Biochemistry

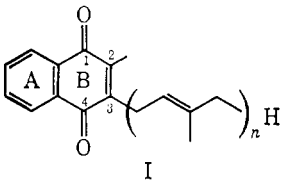
Volume 9, Number 10 May 12, 1970

Biosynthesis of Bacterial Menaquinones. Origin of Quinone Oxygens*

Clinton D. Snyder and Henry Rapoport

ABSTRACT: Utilizing ^{18}O tracer techniques, the biosynthetic origin of the quinone oxygen functions of dihydromenaquinone-9, MK-9 (II, H_2), from *Mycobacterium phlei* has been investigated. This study required development of apparatus for a closed atmosphere growth of aerobic microorganisms in the presence of ^{18}O-enriched oxygen as well as a quinone isolation procedure which is compatible with retention of [^{18}O]quinone label. Total incorporation from [^{18}O]H_2O-labeled media is observed whereas no incorporation of oxygen from $^{18}O_2$ can be detected. That this incorporation reflects the biosynthetic origin of the quinone oxygen functions rather than an *in vivo* exchange reaction of quinone already biosynthesized has been demonstrated in a double-label (^3H and ^{18}O) control experiment. The relevance of this work to current biosynthetic schemes for bacterial menaquinones is discussed.

Although the occurrence of menaquinones (I) in bacteria is widespread, the biosynthetic origin of this interesting class of pigments is only poorly understood. Fortunately, the structural variations of menaquinones observed in the bacterial flora are relatively minor, involving only modifications



I

of the lipophilic periphery such as presence or absence of a 2-methyl group (demethylmenaquinones; Whistance and Threlfall, 1968), variability in the number of isoprene units in the side chain at C-3 (n = 4–10; Cawthorne *et al.*, 1967; Whistance *et al.*, 1969; Campbell and Bentley, 1968, 1969), partial saturation of the side chain (*Mycobacterium phlei* quinone, MK-9 (II, H_2),[1] and others; Azerad *et al.*, 1967; Phillips *et al.*, 1969), or oxidation at the 1′ position of the side chain (chlorobiumquinone; Powls *et al.*, 1968; Bondinell *et al.*, 1969); therefore, there is reason to expect a generalized biosynthetic pathway to prevail.

The biosynthetic origin of the 2-methyl group has already been firmly established in several organisms as L-methionine (Guerin *et al.*, 1965; Jaureguiberry *et al.*, 1966; Jackman *et al.*, 1967) and presumably, although not necessarily, the side chain is constructed from polycondensation of isopentenyl pyrophosphate units to form the appropriate allylic alcohol which can then be introduced into the naphthalenic nucleus. Alternatively, isopentenyl alkylation of the aromatic nucleus followed by stepwise addition of isopentenyl groups to construct the C-3 side chain also is a possibility. Both methylation and prenylation can occur at a stage in which the naphthalenic nucleus already is aromatic and quite possibly also oxygenated at C-1 and C-4. Such a scheme would be consistent with what is known about electrophilic aromatic substitutions.

Biosynthesis of the naphthalenic portion of menaquinones, however, is poorly understood. Shikimic acid, a precursor of aromatic compounds including ubiquinones in bacterial systems, is also observed to be incorporated into menaquinones (Cox and Gibson, 1966; Campbell *et al.*, 1967). Degradative evidence is convincing that shikimate is utilized as a seven-carbon unit introduced intact into ring A and C-1 and/or C-4 (Leistner *et al.*, 1967) although present data do not establish whether or not a symmetrical intermediate

* From the Department of Chemistry, University of California, Berkeley, California. *Received December 29, 1969.* This research was supported by Grant AM 13688 from the National Institutes of Health, U. S. Public Health Service.

[1] For IUPAC rules for nomenclature of these quinones, see IUPAC–IUB Commission (1966).

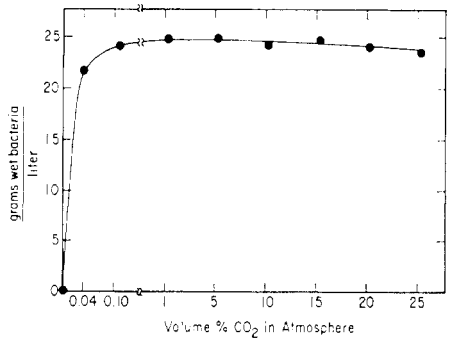


FIGURE 1: Growth of *M. phlei* as a function of CO_2 concentration.

is involved. More recently, the incorporation of 1-naphthol into menaquinone-8 of *Bacillis megaterium* has been determined, leading to the conjecture that 1-naphthol is also on the direct biosynthetic pathway to menaquinone (Leistner *et al.*, 1967). Although degradative evidence again demonstrates that 1-naphthol is incorporated intact with its C-1 located at the 1 and/or 4 position of the quinone, the implication of biosynthetic intermediacy does not necessarily follow since a metabolite of 1-naphthol could have been the species incorporated rather than 1-naphthol itself. The biosynthetic pathway after 1-naphthol is essentially unknown.

From among the many obvious questions posed in menaquinone biosynthesis we have chosen to initiate our investigations with the application of ^{18}O tracer techniques. The necessary analytical tools for this approach are derived from our previous experience in this area (Snyder and Rapoport, 1968) in which we developed a highly accurate and sensitive ^{18}O assay applicable to the limited quantities of high molecular weight menaquinone which must be involved in a biosynthetic study. Primarily the determination of the origin of the menaquinone oxygen functions is relevant to the identification of biosynthetic intermediates and also to the reaction sequence leading to these intermediates. Ultimately, any proposed pathway must be consistent with such evidence. For example, if one or both quinone oxygen functions were derived from atmospheric oxygen, then, by analogy with many observed biological oxygenation reactions (Kaufman, 1962; Nover, 1969), this could implicate an aromatic oxygenation while identification of the locus of incorporation would be further instructive. Alternatively, the carbonyl oxygens could arise from water in which case their insertion by an aromatic oxygenation reaction would be unlikely or in the event that neither water nor atmospheric oxygen proved to be a precursor then naphthalenic biosynthesis from a preoxygenated metabolite would have to be considered.

The system *M. phlei* and its attendant MK-9 (II, H_2) quinone were chosen for study, reflecting the common usage of this organism for quinone studies. For those experiments in which the gaseous phase was to be labeled with ^{18}O-enriched oxygen, construction of a closed atmosphere growth system was necessary. In order to obtain sufficient quantities of quinone for isotope analysis and also to ensure retention of quinone label during extraction from the bacteria, development of a mild but efficient extractive method was required. After growth in the presence of either $^{18}O_2$ or $[^{18}O]H_2O$, ^{18}O analysis of the purified quinones then revealed the extent of incorporation.

An important requirement of this study is that the quinone carbonyl functions be immune to exchange with water under growth conditions since such a background exchange would make inferences concerning their origins meaningless. Fortunately, our experience has confirmed that at least with the exogenous [^{18}O]phylloquinone–*M. phlei* crude extract system such an assumption is valid (Snyder and Rapoport, 1968); however, the necessity for a more relevant control experiment for exchange of the endogenous native quinone under *in vivo*, whole cell conditions is obvious and is considered in this work.

Methods

Closed Atmosphere Growth of M. phlei. A closed system for growing bacteria in the presence of labeled oxygen must employ the following features: (1) operate at 1 atm, 80% N_2 and 20% O_2; (2) employ a CO_2 removal system; and (3) deliver $^{18}O_2$ as needed. The media was contained in two stoppered 4-l. suction flasks with admittance through cotton-plugged glass tubing. This arrangement allowed the flask and its contents to be autoclaved while the rest of the apparatus was not sterilized. The side arm was closed with a serum stopper through which innoculum could be injected and from which gas samples could be removed for mass spectrometric analysis. One of the leads from the flask was attached to an open-ended mercury manometer for continuous monitoring of the internal pressure. The atmosphere above the media was circulated with a closed-circuit Dynapump through a 20% potassium hydroxide solution to effect CO_2 removal. Oxygen gas was contained in a lecture cylinder and connected to the system through a 1-atm diaphragm valve so that oxygen would only be withdrawn as the pressure dropped below 1 atm. Other leads to the flasks were attached to vacuum and nitrogen-flush systems to be used for degassing the media in preparation for the isotope experiments. The flasks themselves were firmly attached with the aid of a wooden yoke to a shaking table to allow maximum swirling.

From early experiments it was determined that little growth occurred with continuous removal of CO_2. Two explanations for this phenomenon seemed plausible: (1) that the bacteria were liberating a volatile toxin which caused poisoning in a closed system or (2) that a certain partial pressure of CO_2 was required for growth as in the case for several other aerobic bacteria (Gladstone *et al.*, 1935). An experiment in which no growth was obtained under a continuous stream of CO_2-free air firmly established a CO_2 growth requirement. In an attempt to optimize growth conditions a CO_2 concentration–growth curve was determined and, as shown in Figure 1, the bacterial yield was found to be remarkably independent of CO_2 concentration as long as it was above a threshold value which is close to the natural atmospheric concentration. This situation actually simplified the isotope experiments in that CO_2 removal was not time critical and it was found satisfactory to let the closed system operate uninterrupted for the first 12 hr and then to activate the CO_2 removal system for 10 min every 4 hr thereafter. Growth in the closed system was never as efficient as that of an open-system control so that self-toxification

could still be involved; however this was not further investigated.

The consumption of oxygen as determined manometrically was 4 l./15 g of wet bacteria. Carbon dioxide evolution was less than oxygen consumption (~70%) during the first 14-hr growth but thereafter it was equal to or slightly greater than oxygen consumption.

Growth of M. phlei in [^{18}O]H_2O-Labeled Media. For those experiments in which the media was [^{18}O]H_2O labeled, a closed atmosphere system was also employed although it was charged with normal oxygen. The principal experimental modification necessary was in the method of sterilization of the media since under autoclaving conditions extensive exchange of the [^{18}O]H_2O label with the steam would have occurred. On the other hand, Millipore filtration of the media into a presterilized suction flask was particularly convenient and circumvented any exchange problem. Filtration through a 0.2 μ cellulose acetate filter was effected by careful suction. Some clogging of the filter occurred and although it was necessary to change the filter several times, a test incubation of the media for 48 hr indicated sterilization had been effective.

Production and Isolation of $^{18}O_2$. Because of the quantities (4 l./experiment) and the enrichment desired (~2 atom %), $^{18}O_2$ was best obtained by electrolysis of [^{18}O]H_2O. Commercially the gas is only available at very high enrichments which were unnecessary for the projected experiments. An electrolysis cell was constructed from two Florence flasks (25 ml) connected at the base by glass tubing (18 mm i.d.); electrodes of platinum foil were connected with platinum wire (20 gauge). The critical features of such a cell were found to be the gauge of the platinum wire, which must be great enough not to overheat, and the diameter of the connection between the two cells, which must be large enough to permit adequate heat dispersion but small enough to prevent mixing of the evolved gases. The cell was charged with 1.8% [^{18}O]H_2O made 4 N in sodium hydroxide by addition of metallic sodium. Electrolysis was limited only by boiling in the tube connecting the two cells, so that with adequate cooling in an ice bath, the cell could evolve approximately 1 l. of O_2/hr while carrying a current of 4–5 A.

The oxygen which was evolved at atmospheric pressure was collected under vacuum in a 6-l. reservoir as described (Samuel, 1962). When the reservoir was filled to 1 atm as indicated by an attached mercury manometer, the gas was transferred *in vacuo* to a lecture bottle which was chilled with liquid nitrogen. By this method 75% of the oxygen was transferred to the cylinder and, although complete transferral was impossible because of the significant partial pressure of oxygen at liquid nitrogen temperature, the loss was constant regardless of the amount of oxygen processed through the system so that repeating the procedure was advantageous. The isotopic content of the oxygen gas so obtained was identical with that in the starting water.

Extraction and Purification of the Quinone. Because of its efficiency (approximately fourfold that of simple organic extraction of wet cells) and purity of the resulting product, the method of choice for extraction of quinone from the bacteria was an alkaline hydrolysis scheme (Azerad *et al.*, 1967). Unfortunately, [^{18}O]phylloquinone subjected to a control extraction suffered complete loss of label. Since reduction of quinone to hydroquinone by the alkaline–pyrogallol system employed as an oxygen scavenger in this procedure was questionable and since any quinone left would surely be subject to base-catalyzed exchange, the extraction was modified to include a prereduction with borohydride before addition of base. Somewhat surprisingly, total exchange of label from the test quinone was again observed so that exchange of the phenolic function of the naphthohydroquinone became suspect. Hydroquinone itself exchanges to a negligible extent under basic conditions (Fesenko and Gragerov, 1955); however, the naphthalenic system could offer different properties. The Bucherer interconversion of 1-naphthylamine and 1-naphthol has been rationalized on the basis of keto contribution to the phenol so that if such a contribution were significant, base-catalyzed exchange through this tautomer could easily occur.

To test this possibility a comparison of the exchange rate of hydroquinone and 2,3-dimethyl-1,4-naphthohydroquinone under alkaline conditions was made. Mass spectral analyses after 1 hr at 80° indicated, as expected, no exchange for benzohydroquinone, but, on the other hand, significant exchange for the naphthohydroquinone; K = 0.97 hr^{-1}, $t_{1/2}$ = 0.71 hr. The latter exchange must reflect the keto character of naphthohydroquinone and, although it does not completely explain the isolation situation where complete exchange is observed under similar conditions in 0.5 hr, other explanations such as an additional catalytic effect by bacterial degradation products are available.

Since retention of [^{18}O]quinone label and the alkaline hydrolysis are incompatible, an alternative extraction procedure was necessary. Both to improve the efficiency of extraction and to remove water which might exchange with the quinone during extraction, the bacteria were subjected to lyophilization. The resulting powder was extracted at reflux with dry benzene and the lipid extract thus obtained was resolved by repeated chromatography on Camag thin-layer chromatography grade Kieselgel using several different solvent systems to yield pure quinone (as determined by ultraviolet extinction and thin-layer chromatography) in yields approaching those of the alkaline hydrolysis. No loss of label was observed in a control extraction of [^{18}O]phylloquinone.

Analytical Techniques. ^{18}O DETERMINATIONS. Organic compounds were pyrolyzed as previously described (Snyder and Rapoport, 1968) to yield carbon monoxide whose ^{18}O content was determined by mass spectrometry. Pyrolysis of an equal amount of unlabeled quinone provided a natural abundance background determination. The ^{18}O content of labeled water was determined by equilibration with a small amount of CO_2 in a sealed tube at 100° followed by mass spectrometry of the gas. $^{18}O_2$ was assayed directly and without purification as in the case of samplings from the atmosphere in the closed growth system. All isotope assays were performed using a Consolidated Electrodynamics Corp. 130 mass spectrometer.

TRITIUM DETERMINATIONS. Water and quinone samples were assayed for tritium by liquid scintillation employing a Nuclear-Chicago Mark I computer. Sample quenching was determined with the external standard attachment for this instrument.

Results and Discussion

Incorporation of ^{18}O into the recovered native quinone, MK-9 (II, H_2), from several experiments is shown in Table

TABLE I: ^{18}O Incorporation into MK-9 (II, H_2) from *M. phlei* Grown in the Presence of Either $^{18}O_2$ Or $[^{18}O]H_2O$.

Expt	Phase Labeled	% ^{18}O in O_2 Or H_2O[a] Before Expt	After Expt[b]	% ^{18}O in MK-9 (II, H_2)[a] Test	Control
1	O_2	1.595	1.437	0.30	0.30
2[c]	O_2	1.470	1.380	0.29	0.28
3	H_2O	2.024	1.990	1.93	0.30

[a] Standard deviation for ^{18}O analysis of O_2 and H_2O is $\pm 0.005\%$ ^{18}O while quinones are determined with an accuracy of $\pm 0.01\%$ ^{18}O. [b] The slight diminution in ^{18}O content of labeled oxygen during the course of expt 1 and 2 must be ascribed to either leakage or dilution from normal oxygen dissolved in the media. A similar but less extensive dilution of labeled water during expt 3 can easily be accommodated by *in vivo* dehydrations and exchange reactions. [c] Glucose omitted from the media.

I. The ^{18}O content of quinone from bacteria grown in the presence of labeled oxygen (expt 1 and 2) is almost identical with that of a natural abundance control. Glucose was omitted from the media in expt 2 in order to maximize the observability of $^{18}O_2$ incorporation. It has been reported that significantly higher values for total incorporation of molecular oxygen into whole cells are obtained when bacteria are grown only on amino acids instead of glucose (Hayaishi, 1962). Thus, omission of highly oxygenated substrate, glucose, leaving casein hydrolysate amino acids, fumarate, and Tween-80 as carbon sources could have permitted observation of an otherwise unobservable pathway to quinone demonstrating incorporation of molecular oxygen. A greater tendency toward agglutination was observed as a morphological change upon omission of glucose and also a drop in bacterial yield (30%) although the total quinone yield remained about the same because of an increase in quinone content. Nevertheless, $^{18}O_2$ incorporation was not observed.

The converse experiment in which the media was labeled gave, as expected, the positive result. As shown in expt 3, the quinone which was 94% pure, incorporated 97% of the isotopic label of the medium, indicating that at some stage in biosynthesis the quinone oxygens are derived from water. Unfortunately, the possible lability of the quinone oxygens to exchange with water leaves us with an ambiguity: Are the quinone oxygens directly derived from water in the biosynthetic pathway or are the quinone oxygens merely in equilibrium with water? A control experiment was therefore necessary to determine the amount of $[^{18}O]H_2O$ incorporation specifically associated with biosynthesis and not with any exchange reaction which might occur under active growth conditions but be irrelevant to biosynthesis.

The experiment conceived to distinguish between these two possibilities involved a short-term exposure of the bacteria to $[^{18}O]H_2O$-labeled media after most of the growth had occurred under normal conditions so that of the total quinone isolated only a small fraction would have been biosynthesized in the presence of $[^{18}O]H_2O$ while the majority of quinone would have been biosynthesized normally. With an independent method of establishing the amount of quinone biosynthesis occurring during the $[^{18}O]H_2O$ exposure, a comparison between this estimate and quinone biosynthesis as measured by $[^{18}O]H_2O$ incorporation can be made. If both estimates are the same then the ^{18}O-labeled quinone can arise only from biosynthesis whereas if the estimate from ^{18}O incorporation is greater than the extent of biosynthesis, this will implicate exchange of previously biosynthesized quinone. An assumption involved at this point is that the older cells are capable of demonstrating the postulated exchange reaction. That exchange might occur only in newly developing cells perhaps associated specifically with quinone biosynthesis and therefore not in the older cells is a possibility, but unfortunately it is not testable.

Assuming a constant quinone concentration during various stages of growth, simple wet yield of bacteria should provide an acceptable measurement of the increment of quinone during $[^{18}O]H_2O$ exposure. Alternatively, the extent of new biosynthesis could be determined by a double-label approach involving simultaneous introduction of a second isotope with the $[^{18}O]H_2O$ media. If the degree of incorporation into actively growing bacteria were known for this isotope then incorporation during $[^{18}O]H_2O$ exposure would provide an independent assay for newly biosynthesized quinone. To minimize considerations involving the linearity of incorporation during the course of an experiment, tritiated water was chosen as a source of label since its concentration will remain constant as a function of growth. However, the degree of incorporation was assumed to be independent of the age of the bacterium. A high degree of incorporation was assured due to carbon–hydrogen exchange of intermediates during mevalonate biosynthesis of the side chain. The stability of aliphatic side chain to exchange ensured that no isotope incorporation would occur into previously biosynthesized quinone.

The experiment was conducted in two parts, the results of which are contained in Table II. Parallel flasks, one of which (flask 1) contained tritiated medium, were inoculated. After 24 hr, the radioactive bacteria were harvested, and the quinone was extracted and purified to determine its specific activity. Incorporation was significant (57%) although not quantitative. The bacteria from flask 2 were harvested, weighed, and resuspended in a $[^3H, ^{18}O]H_2O$-labeled medium. Since incubation was to last only a few hours, sterilization of the media was unnecessary. After 5 hr, the bacteria were reharvested and the increase in weight yield was determined (25%). The purified quinone from these bacteria was then subjected to ^3H and ^{18}O analysis. As can be seen, 51% of the quinone was newly biosynthesized as assayed by tritium incorporation. The fact that only a 25% increase in weight is observed leads to speculation that the isotopic incorporation estimate is falsely high. Reversibility of side-chain addition so that previously biosynthesized quinone could be labeled by addition of a new and therefore radioactive side chain could explain such a discrepancy. One can only say that the real extent of new biosynthesis is most probably bounded between 25 and 50%. The ^{18}O estimate that 36% of the quinone is newly biosynthesized is therefore meaningful. Although the tritium incorporation estimate is obviously excessive, the fact that the ^{18}O estimate is significantly less

TABLE II: Investigation of *in Vivo* MK-9 (II, H_2)–H_2O Exchange.[a]

Flask	Sp Act. (dpm/mm of H)		% ^{18}O			% Newly Biosynthesized Quinone as Estimated by		
	Media	Quinone	Media	Test Quinone	Control Quinone	Wt Yield	T Incorp	^{18}O Incorp
1	1.97×10^5	1.127×10^5						
2	3.78×10^5	1.096×10^5	1.84	0.794	0.20	25	51	36

[a] Experiment 4.

and probably equal to the actual extent of biosynthesis is convincing that no extraneous exchange with water is involved during growth[2] so that the quinone carbonyl oxygens must originate biosynthetically from water.

Conclusions

The conclusion that the quinone oxygen functions arise biosynthetically from water has implications for menaquinone biosynthesis as it is presently proposed. Since shikimate is derived from intermediates of glycolysis (the carboxyl function being formed from phosphoenolpyruvate) and since the lability of the oxygen functions of the various intermediates with respect to water exchange is unknown, the probability that by the time the pathway reaches shikimate the oxygen isotopic content of the carboxylate group will reflect that of the media is high. Also, acceptance of the intermediacy of shikimate to menaquinone probably predetermines that one of the quinone oxygens will arise from the carboxylate oxygen of shikimate so that incorporation of water at that quinone oxygen is not surprising.

If, on the other hand, one accepts the intermediacy of 1-naphthol to menaquinone, the origin of the second and yet to be introduced oxygen function is much more interesting. At this stage, the introduction of the second oxygen must be an aromatic hydroxylation and since aromatic hydroxylations so far examined have been found to proceed with utilization of molecular oxygen, incorporation from $^{18}O_2$ would be expected. The fact that none is observed raises doubts on the proposed involvement of 1-naphthol at least in *M. phlei*. We have observed 1-naphthol incorporation to be very low in this organism (R. M. Baldwin and H. Rapoport, 1969, unpublished observations) and a similar observation has been reported for incorporation into MK-8 of *E. coli* (Ellis and Glover, 1968). At this point alternative proposals for the biosynthesis of the naphthalenic portions of menaquinone would be premature and must await additional experimental evidence.

Experimental Section

2,3-Dimethyl-1,4-naphthohydroquinone–Water-Exchange Reaction. Into one arm of a Y tube was placed [^{18}O]H_2O (1.600% ^{18}O, 1 ml) which was 4 N in NaOH, while the other arm contained benzohydroquinone (10 mg), 2,3-dimethyl-1,4-naphthoquinone (10 mg), PtO_2 (2 mg), and ether (2 ml). Hydrogen was bubbled into the solution until the ether had evaporated. Reduction of the quinone, indicated by a colorless solution, was almost immediate. The Y tube was then connected to a vacuum system, the water was frozen with a Dry Ice–acetone bath, and the system was then evacuated. Degassing of the aqueous solution was accomplished by thawing and refreezing several times. After the tube was sealed, the aqueous alkali was mixed with the hydroquinones resulting in a bright yellow solution without discoloration indicating no oxygen contamination. The tube was immersed for 1 hr at 80° after which it was opened and the contents were immediately neutralized by addition to an ether–1 N HCl mixture. The ether layer was dried and evaporated with oxygen to yield a benzohydroquinone–2,3-dimethyl-1,4-naphthoquinone mixture. Hydroquinone was separated from the mixture in a pure form by simply washing with ether. The naphthoquinone, dissolved in the washings, was purified by column chromatography (eluent: 8% ether in petroleum ether (bp 30–60°)). Both compounds were subjected to ^{18}O analysis by pyrolysis: benzohydroquinone = 0.27% ^{18}O and 2,3-dimethyl-1,4-naphthoquinone = 1.07% ^{18}O. Assuming first order exchange kinetics, $k = 0.97\ hr^{-1}$ or $t_{1/2} = 0.71$ hr.

Growth of Bacteria. M. phlei ATCC 354 was grown with vigorous aeration on a New Brunswick rotary shaker at 37° for 24–48 hr in medium (500 ml/flask) described by Brodie and Gray (1956) which was enriched by the addition of glucose (30 g/l.). The glucose was dissolved in a minimal quantity of water and autoclaved separately to prevent caramelization. Cells were harvested at 0° by centrifugation in a Sorval SS3 centrifuge at 6000 rpm.

Extraction and Purification of Quinone. Wet bacteria (20 g) were suspended in a thick slurry and subjected to lyophilization. Dry benzene (350 ml) was added and the solution was refluxed in the absence of light. A small fraction (50 ml) was collected to remove any residual water and the remainder was refluxed for another 30 min. The bacteria were removed by centrifugation and the benzene extract was evaporated to yield a crude lipid residue. This residue was chromatographed repeatedly on Kieselgel (eluents: 8% ether in petroleum ether and 40% benzene in petroleum ether) to yield pure quinone as judged by ultraviolet light comparison with a standard ($\epsilon^{248\ nm}_{isooctane}$ 19,600).

^{18}O Experiment. EXPERIMENT 1. The experiment was arranged as described in the Methods section. The suction flasks were evacuated to 10 cm and then brought back to 1

[2] This observation is consistent with a previous determination (Snyder and Rapoport, 1968) that in a cell-free system, [^{18}O]phylloquinone suffers no loss of label during oxidative phosphorylation. It is further support, now in the intact cells, that carbon–oxygen bond cleavage is not involved during the quinone's participation in oxidative phosphorylation.

atm with nitrogen. This procedure was repeated several times, and at the end of the last degassing cycle, nitrogen was admitted to only 60 cm. $^{18}O_2$ gas was then admitted from the lecture bottle to bring the internal pressure to 1 atm. A gas sample was removed with a gas-tight syringe for ^{18}O analysis: % ^{18}O = 1.595. The flasks were inoculated from a culture medium and were grown for 36 hr after which time a second gas sample was taken (% ^{18}O = 1.437) and then the bacteria were harvested (15 g). The quinone was isolated (5.1 mg, 95% pure) and a sample of this quinone (4.3 mg) was pyrolyzed for mass spectral assay as was a similar size sample of unlabeled MK-9 (II, H_2); ^{18}O: test = 0.30%; control background = 0.30%.

EXPERIMENT 2. The procedure was as in expt 1 except that glucose was omitted from the media. The ^{18}O content of the oxygen before inoculation was 1.47% ^{18}O and after 48-hr growth it was 1.38%. The bacterial yield was 10 g from which 3.1 mg (96% pure) of quinone was obtained. ^{18}O assay was then performed on a 2.7-mg sample; ^{18}O: test = 0.29%; MK-9 (II, H_2) control = 0.28%.

EXPERIMENT 3. The medium ingredients were dissolved in [^{18}O]H_2O (2.050% ^{18}O). Sterilization was accomplished by Millipore filtration and a sample of the medium before growth was taken for another ^{18}O analysis (2.024% ^{18}O). After 36-hr growth, a similar assay of medium was made (1.990% ^{18}O). The bacteria were harvested (12 g) and the quinone was obtained (4.1 mg, 94% pure). A sample of this quinone (3.7 mg) and an identical comparison sample of MK-9 (II, H_2) were assayed for ^{18}O content; ^{18}O: test = 1.930%, control MK-9 (II, H_2) = 0.30%.

EXPERIMENT 4. The medium (not labeled) was divided between two flasks and tritiated water (1 ml, 4.75 mCi/ml) was added to one (flask 1). An aliquot from this flask was removed for ^3H assay (1.97×10^5 dpm/mm of H) and then both flasks were inoculated. After 24-hr growth, both flasks were harvested. Pure quinone (5.1 mg, 100% pure) was obtained from the tritiated bacteria (15 g). The specific activity of this quinone was 1.127 dpm/mm of H, corresponding to 57% incorporation assuming complete exchange at all hydrogen positions. The bacteria from the other flask were weighed (16.7 g) and resuspended in fresh media (500 ml) which was tritium and ^{18}O labeled *via* H_2O (3.78×10^5 dpm/mm of H and 1.840% ^{18}O). The bacteria were grown for another 5 hr after which time they were harvested (22.2 g) and the quinone was isolated (7.4 mg, 97% pure). ^3H assay of the quinone (1.096×10^5 dpm/mm of H) corresponded to 29% incorporation. Thus, assuming 57% incorporation as theoretical, this quinone was $^{29}/_{57} \times 100$ or 51% newly biosynthesized. Also, the ^{18}O content of the quinone was determined (0.794% ^{18}O). If x = fraction of newly biosynthesized quinone and $1 - x$ is the fraction of quinone biosynthesized under previous nonisotopic conditions, then $0.794 = x(1.84) + (1 - x)(0.20)$ or $x = 0.36$. Therefore, 36% of the quinone is newly biosynthesized, based on its ^{18}O content.

References

Azerad, R., Cyrot, M.-O., and Lederer, E. (1967), *Biochem. Biophys. Res. Commun. 27*, 249.

Bondinell, W. R., Snyder, C. D., and Rapoport, H. (1969), *J. Amer. Chem. Soc. 91*, 6889.

Brodie, A. F., and Gray, C. T. (1956), *J. Biol. Chem. 219*, 853.

Campbell, I. M., and Bentley, R. (1968), *Biochemistry 7*, 3323.

Campbell, I. M., and Bentley, R. (1969), *Biochemistry 8*, 4651.

Campbell, I. M., Coscia, C. J., Kelsey, M., and Bentley, R. (1967), *Biochem. Biophys. Res. Commun. 28*, 25.

Cawthorne, M. A., Jeffries, L. R., Harris, M., Price, S. A., Diplock, A. T., and Green, J. (1967), *Biochem. J. 104*, 35c.

Cox, G. B., and Gibson, F. (1966), *Biochem. J. 100*, 1.

Ellis, J. R. S., and Glover, J. (1968), *Biochem. J. 110*, 22p.

Fesenko, V. V., and Gragerov, I. P. (1955), *Dokl. Akad. Nauk SSSR 101*, 695.

Gladstone, G. P., Tildes, P., and Richardson, G. M. (1935), *Brit. J. Exptl. Pathol. 16*, 335.

Guerin, M., Azerad, R., and Lederer, E. (1965), *Bull. Soc. Chim. Biol. 47*, 2105.

Hayaishi, O., Ed. (1962), *in* Oxygenases, New York, N. Y., Academic, pp 25ff.

Jackman, L. M., O'Brien, I. G., Cox, G. B., and Gibson, F. (1967), *Biochim. Biophys. Acta 141*, 1.

Jaureguiberry, G., Lenfant, M., Das, B. C., and Lederer, E. (1966), *Tetrahedron, Suppl. 8*, 27.

Kaufman, S. (1962), *in* Oxygenases, pp 129ff.

Leistner, E., Schmitt, J. H., and Zenk, M. H. (1967), *Biochem. Biophys. Res. Commun. 28*, 845.

Nover, L. (1969), *Pharmazie 7*, 361.

Phillips, P. G., Dunphy, P. J., Servis, K. L., and Brodie, A. F. (1969), *Biochemistry 8*, 2856.

Powls, R., Redfearn, E., and Trippett, S. (1968), *Biochem. Biophys. Res. Commun. 33*, 408.

Samuel, D., (1962), *in* Oxygenases, Hayaishi, O., Ed., New York, N. Y., Academic, pp 31ff.

Snyder, C. D., and Rapoport, H. (1968), *Biochemistry 7*, 2318.

Whistance, G. R., Dillon, J. F., and Threlfall, D. R. (1969), *Biochem. J. 111*, 461.

Whistance, G. R., and Threlfall, D. R. (1968), *Biochem. J. 108*, 505.