (*ubiB*, *ubiH*, or *ubiF*) are each able to synthesize ubiquinone 8 anaerobically, whereas mutants lacking the octaprenyltransferase (*ubiA*), carboxy-lyase (*ubiD*), or methyltransferases (*ubiE* or *ubiG*) of the aerobic pathway remain blocked anaerobically. The demonstration that *E. coli* possesses a special mechanism for the anaerobic biosynthesis of ubiquinone suggests that this quinone may play an important role in anaerobic metabolism.

The effects of aerobic or anaerobic conditions of growth on the relative levels of ubiquinone and menaquinone synthesized by members of the *Enterobacteriacea* have been examined by several groups of workers (Lester and Crane, 1959; Kashket and Brodie, 1960; Bishop et al., 1962; Polglase et al., 1966; Whistance and Threlfall, 1968; El Hachimi et al., 1974). Although the results of these studies are to some extent contradictory, most reports indicate that ubiquinone is formed in much higher levels aerobically than anaerobically, whereas the menaquinones predominate under anaerobic conditions.

We have recently reported a role for ubiquinone 8 in the anaerobic electron-transport chain to nitrate in *E. coli* K12 (Wallace and Young, 1977b). The strains used in this study formed 10–20% of the aerobic level of ubiquinone 8 when grown anaerobically with nitrate. Since molecular oxygen is utilized for the three aromatic hydroxylations in the aerobic biosynthesis of ubiquinone 8 (see Alexander and Young, 1978), the above result suggests that *E. coli* must possess special mechanisms for the synthesis of ubiquinone 8 anaerobically.

The following study shows that appreciable quantities of ubiquinone 8 (50–70% of aerobic levels) are synthesized by a wide variety of strains of *E. coli* K12 when grown anaerobically in the presence of fumarate. Using mutants blocked in the various reactions of the aerobic pathway (Young et al., 1973), it is shown that three alternative hydroxylases are utilized under anaerobic conditions.

† From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra City, A.C.T. 2601, Australia. *Received June 16, 1978.*

0006-2960/78/0417-4750$01.00/0