The Role of the Quinone in Oxidative Phosphorylation. Evidence against Carbon–Oxygen Bond Cleavage*

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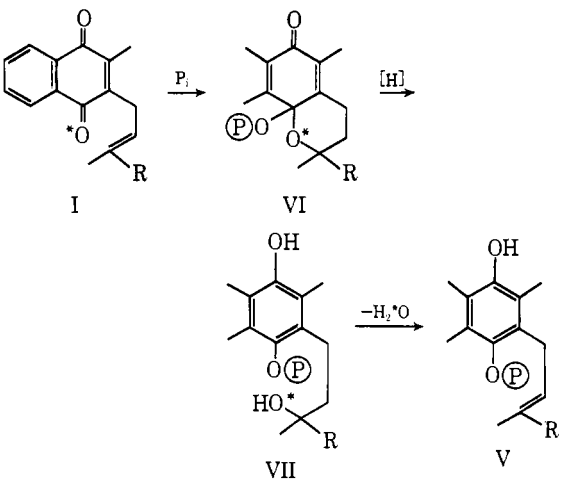
ABSTRACT: The use of ^{18}O to test mechanisms proposed to explain the function of the quinone in oxidative phosphorylation is reported. The study involved reconstituting light-inactivated extracts of *Mycobacterium phlei* with ^{18}O-labeled phylloquinones. The synthesis of both specifically and uniformly ^{18}O-labeled phylloquinones necessary for the study is presented. Conventional ^{18}O analytic techniques are critically evaluated and the description of a highly accurate ^{18}O analytical procedure is given. In the experiments reported, no isotope loss from the exogenous quinones which was specifically associated with oxidative phosphorylation could be detected.

The implications of this study for mechanistic considerations of quinone involvement in oxidative phosphorylation are discussed.

Involvement of the quinone in oxidative phosphorylation has recently been studied in the bacterial system *Mycobacterium phlei*, focusing in detail on the question of carbon–hydrogen bond cleavage at different sites within the quinone molecule under *in vivo* conditions (Di Mari *et al.*, 1968). Little attention has been given to the question of lability of the carbon–oxygen bond as a possible indication of quinone involvement in oxidative phosphorylation.[1] Evidence for a role for quinone in oxidative phosphorylation is strong, and of the many currently proposed schemes[2] several require bond lability at the carbonyl function.

For example, a common postulate is 1,2 addition of orthophosphate to either carbonyl function of the quinone followed by reductive dehydration to a quinol phosphate with or without concomitant chromanol formation (Clark and Todd, 1961) (Scheme I). Oxidation will then regenerate the quinone and a metaphosphate species with the result that the original quinone oxygen is lost to the water pool. At least in theory, the operation of such a cycle *in vivo* could be tested either by introduction of an ^{18}O label from inorganic phosphate or loss of label from quinone-^{18}O.

Because of the apparently specific requirement for a β,γ unit of unsaturation in the quinone side chain, several other mechanisms have been invoked to involve this feature. Addition of orthophosphate across the 4-carbonyl double bond coupled with addition to the side-chain double bond will give VI which then can be reduced to yield VII, whereupon the corresponding quinol phosphate (V) is formed by subsequent dehydration (Clark and Todd, 1961). From this point the cycle can proceed as before, again with the result that the quinone oxygen is lost to the medium. A test for its operation would be loss of label from quinone or introduction *via* phosphate.



Biological conversion of quinone into a quinone methide form (IX) is an integral part of several postulated quinone cycles (Chmielewska and Cieslak, 1960; Vilkas and Lederer, 1962; Erickson *et al.*, 1963). Since this ring closure has been effected *in vitro* under conditions of strong acid catalysis, it presumably involves a protonated intermediate, VIII. However, one could conceive of an *in vivo* cyclization proceeding *via* hydration, hemiketal formation (XI), and then dehydration. If this mechanism were valid, quinone methide formation would result in loss of label at the 4 position of the quinone. If ring closure were to occur similarly to the acid-catalyzed reactions, the original quinone oxygen would

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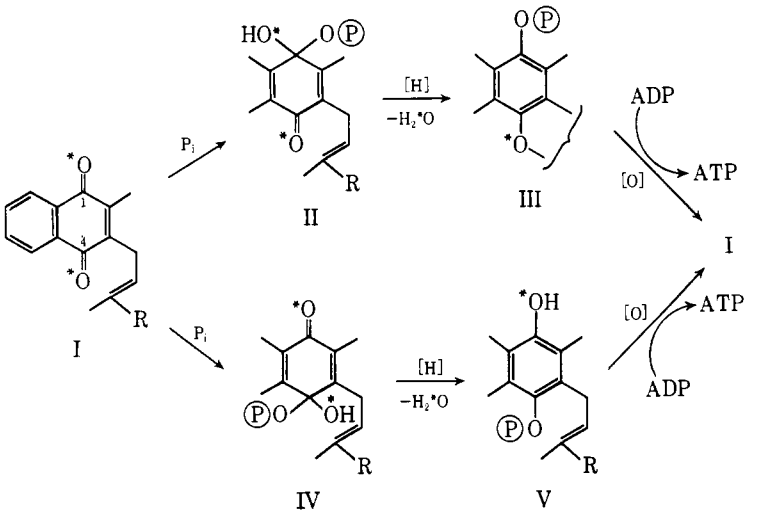
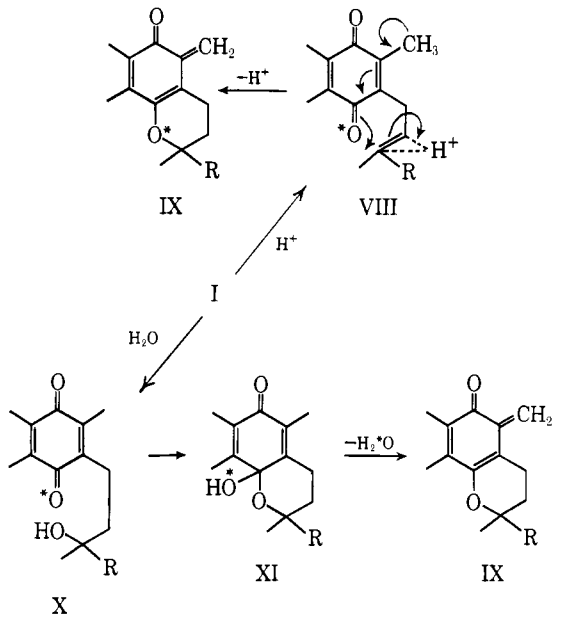
[1] The only comments on this subject are references to unpublished observations that inorganic phosphate oxygen does not exchange with quinone oxygen (Vilkas and Lederer, 1962; Brodie, 1965).

[2] A more comprehensive discussion of these points is presented in the preceding paper (Di Mari *et al.*, 1968).

SCHEME I





remain intact. Operation of the proposed equilibrium in ^{18}O-labeled aqueous medium would result in incorporation at the 4 position.

Finally, it should be noted that except for the possibility of oxygen exchange associated with quinone–methide formation, the mechanisms of Vilkas and Lederer (1962) and Erickson *et al.* (1963) involve no possibility of additional exchange, particularly at the 1 position.

Discussion

Using the bacterial system *M. phlei* and the approach of previous isotope studies[2] two investigative techniques relevant to the question of carbon–oxygen bond cleavage are available: (a) introduction of isotope into endogenous native quinone (MK-9(2H)) or exogenous phylloquinone *via* $H_2{}^{18}O$ and/or ^{18}O-labeled inorganic phosphate and (b) loss of isotope from exogenous phylloquinone-^{18}O.

From several points of view the former approach would be superior. The fact that the study could be performed with the native, undisturbed system rather than with the light-inactivated, phylloquinone-reconstituted system would be advantageous. In addition, although the mode of quinone involvement in both would be the same, the question arises as to what fraction of exogenous phylloquinone would be involved in a cyclic manner similar to that of the native quinone since, for analytical purposes and for maximal biological activity, the exogenous quinone must be in approximately 70-fold excess relative to the native quinone and since the fraction of native quinone itself involved in oxidative phosphorylation could be vanishingly small.

Clearly, for either study, maximizing the analytical sensitivity to detection of changes in isotopic content maximizes the significance of the experiment, and one should pursue this approach by examining the oxygen involvement of the native quinone. However, other limitations intervene. Because of the scale of the experiment, the enrichment of crude bacterial extract in $H_2{}^{18}O$ must necessarily be low so that any changes in ^{18}O content of the quinone would be measured at near natural abundance level where experimental accuracy is minimal.

Studying isotope incorporation *via* ^{18}O-labeled phosphate is not as sensitive to this criticism, for high ^{18}O enrichment in this species is easily attained on the scale contemplated; however, other complications to this approach are involved. Whereas, considering the time involved per experiment, uncatalyzed $P^{18}O_4 \rightleftarrows H_2O$ exchange is small, evidence for this exchange under biological conditions specifically involving operation of oxidative phosphorylation has been presented (Cohn and Drysdale, 1955; Boyer *et al.*, 1956). Not only would this serve to dilute the starting $P^{18}O_4$, adding an additional complication, but also it opens a whole new question which could ultimately result in a false, negative

experiment. That is, if such an exchange can occur with phosphate, why not a similar exchange with quinone so that ^{18}O introduced by the phosphate could be lost by quinone exchange with water? Thus an important control would be lacking. Also, it should be noted that isotope incorporation *via* phosphate does not investigate all possible mechanisms since it excludes those involving water. Finally, isotope incorporation *via* water or phosphate will not directly locate the site of incorporation since the analytical method of choice cannot distinguish between O-1 and O-4 incorporation; only loss of isotope from specifically labeled compounds can illuminate this aspect.

The advantages of approach b now become evident. These are (1) optimizing the sensitivity of the analytical method by having the option of working at sufficiently high levels of ^{18}O enrichment, (2) availability of a simple control for exchange not associated with oxidative phosphorylation, (3) simultaneous examination of mechanisms involving exchange *via* water and phosphate, and (4) selective examination of mechanisms involving 1- and 4-oxygens. Thus, from all considerations except disruption of the native system and fraction of quinone involvement, examination of exchange out of ^{18}O-labeled phylloquinone is preferable.

In consideration of the latter criticism of the method chosen, *i.e.*, fraction of quinone involved, every effort was made to enhance the sensitivity to detection of biological exchange.[2] Primary efforts have been directed toward the analytical methods; however, other considerations are important. Because the site of quinone action is presumably associated with the particulate (electron-transport particles) phase, separation at the end of an experiment of the particulate phase, which contains 10–20% of the exogenous quinone, from the supernatant, in which most of the remainder of the exogenous quinone is present as an unsolubilized emulsion, should significantly increase sensitivity to detection of exchange associated with either phase. To support this assumption, additional studies have shown that equilibration between the two pools is relatively slow, having a half life of ~2.5 hr. Assuming only one mechanism involving either the 1 or the 4 position of the quinone is operating, then separate examination of the 1 and 4 positions specifically labeled adds a factor of two in sensitivity in addition to identifying the quinone position involved.

Another attempt to increase sensitivity to quinone involvement is examination of the anaerobic–aerobic cycle. Using the *M. pheli* system reconstituted with phylloquinone, a naphthotocopherol-like compound containing organically bound phosphate was found to accumulate under anaerobic conditions in up to 20% yield (Russell and Brodie, 1961).[3] Subsequent oxidation yielded phylloquinone and inorganic phosphate in stoichiometric amounts. For the isotope experiments the effect of such a cycle would be to increase the fraction of exogenous phylloquinone involved in oxidative phosphorylation relative to the steady-state aerobic situation, thus increasing the probability of detection *via* isotope exchange.

The experiments were performed as previously described.[2] Crude dialyzed extract of *M. phlei* was exposed to ultraviolet irradiation in order to destroy the native quinone, and the irradiated extracts were then reconstituted with a sonically prepared emulsion of the proper ^{18}O-labeled test quinone. The oxidative phosphorylation activity of a portion of this extract was assayed in a Warburg system, employing a pyruvate substrate, while the major portion was aerated in the presence of the same substrate in the macro system. After most of the phosphate had been consumed in the macro system, the extract was separated into particulate and supernatant phases by centrifugation (100,000*g*), whereupon each phase was denatured and extracted separately with ethanol.

The quinones recovered from the ethanol extracts were purified by solvent partition and extensively chromatographed, including chromatography on a 5% $AgNO_3$–Kiesel gel column designed to remove any residual native quinone. A sample of the test quinone not subjected to the biological system (standard quinone) was chromatographed in the same manner as a control for any exchange associated specifically with the chromatographic procedure. After the recovered quinones were judged sufficiently pure (>99%) by ultraviolet comparison with the standard quinone, samples were pyrolyzed to yield carbon monoxide, and the per cent retention of isotope was calculated by comparison with the standard. A control in which oxidative phosphorylation activity was inhibited with potassium cyanide was performed to detect any irrelevant exchange not specifically associated with oxidative phosphorylation.

Materials and Methods.

^{18}O Determination. OXIDATIVE PYROLYSIS TO CARBON DIOXIDE. The requirements for an analysis in this study were that the sample size needed to be minimal ($\leq$5 mg) and that the reproducibility be maximal. These restrictions eliminated such techniques as quantitative infrared analysis, mass spectrometry of the intact quinone molecule, and activation analysis. Because of its ease of operation and widely reported applicability, ^{18}O analysis of the quinones was attempted by oxidative pyrolysis to carbon dioxide (Rittenberg and Ponticorvo, 1956, as modified in Yamamoto *et al.*, 1962) followed by mass spectrometric analysis of the carbon dioxide derived *via* reaction 1. Preliminary determinations using 2-methyl-

$$\text{C, H, O} \xrightarrow[500^\circ]{HgCl_2} \text{C} + CO_2 + HCl \downarrow_{Ag_2SO_4} \qquad (1)$$

1,4-napthoquinone (menadione) and phylloquinone indicated immediately the need for several modifications. Because of the difficulty in obtaining reproducible anhydrous mercuric chloride, the chlorine oxidizing agent was added as molecular chlorine in addition to a stoichiometric amount of mercury, thereby eliminating a

[3] After completion of this work, a publication appeared in which the yield of this phosphorylated naphthotocopherol-like derivative was only ~0.5% (Watanabe and Brodie, 1966).

TABLE I: Analysis of Quinones-^{18}O by Oxidative Pyrolysis.

Compound	^{18}O Found (%)	^{18}O Theor (%)
Menadione	16.2 ± 0.4[a]	
Phylloquinone[b]	13.4 ± 0.7[a]	16.2
Menadione + HCl	13.7	16.2

[a] Each value is an average of five determinations. [b] Phylloquinone synthesized from the 16.2% menadione-^{18}O. No isotope loss should occur during the synthesis.

TABLE II: Modifications of ^{18}O Analysis of Phylloquinone by Oxidative Pyrolysis.

Pyrolysis Tube	Modification	% ^{18}O[a]
Pyrex		19.4 ± 0.8
Vycor		19.3 ± 0.8
Quartz		22.6 ± 0.7
Quartz	No Hg	22.4 ± 0.7
Pyrex	No Ag_2SO_4	19.9 ± 0.9

[a] Average of at least five determinations per experiment.

large source of scatter. In terms of purity and yield the carbon dioxide gas derived from pyrolysis of menadione was quite sufficient for analysis; however, when the method was applied to an equimolar amount of the higher molecular weight phylloquinone, the carbon dioxide yield dropped dramatically and gross contamination of the sample with propane (*m/e* 44) was obvious. From consideration of the chemistry involved the two observations were related: the amount of added chlorine was insufficient to oxidize completely the organic hydrogen present so that thermally derived hydrocarbons (*e.g.*, propane) were not oxidized and, in addition, excess hydrogen (also thermally derived) reduced the product carbon dioxide, $CO_2 + H_2 \rightarrow CO + H_2O$. A simple increase to a stoichiometric amount of chlorine relative to hydrogen solved both problems, but this modification only served to make the real deficiencies of the analysis apparent. These difficulties are illustrated in Table I.

The scatter shown was observed with both menadione and phylloquinone, although it was invariably greater with the latter. In order to maximize the significance of the experiment to which this analytical method would be applied, such scatter should preferably be limited only by the mass spectral capabilities (in this case ±0.2%). The second observation from Table I is that the ^{18}O content of phylloquinone was invariably 15–20% lower than the menadione from which it was prepared. While such an effect is not particularly significant in terms of this study since the major interest is in reproducibility and not accuracy, it is nonetheless symptomatic of the primary problem. In considering the differences between the two samples, it is obvious that on a mole basis pyrolysis of phylloquinone should yield approximately six times as much hydrogen chloride as its precursor menadione. To test the suspicion that this additional hydrogen chloride was responsible for the apparent isotope loss, menadione was pyrolyzed in the presence of a 40-fold molar excess of hydrogen chloride with a resultant decrease in apparent ^{18}O content similar to that observed with phylloquinone.

Relevant to the major criticism of the method, namely the intolerable scatter, several studies, as reported in Table II, were made in an attempt to identify and minimize the source of this scatter. Variation in the material of construction of the pyrolysis tubes from Pyrex to vycor to quartz, while resulting in an increase in the apparent ^{18}O content with quartz, left the scatter essentially unaffected. The removal of the mercury as a potential catalyst for exchange had no effect. Concern that traces of water adsorbed on the silver sulfate might exchange with carbon dioxide under the acidic conditions led to a removal of that step and substitution of a gas–liquid partition chromatography purification step to remove hydrogen chloride, but again there was no improvement. We finally concluded that the exchange was associated with the glass and acidic conditions and therefore unavoidable. Thus, this analytical method suffers from an unavoidable scatter and a measurement of ^{18}O content which is a function of the amount of hydrogen present in the molecule.[4]

PYROLYSIS TO CARBON MONOXIDE. An alternative which involves conversion of the quinone oxygen into a simple molecule whose ^{18}O content may be assayed by mass spectrometry is the nonoxidative pyrolysis to yield carbon monoxide. Difficulties anticipated were (1) the presence of background nitrogen and (2) ethane and ethylene contamination in the *m/e* 28–30 region. Samples (10–20 μmoles) were pyrolyzed for 3 hr at 500°. The pyrolysate gases were cooled with liquid nitrogen and the noncondensable gases (mostly carbon monoxide, methane, and hydrogen) were examined by mass spectrometry. Background nitrogen contamination of the *m/e* 28 peak was slight (<3%) and could be quantitatively monitored by assuming a constant ratio to the background oxygen peak. Hydrocarbon contamination which could be monitored from the *m/e* 27 and 26 region was usually minimal (<1%) when the pyrolysis gases were carefully frozen. Table III illustrates both the precision and accuracy of the method.

Synthesis of Test Quinones. KINETIC EXCHANGE STUDY. In order to determine the feasibility of selectively labeling menadione at the 1 and 4 positions by simple exchange, a study of the exchange properties of 1,4-naphthoquinone and 2,3-dimethyl-1,4-naphthoquinone was

[4] Similar criticisms will apply to a recently published deuterium analysis employing the same oxidative pyrolysis (Eisenberg, 1966).

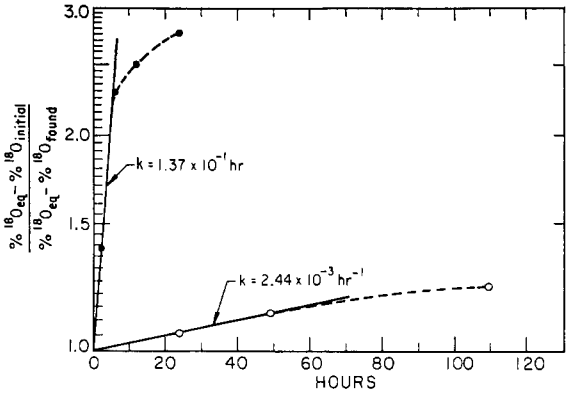


FIGURE 1: Carbonyl-oxygen exchange rates of 1,4-naphthoquinone (—●—) and 2,3-dimethyl-1,4-naphthoquinone (—○—).

conducted. A comparison of these model compounds should give at least a measure of the relative exchange rates at the two carbonyl functions in menadione. The quinones and $H_2{}^{18}O$ (1.7%) were solubilized in tetrahydrofuran and exchange was initiated by the addition of a catalytic amount of sulfuric acid. Exchange did not occur to a measureable extent under neutral conditions. The quinones were recovered, purified by sublimation, and assayed for ^{18}O content by pyrolysis to carbon monoxide. From Figure 1 it can be seen that neither of the two model compounds obeys theoretical first-order kinetics and that 1,4-naphthoquinone deviates to a greater extent than 2,3-dimethyl-1,4-naphthoquinone. Presumably, the acid catalyst is being consumed by the quinones and possibly by the solvent. However, from a comparison of initial rates it is seen that the unhindered carbonyl function exchanges over 50 times faster than the hindered one, a difference which for this study was sufficient to be exploited for the preparation of essentially specifically labeled quinone.

SYNTHESIS OF PHYLLOQUINONE-4-^{18}O. Menadione-4-^{18}O was prepared under exchange conditions identical with those of the preceding study. The exchange was allowed to proceed for 24 hr, at the end of which time the recovered quinone had a total of 37.5% ^{18}O. Calculation of the relative isotope distribution between the two carbonyl functionalities was based on the exchange rate ratio of 56 for unhindered to hindered carbonyl; at position O-1, per cent ^{18}O = 1.3 and per cent of label = 1.8; at position O-4, per cent ^{18}O = 73.7[5] and per cent of label = 98.2. The menadione was then converted to the corresponding phylloquinone by boron triflouride–etherate-catalyzed condensation of the hydroquinone with phytol. As shown in Table III, the reaction proceeded without loss of isotope.

SYNTHESIS OF PHYLLOQUINONE-1-^{18}O. By a simple permutation of the previously described exchange sequence, menadione predominately labeled in the 1 position was obtainable. If uniformly ^{18}O-labeled menadione were available, then a washing-out procedure with water would yield the desired enrichment. Test studies of exchange conditions indicated that tetrahydrofuran–water–sulfuric acid exchange conditions were insufficient to reach equilibrium at the hindered positions; however, exchange of both oxygen functions in the molecule was complete after 3 hr in refluxing tetrahydrofuran with boron trifluoride–etherate as catalyst. Using this process and $H_2{}^{18}O$ (97%), menadione uniformly labeled to the extent of 76.0% ^{18}O was prepared. This was subsequently exchanged twice with water under the conditions of the kinetic study. Calculation of the isotope distribution in the resulting quinone (39.5% ^{18}O) was again accomplished by simultaneous consideration of the previously established rate ratio for hindered to unhindered carbonyl exchange and the initial and final ^{18}O contents;[6] at position O-1, per cent ^{18}O = 74.8 and per cent label = 94.7; at position O-4, per cent ^{18}O = 4.2 and per cent label = 5.3. The menadione was then converted into the corresponding phylloquinone without loss of isotope as assayed by pyrolysis to carbon monoxide.

TABLE III: Analysis of Quinones-^{18}O by Pyrolysis to CO.

Compound	^{18}O Found (%)[a]	^{18}O Theor (%)
Menadione-4-^{18}O	37.5 ± 0.2	
Phylloquinone-4-^{18}O[b]	37.6 ± 0.2	37.5 ± 0.2

[a] Average of five determinations per sample. [b] Synthesized from the 37.5% menadione-^{18}O.

SYNTHESIS OF PHYLLOQUINONE-1,4-$^{18}O_2$. For those experiments in which the added sensitivity gained by using specifically labeled quinone was deemed unnecessary, uniformly labeled phylloquinone was used. Because of the poor yield involved in synthesis from menadione, the most efficient means of preparation of this compound might be *via* direct exchange on phylloquinone itself. Since both carbonyls are hindered, conditions at least as severe as those used to prepare uniformly labeled menadione were necessary. To achieve maximum solubility, the solvent was changed to dioxane, and using $H_2{}^{18}O$ (~8%) exchange was found to be complete after 3 hr at reflux. Although some decomposition occurred the product phylloquinone (7.0% ^{18}O) was obtainable pure after chromatography.

Results

The values for phosphate fixation coupled to oxidation in the isotope experiments are presented in Table IV and the extent of retention of ^{18}O label is found in Table V.

[5] Theoretical for complete exchange was 81%, *i.e.*, exchange proceeded to 91% of completion.

[6] A small difference between the distribution of label in these quinones as presented here and in the preliminary communication results from a slightly different interpretation of the rate data.

TABLE IV: Oxidative Phosphorylation with Intact and Light-Inactivated Reconstituted[a] Extracts[b] of *M. phlei*.

Expt[c]	System	Extract	Quinone Added	Duration	ΔP_i (μmoles)	O_2 (μ-atoms)	P/O
1	Warburg[d]	Standard	None	12 min	10.1	11.4	1.0
		Inactivated	None	12 min	3.9	4.0	1.0
		Inactivated	Phylloquinone-4-^{18}O	12 min	11.8	10.9	1.1
	Macro[e]	Inactivated (76 ml)	Phylloquinone-4-^{18}O	1.5 hr	620		
2	Warburg	Standard	None	12 min	10.0	10.2	1.0
		Inactivated	None	12 min	2.6	1.2	
		Inactivated	Phylloquinone-1-^{18}O	12 min	8.8	7.0	1.3
	Macro	Inactivated (100 ml)	Phylloquinone-1-^{18}O	2.5 hr	1170		
3	Warburg	Standard	None	12 min	8.7	12.6	0.7
		Inactivated	None	12 min	1.8	2.1	0.9
		Inactivated	Phylloquinone-1,4-$^{18}O_2$	12 min	5.2	7.7	0.7
	Macro[f]	Inactivated (91 ml)	Phylloquinone-1,4-$^{18}O_2$	3 hr (N_2), then 3 hr (O_2)	180 750		
4	Warburg	Standard	None	12 min	16.2	15.0	1.1
		Inactivated	None	12 min	8.1	9.2	0.9
		Inactivated	Phylloquinone-1,4-$^{18}O_2$	12 min	14.0	14.2	1.0
		Inactivated + KCN	Phylloquinone-1,4-$^{18}O_2$	12 min	3.0	0	
	Macro	Inactivated + KCN (38 ml)	Phylloquinone-1,4-$^{18}O_2$	24 hr	130		

[a] Extracts (20–28 mg of protein/ml) at 0° were inactivated by 25-min exposure to two 15-W General Electric black lights (long-wavelength ultraviolet). Phylloquinone (1 mg/ml) was incorporated as an emulsion formed by sonication in a portion of light-inactivated extract at 0°. [b] All systems in each experiment were aliquots of the same bulk cell-free bacterial extract; inactivated systems were aliquots of a light-treated portion of this bulk extract. [c] Substrate was pyruvate in expt 1, 2, and 4, and malate in expt 3. [d] Warburg systems contained components described previously (Brodie and Gray, 1956) (extract volume 2.4 ml). [e] Same components as above in amounts proportional to indicated volume; the reaction flask was shaken at 30°. The reaction was followed by periodic phosphate analyses and terminated by centrifugation to separate particles and supernatant and addition of 95% ethanol. [f] Proceeded anaerobically for 3 hr in a nitrogen atmosphere, then aerobically for 3 hr.

Several observations should be made before reaching any conclusions from the results. Examination of Table IV will show a time discrepancy between the macro and Warburg system in that the macro system usually took five to ten times as long to consume the same fraction of phosphate as did the Warburg system. This difference is ascribed to a smaller surface to volume ratio requiring, in the macro system, that aeration proceed at a slower rate than in the Warburg. This should not be detrimental since presumably the phosphate is fixed by the same time-independent mechanism in both cases and may even be advantageous in that it would allow more time for phylloquinone exchange at the active site.

The anaerobic–aerobic experiment was performed using uniformly ^{18}O-labeled phylloquinone since the fraction of quinone involved was expected to be great enough (~20%) so that added sensitivity gained from selective labeling was unnecessary. It should be noted that during the anaerobic portion of the cycle, 15–20% of the available phosphate was consumed; this phosphate uptake could have occurred *via* electron transport because of the availability of substrate amounts of phylloquinone to act as an electron acceptor, thus allowing part of the oxidative phosphorylation cycle to proceed. A similar observation can be made for the potassium cyanide control (expt 4).

Although not reported in Table IV, no difference in rate of oxidative phosphorylation activity could be observed for the ^{18}O-labeled phylloquinones relative to phylloquinone itself. The possibility of a discriminatory isotope effect in these experiments was felt to be negligible.

As can be seen from Table V, no significant loss of isotope occurred in expt 1, 2, or 3. The small loss observed was roughly proportional to the time of exposure of quinone to the aqueous medium. Using 2,3-dimethyl-1,4-naphthoquinone and a tetrahydrofuran–water solvent system, no exchange of the hindered carbonyls could be detected at room temperature after a week in the absence of an acid catalyst. However, such results cannot be transferred directly to the phylloquinone–*M. phlei* system since (1) the concentration of water surrounding the quinone could be much greater, especially in the case where quinone has essentially been sol-

TABLE V: Retention of ^{18}O Label of Quinone during Oxidative Phosphorylation with Extracts of *M. phlei*.

Expt	Quinone Isolated	Duration (hr)	Phase	% Purity[a]	% Retention of ^{18}O[b]
1	Phylloquinone-4-^{18}O	1.5	Particles	99.5	100
			Supernatant	100.5	100
2	Phylloquinone-1-^{18}O	2.5	Particles	99.6	98.4
			Supernatant	100.0	99.3
3	Phylloquinone-1,4-$^{18}O_2$	6	Particles	100.7	98.9
			Supernatant	100.9	98.7
4	Phylloquinone-1,4-$^{18}O_2$	24	Particles + supernatant	99.6	94.9

[a] Standard deviation is ±0.4%. Both the purity and percentage retention of ^{18}O were determined by comparison with a standard quinone sample which was subjected to purification procedures identical with the test quinones. [b] Standard deviation is ±0.3%; ^{18}O: phylloquinone-4-^{18}O, 74%; phylloquinone-1-^{18}O, 75%; phylloquinone-1,4-$^{18}O_2$, 7%.

ubilized by the particles, and (2) some protein factors could act as nonspecific catalysts for this type of exchange. Indeed expt 4 indicates a background exchange not associated with oxidative phosphorylation of a magnitude sufficient to explain that observed in the previous three experiments. To enhance this effect, the control experiment was run for 24 hr; the particulate and supernatant phases were not separated since by that time equilibration between the two phases would have occurred. Because of the uncertainities involved with the ^{18}O determinations themselves, the rate of quinone $\rightleftarrows$ water exchange associated with the particles *vs.* supernatant, and the rate of phylloquinone exchange between the two pools, it is particularly difficult to put such a small-order correction factor on a quantitative basis. However, one can easily estimate that if any quinone exchange is involved in expt 1, 2, and 3, it is less than 1%.

Conclusions

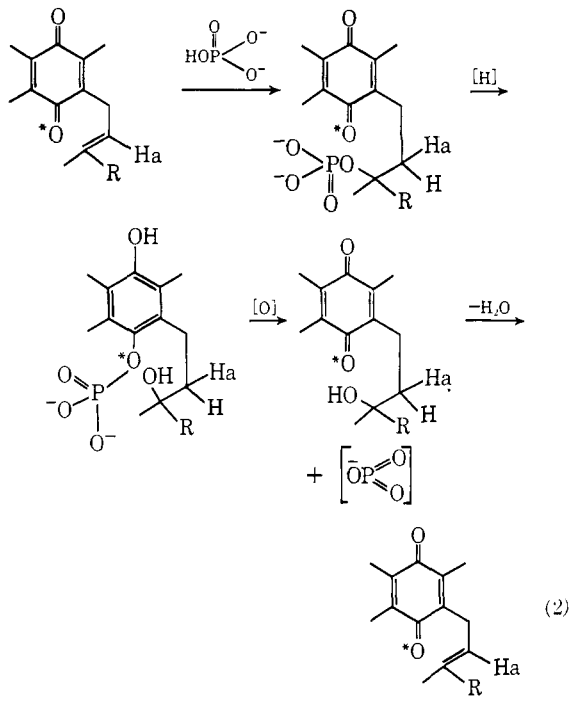
The results of these experiments indicate that if quinone is involved in oxidative phosphorylation in such a way as to require carbon–oxygen bond cleavage, it is so involved to an extent of <1%. To obtain an over-all picture of the direct quinone involvement in phosphate fixation in oxidative phosphorylation, these results should be compared to the analogous findings of the preceding paper in which carbon–hydrogen bond breakage of phylloquinone as measured by deuterium loss is less than 1% and as measured by tritium incorporation is less than 0.1%. Taken in concert these results require that either (1) if quinone is involved *via* a mechanism requiring carbon–hydrogen or carbon–oxygen exchange it must be involved to an extent of less than 1%,[7] or (2) quinone is involved in oxidative phosphorylation but in a manner not requiring carbon–hydrogen or carbon–oxygen bond cleavage, or (3) quinone is not directly involved in phosphate fixation in oxidative phosphorylation.

Of these three possibilities, the last seems most unlikely since, as previously discussed,[2] other evidence for quinone involvement is strong. The possibility of only a very small fraction of either exogenous or endogenous quinone being involved in oxidative phosphorylation is real although the presence of a large amount of native quinone seems to argue for its involvement. One can speculate concerning the experimental detection of less than 1% of quinone involvement by isotopic techniques; however, the difficulties involved are mammoth. This would require not only more sensitive isotopic analyses, purification techniques, and purity criteria but also more sophisticated controls for quinone involvement not associated with oxidative phosphorylation. In the deuterium and ^{18}O experiments where a difference between a standard and test quinone is being measured, an increased sensitivity to isotopic exchange is dependent upon both increased sensitivity to isotopic change and increased assurances of purity since the two are related in any isotopic analysis which is sensitive to contamination. In attempting to push the limits of this analysis the need for a careful and quantitative control for irrelevant exchange becomes obvious as illustrated in the ^{18}O work. Probably the most sensitive experiments in this series are the studies of tritium incorporation into the native quinone, and the significance of the 0–0.5% apparent incorporation observed here can be challenged by the possibility of undetectable contamination or a background of *de novo* native quinone biosynthesis. Control for the latter would be particularly difficult since biosynthesis would require the operation of oxidative phosphorylation. Thus, in the face of negative results at the 1% or less level, further applicability of these isotopic techniques is ineffectual.

All proposed mechanisms for quinone involvement require carbon–hydrogen or carbon–oxygen bond cleav-

[7] As pointed out in the preceeding paper (Di Mari *et al.*, 1968) still admissible is stereospecific carbon–hydrogen bond cleavage with no hydrogen exchange at the side-chain β carbon.

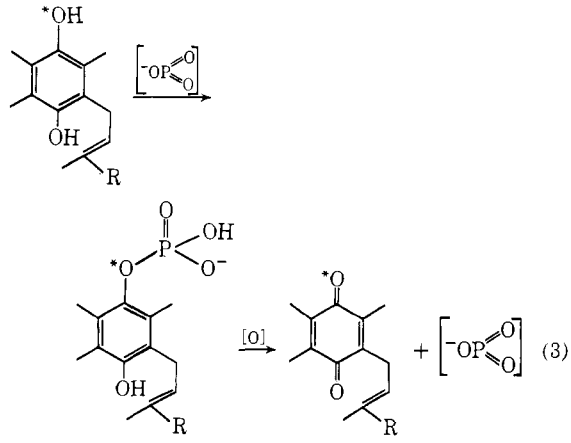
age, and since our experiments cannot detect such cleavage, the alternative of other mechanisms should now be considered. Such a possible mechanism for involvement of quinone as an active intermediate is outlined in reaction 2 in which inorganic phosphate adds across the β,γ-



(2)

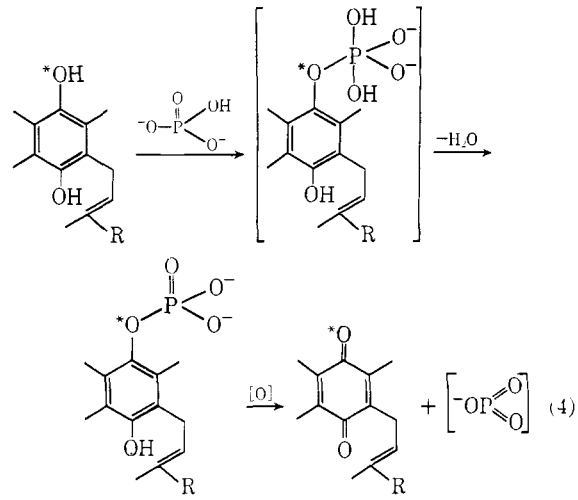
double bond, migrates to the 4-oxygen upon reduction, and then separates as metaphosphate upon oxidation of the quinol to a γ-hydroxyquinone. The γ-hydroxyquinone can be stereospecifically dehydrated to quinone to complete the cycle. Since all quinone bonds remain intact through the cycle, the involvement of the 4-oxygen or the β-methine hydrogen could not be detected by isotopic methods.

Other consistent alternatives could involve the quinone simply as a phosphate carrier which accepts metaphosphate already generated from another source. Quinol could be esterified with metaphosphate and then oxidized to regain metaphosphate and quinone with the result that the original quinone bonds remain intact (reaction 3). Or similarly, quinol could be phosphorylated



(3)

chemiosmotically so that the elements of water would be lost from the phosphate moiety (reaction 4). However



(4)

no evidence is presently available to support any of these alternatives and the answer to the question of quinone involvement in oxidative phosphorylation *via* these and other yet to be envisioned mechanisms will have to await the application of other more sensitive and sophisticated techniques.

Experimental Section

Bacterial Test System. The preparation of extracts, reconstitution of light-treated extracts, methodology of the biological experiments, and isolation and purification of the added quinones were performed as before.[2]

^{18}O Analysis. OXIDATIVE PYROLYSIS TO CARBON DIOXIDE. Modification using chlorine gas. Phylloquinone (5 mg) or menadione (2 mg) and mercury (5 μl) were added to a pyrolysis tube which was evacuated to less than 1 μ. Research grade chlorine gas (10 cc) (Matheson) (at atmospheric pressure) was then added by vacuum transfer techniques and the pyrolysis tube was sealed. Analysis proceeded as described (Rittenberg and Ponticorvo, 1956) employing a Consolidated Electronics Corp. 130 mass spectrometer.

Modification using no mercury. Phylloquinone (5 mg) was added to a pyrolysis tube and evacuated to less than 1 μ. Chlorine (6 cc) was added *in vacuo* and the pyrolysis tube sealed. Mass spectrometric examination showed no excess of chlorine after pyrolysis.

Modification using no Ag_2SO_4. Menadione or phylloquinone was pyrolyzed using excess chlorine with mercury as a scavanging agent. The pyrolysate gases were chromatographed by gas–liquid partition chromatography (6 ft × 0.25 in. column of 20% *n*-nonane on 60–80 mesh firebrick, −78°) to yield pure CO_2 which was assayed for ^{18}O content. Mass spectrometric examination of pyrolysis gases from a menadione sample before and after chromatography showed the same isotopic content, indicating no exchange associated with the column. A similar test using an alternate column of 60–80 mesh silica gel poisoned with 1% dioctyl phthlate at room temperature showed extensive exchange.

NONOXIDATIVE PYROLYSIS TO CARBON MONOXIDE. A 7 × 0.9 cm Pyrex pyrolysis tube was constructed with a breakseal on one end and a ground-glass joint on the other. The quinone sample (10–20 μm) was placed in the tube which was evacuated to less than 1 μ and sealed. After a 3-hr pyrolysis at 500°, the cooled pyrolysis tube was placed in the inlet tube to the mass spectrometer, the breakseal was opened, and the pyrolysis gases were carefully frozen with liquid nitrogen. The uncondensed gases remaining were then admitted into the mass spectrometer. The ^{18}O content of the carbon monoxide was calculated from eq 5, where [30], [29], ... are measures

$$\% \ ^{18}O = \frac{1.011[30] - 0.01\{[29] - 0.01[28]\}}{[28] + [29] + [30] + [31]} \times 100 \quad (5)$$

of the relative intensities of *m/e* 30, *m/e* 29, ...

Synthesis of Isotopically Labeled Quinones. EXCHANGE RATE COMPARISON BETWEEN 1,4-NAPHTHOQUINONE AND 2,3-DIMETHYL-1,4-NAPHTHOQUINONE. The test quinones, obtained commercially, were purified by sublimation before use. Each quinone (2.9 mmoles) was dissolved in dry tetrahydrofuran (5.5 ml) and $H_2{}^{18}O$ (1.67%, 1.0 ml) and the exchange was then initiated by the addition of sulfuric acid (25 μl). At various intervals, aliquots (0.6 ml) of the solutions were removed and added to petroleum ether (bp 30–60°) (15 ml). The petroleum ether phase was dried over magnesium sulfate, and then the solvent was removed and the quinones were purified by sublimation. The ^{18}O content of the quinones was determined by pyrolysis to carbon monoxide.

SYNTHESIS OF PHYLLOQUINONE-4-^{18}O. Menadione (1010 mg) was exchanged for 24 hr with 98% $H_2{}^{18}O$ (0.455 ml), tetrahydrofuran (10 ml), and sulfuric acid (23 μl). The recovered quinone was purified by sublimation to yield 960 mg (95%). The menadione-4-^{18}O was condensed with phytol to yield phylloquinone-4-^{18}O (Woods and Taylor, 1957). The ^{18}O content was menadione-4-^{18}O, 37.5%; phylloquinone-4-^{18}O, 37.6%

SYNTHESIS OF PHYLLOQUINONE-1-^{18}O. A solution of menadione (1030 mg), 97.5% $H_2{}^{18}O$ (1 g), BF_3–Et_2O (0.5 ml), and tetrahydrofuran (6 ml) was refluxed for 3 hr. The yield of menadione-1,4-$^{18}O_2$ after purification was 1010 mg (98%) and its ^{18}O content was measured as 76.0% (theoretical, 78.7%). The product was then subjected to a washout procedure in which it was dissolved in tetrahydrofuran (11 ml), water (2 ml), and sulfuric acid (50 μl) and allowed to stand at room temperature for 24 hr. The quinone was then reisolated and resubjected to the same treatment. The quinone was purified (yield, 975 mg, 97%) and its ^{18}O content measured (% ^{18}O = 39.5).

SYNTHESIS OF PHYLLOQUINONE-1,4-$^{18}O_2$. A solution of phylloquinone (1 g), 8.0% $H_2{}^{18}O$ (1 g), BF_3–Et_2O (0.5 ml), and dioxane (10 ml) was refluxed for 3 hr. The quinone was recovered and purified by chromatography on Kiesel gel (eluent: 10% Et_2O in petroleum ether) to yield 750 mg (75%) of pure phylloquinone-1,4-$^{18}O_2$ (% ^{18}O = 7.0).

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