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

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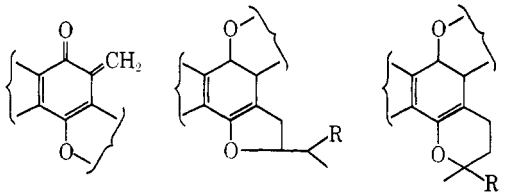
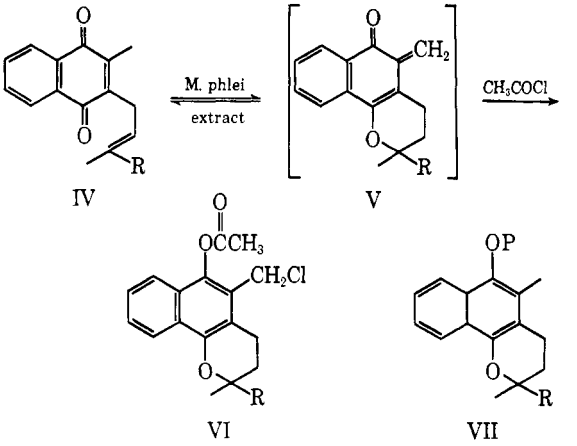
ABSTRACT: The use of hydrogen isotopes to test the mechanisms proposed to explain the function of the quinone in oxidative phosphorylation is reported. The study included the incubation in bacterial extracts prepared from *Mycobacterium phlei* of (1) both exogenous phylloquinone and the endogenous menaquinone with T_2O and (2) the synthetic phylloquinones 2-methyl-d_3-3-phytyl-1,4-naphthoquinone, 2-methyl-3-phytyl-α-d_2-1,4-naphthoquinone, and 2-methyl-3-phytyl-β-d-1,4-naphthoquinone. The syntheses of the latter quinones as well as of other isotopically labeled quinones used are described in detail. Conventional methods for analysis of isotope content are evaluated; a description of a new and highly accurate pyrolysis–mass spectral analysis for deuterium is presented. In the experiments reported, neither isotope incorporation into added or native quinones nor isotope loss from synthetic quinones was observed. The effect of these findings upon the acceptability of current mechanistic proposals for the quinone's role in oxidative phosphorylation is discussed in this paper.

Various mechanisms have been proposed to explain the role of the quinone in oxidative phosphorylation.[1] These mechanisms postulate as the active species involved in binding inorganic phosphate an isomeric form of the quinone, characterized by the structural features referred to as quinone methide (I) or dihydrofurano (II) or dihydropyrano (III) ring systems.



I II III

Several attempts have been made to demonstrate the existence of these quinone forms *in vivo*. Wagner *et al.* (1962) isolated VI after the addition of acetyl chloride to a water-free (removed *in vacuo*) *Mycobacterium phlei* system which had been light treated, reconstituted with vitamin $K_{1(20)}$, and maintained anaerobically to allow accumulation of the quinone intermediates. Consequently, VI was interpreted as the acetyl chloride adduct of enzymically formed quinone methide V. However, VI can also arise *via* reaction of quinone with acetyl chloride and a trace of water *in vitro*, and it was subsequently shown that the origin of the isolated compound was chiefly nonenzymic (Wagner *et al.*, 1963).



IV V VI VII

To avoid ambiguities arising from chemically induced artifacts, attempts were made to isolate quinone intermediates from reconstituted *M. phlei* systems without the use of trapping agents. From light-treated, vitamin $K_{1(20)}$-reconstituted bacterial systems which had been maintained anaerobically, a reduced form of the quinone was isolated (Russell and Brodie, 1961). When this compound was allowed to oxidize in air, quinone was regenerated and inorganic phosphate was released. When used as the sole source of phosphate to reconstitute a light-treated extract system, this compound supported the formation of ATP.[2]

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[1] For comprehensive presentation and discussion of the mechanisms proposed for quinone participation in oxidative phosphorylation, see Lederer and Vilkas (1966) and Brodie (1965).

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: NABS, *p*-nitroazobenzoate; TCA, trichloroacetic acid; THF, tetrahydrofuran.

On the basis of spectroscopic comparison, the compound was proposed to be similar in structure to the chromanol phosphate VII. A subsequent publication (Watanabe and Brodie, 1966) modified these findings in that (1) the yield of this reduced species isolated from the bacterial system decreased from the initially reported 20% of total added quinone to 0.3% and (2) further comparison between the material isolated and synthetic VII showed them to be different. The bulk of reduced quinone isolated was phyllohydroquinone; the identity of the phosphorylated quinone species remains unknown.

Thus there is no definite, unambiguous isolation of a quinone species containing, or derived from, the structural features of I, II, or III from either a bacterial or a mitochondrial source. If such intermediates exist, there are plausible explanations which would account for their elusiveness. For instance, even though there is a relatively high concentration of quinone in the systems studied, under steady-state conditions the amount actually participating in phosphate fixation may be extremely small. A proposal (Hatefi, 1959a,b) which would doom any attempt to isolate such intermediates no matter how extensively formed is that the function of the large amount of phospholipid associated with electron transport and oxidative phosphorylation is to insulate such intermediates from contact with water. Once this protective envelope is disrupted, *e.g.*, by solvent extraction during isolation procedures, such intermediates are immediately hydrolyzed. If this were true, there is little hope for the isolation of enzymatically altered quinones from biological systems.

Discussion

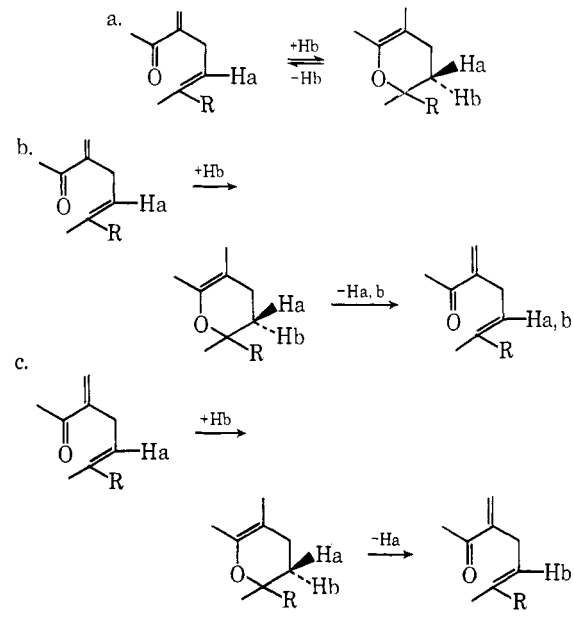
Consideration of the difficulties inherent in the isolation of reactive intermediates made it clear that an alternate approach was required to elucidate the precise role of the quinone in oxidative phosphorylation. It becomes obvious on examining structures I, II, and III that in order for the quinone molecule to assume any of these forms a cleavage of C–H bonds must occur. Consequently the use of hydrogen isotopes would serve as a direct test of those proposed mechanisms, and of any others, which require carbon–hydrogen bond cleavage and formation in the quinone molecule during respiration. In addition to its analytical sensitivity, this approach has the advantages that (1) isotopically labeled variations of the same quinone can be used, thereby eliminating ambiguities from solubility differences encountered when different quinone analogs are used, and (2) quinone, rather than an unstable or derivatized form, is isolated and analyzed, thus eliminating ambiguities arising from the possibility of artifact formation.

The experiments proposed to exploit this approach were designed to examine both the removal of isotope from a synthetic, isotopically labeled quinone molecule and the incorporation of isotope into the quinone from a source of isotope in the medium. For example, incorporation of tritium from the medium during respiration would indicate general carbon–hydrogen bond alteration within the quinone while the loss of deuterium from the synthetically labeled quinone would indicate specific sites of such alteration. The positions suspect as sites of such exchange are the 2-methyl group and the α-methylene and β-methine of the 3-side chain.

This approach eliminates difficulties encountered in previous attempts to elucidate the quinone's role in oxidative phosphorylation. However, factors which must be considered, especially in the interpretation of negative results, are (1) stereospecificity in the addition and removal of hydrogen at the double bond in the side chain, (2) the source of the hydrogen, (3) an isotope effect, and (4) the mole fraction of quinone possibly involved.

Hydrogen may be introduced into the side chain o the quinone molecule during reduction and subsequently removed upon oxidation *via* a stereospecific addition and removal of the same hydrogen (Scheme Ia), a nonstereospecific addition and removal (Scheme Ib), and a stereospecific addition of hydrogen followed by the removal of the hydrogen originally present (Scheme Ic).

SCHEME I



When H_b is tritium one should observe no incorporation of isotope if process a is the mode of reduction–oxidation, indeterminate incorporation of isotope from process b since several steps of unknown mechanism are involved, and a maximum incorporation of isotope *via* process c. Analogous considerations apply to removal of isotope from 2-methyl-3-phytyl-β-*d*-1,4-naphthoquinone. If process a is operative, no loss of deuterium from the side chain will be observed since protium added during respiration will be lost upon oxidation. If the addition and removal of hydrogens

at the site of activity is nonstereospecific as in process b, the isotope composition would be indeterminate. The exclusive operation of process c would provide the maximum loss of isotope.

In the tritium experiments the hydrogen introduced into the quinone side chain must ultimately be derived from water if incorporation of isotope is to be possible. However, other possible sources of this reducing hydrogen in the system must be considered. The concept of the electron-transport complex which depicts the various cofactors as being insulated from the surrounding aqueous milieu by lipid may require the reducing hydrogen to enter the chain by a vehicle other than water, *e.g.*, substrate. If the hydrogens used in quinone reduction are exclusively those released during oxidation of certain substrates and carried through the electron-transport system, they must equilibrate with the isotopically labeled water in order for tritium to be introduced into the quinone.

Those substrates, *e.g.*, pyruvate, which cannot donate formal hydrogen upon oxidation, can be visualized as electron donors to a possible localized protium pool which may or may not be in equilibrium with the aqueous medium. If this pool is insulated from the surrounding aqueous medium and if it is the exclusive source of reducing hydrogen, then no incorporation of tritium will be observed. The proposed deuterium experiments, in addition to identifying the site of any observable exchange, will not be subject to this ambiguity concerning the source of exchangeable hydrogen since isotope migration out of the quinone will be the observed phenomenon.

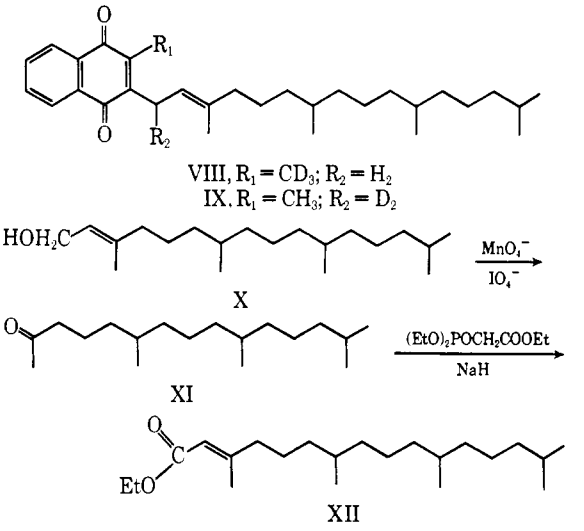
In either experiment the possibility of an isotope effect exists, and although the effect will be more discriminatory in the case of tritium it will also be nonobservable since the process is cyclic and an isotope effect which discriminates against incorporation on reversal will favor retention. The possibility of different isotope effects for addition and removal of isotope exists; however, the magnitude and direction of such a net effect are unpredictable. However, since the deuterated test quinones are all >99% D, operation of, and conditions for observing, such an isotope effect should be maximal. If carbon–hydrogen bond cleavage is the rate-determining step, the isotope effect should be reflected in a decrease in oxidative phosphorylation activity. Also, in considering the isotope effect in the experiments with the 2-CD_3 and α-CD_2 quinones, no isotopic choice is operable. Therefore, carbon–hydrogen bond breaking and making must lead to loss of deuterium. The lack of observation of such effects could occur if only a small nonequilibrating pool of the deuterated quinone were involved in oxidative phosphorylation, so that after the first cycle of respiration the deuterium label would be lost and recycling would occur with the protium compound.

In view of the above discussion, it becomes clear that neither the tritium nor the deuterium experiments can individually provide conclusive evidence on the quinone's role in oxidative phosphorylation in the advent of no observable isotope loss or incorporation. These experiments involve several factors which by their very nature could account for such negative results. However, when these two sets of experiments are considered jointly, they are complementary; the number of variables in this combination is less than in either of the component experiments. Unfortunately, even this combination does not offer definite evidence for the nonparticipation of the quinone in the biological process since there are still variables, *e.g.*, the possibility of process a, which are common to both and which could be invoked to explain negative results.

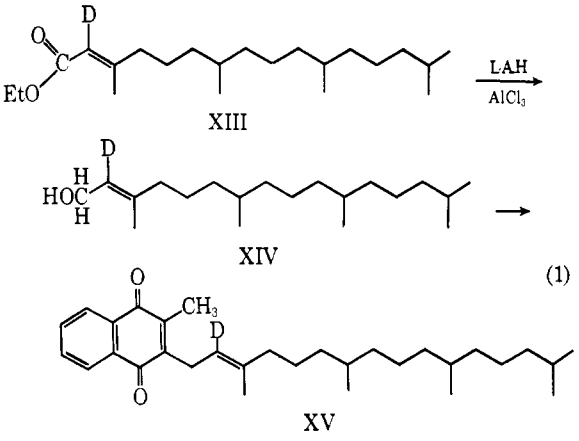
Synthesis of Test Quinones

All of the deuterium- and tritium-labeled quinones used in this study were prepared *via* modifications of standard synthetic procedures.

The syntheses of 2-methyl-d_3-3-phytyl-1,4-naphthoquinone (VIII) and of 2-methyl-3-phytyl-α-d_2-1,4-naphthoquinone (IX) have been described elsewhere (Di Mari *et al.*, 1966). An alternate route was also used to produce ethyl phytenate (XII) from phytol. This consisted in the periodate–permanganate cleavage of phytol followed by condensation of the resulting C_{18} ketone, XI, with triethyl phosphonoacetate-d_2. With the previously reported (Jackman *et al.*, 1965) use of excess sodium hydride the β,γ-unsaturated isomer was formed as a by-product. However, by using an excess of the phosphonate only the desired α,β-unsaturated compound was formed as a mixture of *cis* and *trans* isomers (2:3).



The synthetic route devised for the preparation of 2-methyl-3-phytyl-β-*d*-1,4-naphthoquinone (XV) is shown below in reaction 1. Two different routes were used to prepare the required ethyl phytenate-α-*d* (XIII), one *via* triethyl phosphonoacetate-α-d_2 and the other *via* ethoxyacetylene-1-*d*. In the phosphonoacetate approach, the α-methylene protons of XVI are sufficiently acidic to exchange with deuterium from D_2O. This exchange occurs at room temperature rapidly under mild alkaline catalysis (Na_2CO_3, $(CH_3)_3N$), slowly at neutral pH (10% isotope incorporation after 18 hr), and to no observable extent at acidic pH. The criterion used for complete equilibration of the triethyl



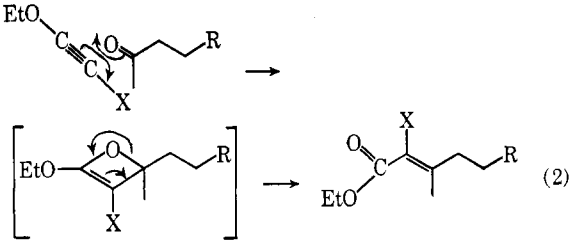
(1)

$$(EtO)_2POCH_2COOEt \xrightarrow{D_2O} (EtO)_2POCD_2COOEt$$
XVI XVII

phosphonoacetate with D_2O was the disappearance of the α-methylene protons of the reagent (δ 2.85, d, J = 21 cps) from the nuclear magnetic resonance spectrum. By repeated additions of D_2O and carbonate (to maintain pH since alkali was being consumed by slow ester hydrolysis) phosphonate reagent was prepared from which the methylene absorption in the nuclear magnetic resonance had completely disappeared. Using this phosphonate and sodium hydride or sodium to effect condensation with the C_{18} ketone, XI, consistently produced ethyl phytenate (XIII) of 98–99 mole % D.[3] The protium which is still found in the product (since the D_2O used was 99.8 mole % D, complete exchange should have given 99.8 mole % D) is believed to have arisen, not from incomplete labeling of the phosphonate reagent, but from H_2O produced *via* aldol condensation of the ketone under the basic conditions of the condensation.[4]

In the ethoxyacetylene route, isotopic labeling of ethoxyacetylene was in direct analogy to the reported preparation of DC≡CSEt (Hogeveen and Drenth, 1963). The ethoxyacetylene was added to a solution of butyllithium in toluene and the precipitated lithium salt was hydrolyzed with D_2O. Distillation produced ethoxyacetylene-1-*d* in a solution of hexane (present in the butyllithium reagent) which was used in this form for the ensuing condensation with the C_{18} ketone, XI.

One of the mechanisms proposed for the BF_3–etherate-catalyzed formation of esters directly from the condensation of ethoxyacetylene with carbonyl compounds (Vieregee *et al.*, 1966) is reaction 2. If this mechanism is correct, condensation of ethoxyacetylene-1-*d* (X = D) with the C_{18} ketone should produce the desired deuterium labeled ester, XIII. This was the



(2)

case. Reaction of the deuterium-labeled acetylene with the ketone yielded ethyl phytenate (XIII) with a deuterium content of 99.5 mole % in the vinylic position. The high degree of isotope present in the ester obtained from this reaction is probably a direct result of the acidic character of the condensation reaction. Under these conditions, the possibility of the presence of labile protium sources generated by ketone aldol condensations is minimized.

Ester produced *via* this condensation was used to complete the synthesis of 2-methyl-3-phytyl-β-*d*-1,4-naphthoquinone (XV) by reduction with lithium aluminum hydride and condensation of the resulting phytol-2-*d* (XIV) with 2-methyl-1,4-naphthoquinone. The resulting quinone had the same isotope content as the starting ester.

Two additional deuterated phylloquinones were prepared, the 2-methyl-*d* and the 2-methyl-d_2 compounds. These were used in the development of analytical nuclear magnetic resonance techniques for analysis of the deuterium content of the 2-methyl group. The synthetic routes and conditions were the same as those previously reported (Di Mari *et al.*, 1966), using lithium aluminum hydride or deuteride at the appropriate steps.

Also, tritiated phylloquinone-5,6,7,8-t_4 was synthesized in the same manner and under the same conditions reported for phylloquinone-5,6,7,8-d_4 (Di Mari *et al.*, 1966), using T_2O in place of D_2O.

Separation of cis and trans Isomers. During the course of these syntheses, *cis–trans* isomer mixtures were encountered at three stages in the sequence, *viz.*, ethyl phytenate, phytol, and phylloquinone. Separations were effected at each stage in order to evaluate the best procedure for reaching our ultimate goal, isomerically pure quinone.

1. ETHYL PHYTENATE. The reported (Jackman *et al.*, 1965) separations of *cis*- and *trans*-ethyl phytenate using gas–liquid partition chromatography and alumina chromatography were found to be unsuitable for our large-scale needs. A convenient system was devised that consisted in chromatographing the ester mixture in 2% ethyl ether in pentane on a Kiesel gel column containing 1% each of Mn-activated zinc silicate and Ag-activated ZnS–CdS (Silvania phosphors 160 and 122, respectively). The esters were clearly visible under ordinary light as a broad, bright white band, the forefront of which contained the *cis* isomer. The progress of the esters could be followed during the chromatography, and the broad band obtained was readily fractionated into pure isomers.

2. PHYTOL. The procedure devised for the separation

[3] The mole % D reported for ethyl phytenates in this series was determined by adding a known mole % of a standard compound, ethyl β-methylcinnamate (vinyl proton, δ 6.02), to a sample of the ester followed by planimetric comparison of the vinylic absorptions observed in nuclear magnetic resonance spectra of the mixtures.

[4] A similar observation has been made in the case of ketones used in Wittig reactions (W. Bondinell and H. Rapoport, these laboratories).

TABLE I: Partition of Phylloquinone between Supernatant and Particulate Phases of *M. phlei* Extracts.

System	Vol of Extract (ml)	Amt of[a] Quinone Added (mg)	Amt of Quinone Recov from Particles (mg)	% Yield from Particles	% Yield from Supernatant[b]
I	5.0	11.5	0.9	8	92
II	5.0	5.0	0.8	16	84
III	5.0	1.0	0.4	44	56

[a] Tritiated phylloquinone was used for these experiments; weights of recovered quinone were determined by liquid scintillation counting. [b] Recovery of added quinone was *ca.* 80%.

of the isomeric phytols was (a) conversion of the mixture into the *p*-nitroazobenzoate (NABS) esters by reaction with the acid chloride and pyridine; (b) chromatography of the bright orange derivatives on activity III neutral alumina, impregnated with 5% $AgNO_3$; and (c) removal of the NABS residue *via* alkaline hydrolysis. Both the formation and the hydrolysis of the NABS derivative of phytol were quantitative, and neither of these operations caused isomerization of the carbon–carbon double bond in the alcohol. The isomeric NABS esters were not separated into discrete bands on the chromatographic system used; however, they were well fractionated within the broad band obtained.

QUINONES. The various quinones which were used in the bacterial test system were separated into their *cis* and *trans* isomers *via* preparative thin-layer chromatography using the Kiesel gel, *n*-butyl ether–hexane system described by Jackman *et al.* (1965). The *trans* isomers were removed from the plate and eluted from the adsorbant with acetone, the solvent was removed *in vacuo*, and the resulting quinone was chromatographed on a Kiesel gel column (pentane–5% ethyl ether–pentane) to remove last traces of *n*-butyl ether.

Analytical Techniques

A. Isolation of Quinone from the Biological System. The *M. phlei* extract system used is composed of a supernatant and a particulate phase into which exogenous test phylloquinones were introduced and dispersed as an emulsion by sonication. Two distinct pools, the supernatant and the particulate, are formed. The assumed site of quinone action is the particulate phase since this is the phase with which the natural quinone is mostly associated. Therefore, to enhance the detectability of exchange in either phase, the extract was separated into its two components at the conclusion of each experiment, and quinone from each was purified and analyzed. However, two questions concerning the necessity of this separate examination of the two phases immediately arise: (1) what is the relationship between added exogenous quinone and endogenous native quinone in the particulate phase and (2) what is the nature of quinone equilibration between supernatant and particulate pools?

The rationale for the separate analysis of supernatant and particulate quinones was that if quinone action were restricted to the particulate fraction alone, the excess, nonactive quinone present from the supernatant would only dilute any transformed species. However, the two types of quinone present in the particulate phase itself, *i.e.*, enzymatically bound and randomly adsorbed, must also be considered. If, within the particle, there is equilibration between these two types of quinone, any exogenous phylloquinone which penetrates the particle can participate in the process and little dilution of isotopically altered species present in this phase would occur. However, if the molar concentration of native quinone originally present in the particulate phase represents the maximum quinone which can act and if there is no equilibration between enzyme-bound and randomly oriented molecules, the excess exogenous quinone which penetrates the particle becomes important since it would tend to serve as a diluting factor in the recovered material.

Table I presents the yields of quinone recovered from each phase of three separate extracts which had been reconstituted with different amounts of phylloquinone. The data for system I show that only 8% of the quinone recovered was associated with the particles; when the amount of initially added quinone was reduced by a factor of ten (system III), this figure increased to 44%.

Several factors were considered before choosing system II for the isotopic experiments. For reasons discussed above, it would be of value to minimize the amount of quinone associated with the particulate fraction. Factors limiting the minimal amount of quinone which can be added to test systems are: (1) a sufficient amount of quinone must be recoverable from the particulate phase to allow purification and analysis and (2) oxidative phosphorylation activity must be maximal. The first condition essentially eliminated system III since the experimental scale required to obtain sufficient particulate quinone for subsequent operations would be prohibitive. Early experiments had shown that oxidative phosphorylation activity was maximal at a quinone concentration of 1 mg/ml and, at this concentration, sufficient pure quinone could be recovered from the particulate phase of a moderate quantity of extract; con-

sequently, system II was chosen as the model for all of the experiments conducted in this series.

To determine the relationship between the concentration of added phylloquinone which is ultimately absorbed into the particles and the native quinone originally present, a large batch of nonlight-treated bacterial extract was processed to remove native quinone in a manner similar to that used for the recovery of added phylloquinone. The average concentration of native quinone found to be associated with the particulate phase of this extract was 25 μg/ml of crude extract (0.032 μmole/ml). Since the yield of phylloquinone from the particles in system II was 160 μg/ml (0.35 μmole/ml), added quinone is present in an 11-fold excess over native quinone. Thus 10% of the exogenous quinone is the maximum amount of added quinone which could be active, assuming complete destruction of native quinone by light treatment and nonequilibration of quinone at the active sites.

If only the quinone which is associated with the particulate phase is involved in oxidative phosphorylation and if there is no rapid equilibration between supernatant and particulate pools, then the separate analysis of supernatant and particulate quinones is valid. If, on the other hand, the active quinone becomes randomly distributed between these two pools during the experiment, such a separation is pointless. To investigate this question, we studied the extent of equilibration between the two observable quinone pools formed upon sonication of phylloquinone into an extract of *M. phlei*. The experiment involved preparation of electron-transport particles with which exogenous, tritium-labeled phylloquinone was associated and resuspension of these labeled particles in a supernatant fraction into which unlabeled phylloquinone had been sonicated. By separating this system after an appropriate time, the equilibration of isotopically labeled quinone between these two pools could be measured. An assay of the total quinone present in the supernatant after 1.5-hr equilibration with labeled particles showed that tritiated phylloquinone was present to the extent of 6.1% (the theoretical value for complete equilibration is 18.4%). Using the first-order exchange law, this corresponds to an equilibration rate of 0.27 hr^{-1} or a half-life of 2.5 hr. Although this indicated rapid exchange, there definitely was not complete exchange of quinone between the two pools in this time period. Thus separate examination of supernatant and particulate quinone could increase the ability to detect isotopic change. The unanswerable question remaining is whether or not that quinone which is physically associated with the active site(s) within the particle would be involved in such an exchange.

As a consequence of these results, the routine isolation procedure adopted for all experiments undertaken was (1) separation of test systems into supernatant and particulate components, (2) removal of protein and lipid factors from each by the addition of cold ethanol followed by centrifugation, (3) dilution of supernatants obtained with water and extraction with pentane, and (4) chromatography.

B. Purification of Quinones. Purification of the reisolated test quinones was pursued rigorously since the presence of impurities would confuse the analytical data and could lead to false positive results. In the tritium studies, for instance, quinone-contaminating lipid material arising from *de novo* biosynthesis during the experiment[5] could give rise to a large radioimpurity. Even a small mass of such an impurity would have an effect upon the results since a much larger portion of the hydrogen per molecule of lipid would be tritium labeled than in the recovered test quinone where isotope incorporation was anticipated at only a few sites in the molecule. For example, a 0.1% mass impurity could easily produce an apparent incorporation of several per cent in the recovered phylloquinone. Impurities would similarly cause an apparent loss of isotope in the deuterated phylloquinone studies since the analytical method of choice examines the gross sample for deuterium content. In this case, however, simple ultraviolet extinction measurements were sufficient to ascertain the purities of quinone samples (ultraviolet determinations in isooctane were performed on a Cary 14 spectrophotometer to 0.3% standard deviation).[6]

Quinones recovered from the bacterial test systems were purified repeatedly by column chromatography on Camag thin-layer-chromatography-grade Kiesel gel using several different solvent systems. Because of the fine particle size of this adsorbant, chromatographies were conducted under nitrogen pressure to ensure a reasonable flow rate. The resolution on carefully prepared columns with small ratios of compound to adsorbant (1:500) was comparable to that obtained on thin-layer chromatography. Use of column rather than preparative plates has the further advantage that contaminants associated with the Kiesel gel adsorbant are not carried with the quinone sample (upon elution of the quinone band from thin-layer-chromatography plates one frequently observes a relatively high molecular weight impurity in the quinone sample).

A particularly elusive impurity was found to be the natural menaquinone, MK-9(2H), which was not completely destroyed during light treatment. Its presence is not detected in the ultraviolet purity determination used since its ultraviolet spectrum is the same as that of $K_{1(20)}$. However, it was found that Kiesel gel impregnated with 5% silver nitrate and developed with 8% ethyl ether–petroleum ether (30–60°) completely retained the native quinone at the origin while allowing the phylloquinone to chromatograph normally.

The validity of each step used in the isolation of quinone from the initial introduction into the bacterial system to the final chromatography was checked by using the nuclear tritium-labeled phylloquinone. The maxi-

[5] The *de novo* biosynthesis of lipid material by cell-free *M. phlei* extracts during the feeding of ^{14}C-labeled methionine has been reported (Azerad *et al.*, 1967).

[6] An observation associated with these determinations was that phylloquinone, stored in the dark in the presence of air for several days, formed several per cent of a hydroperoxide (C. Snyder and H. Rapoport, these laboratories). This species has an extinction which is greater than that of phylloquinone in the 250-mμ region, thus indicating for the purified quinones, in some instances, greater than actual purity. Storage of samples under nitrogen eliminated this problem.

mum yield of purified quinone obtainable from the various experiments undertaken was *ca.* 85%. One possible explanation for the loss of about 15% was that the enzymatic system had converted this amount into an alternate form which had either not been extracted by the pentane or had remained at the top of a chromatography column as a polar residue. Therefore, tritium-labeled quinone was added to a light-treated system in the same manner used for test quinones and the system was allowed to respire. At the conclusion of the experiment, the quinone was reisolated and purified *via* the above procedure. All residues including precipitated protein and lipid and spent column materials were extracted with boiling chloroform to remove organic material. All were evaporated and then were counted in scintillation solution; none showed any significant radioactivity. As a result it appears that unrecovered quinone was not left behind in any one place but was lost to a small extent at various steps in the purification procedure.

C. Tritium Analysis. Quinone samples were assayed for tritium using a Nuclear-Chicago Mark I liquid scintillation computer. Sample quenching was determined by the external standard attachment of this instrument. Low-activity samples were compared with a background sample which contained an equimolar amount of unlabeled phylloquinone and were counted for at least 10 hr each to ensure a standard deviation per sample of less than 1 cpm.

D. Deuterium Analysis. Various methods were evaluated in order to find a highly accurate method for deuterium analysis of the quinones. These included nmr, mass spectrometry of the intact quinone molecule, and mass spectrometry of degradation products of the quinone.

NUCLEAR MAGNETIC RESONANCE. The use of nuclear magnetic resonance was hampered by the size of the sample available after recovery from the biological test system. Spectra obtained with samples of this size (~8 mg) were not definitive because of the high noise levels. Using standard samples of 2-methyl-d_2-3-phytyl-1,4-naphthoquinone in 2-methyl-d_3-3-phytyl-1,4-naphthoquinone to evaluate the efficiency of protium detection, the best results were obtained with an HR-60 spectrometer with an NMR Specialities heteronuclear decoupler. As seen in Figure 1, 10% protium could be readily detected.

MASS SPECTROMETRY OF VITAMIN $K_{1(20)}$. Attempts to analyze the intact vitamin $K_{1(20)}$ molecule were largely unsuccessful since the ready loss of side-chain protons obscured the areas of analytical interest centered on either side of the molecular ion. Best results were obtained by using a combination of low voltage (10 eV electrons) and direct insertion (Di Mari *et al.*, 1966); by averaging the results of several scans, a minimum of 5% protium could be detected in standard mixtures of deuterated and partially deuterated quinone.

Both the nuclear magnetic resonance and the mass spectral methods could be used to detect the presence of protium in deuterium-labeled quinones; however, isotope loss from quinones reisolated from the biological test system could easily be less than the 5% lower limit. Therefore, an alternate method of analysis was devised to increase the sensitivity of detection.

MASS SPECTROMETRY OF DEGRADATION PRODUCTS. Since mass spectrometry of the intact quinone proved unsuccessful, pyrolysis of the quinone followed by mass spectral analysis of the pyrolytic products was examined. The reported oxidative pyrolysis of compounds to water followed by reduction of the water to produce hydrogen (Graff and Rittenberg, 1952) was rejected as an analytical procedure because of difficulties involving sample size, memory effects, sample handling, mass spectral correction factors, and limited precision. A recently reported oxidative degradation using chlorine to produce hydrogen chloride which is then reduced to hydrogen (Eisenberg, 1966) was also insufficient for our purposes because of the large correction factors required and the reported imprecision.

A technique which was then examined was the nonoxidative pyrolysis to yield a smaller, hydrogen-containing species which would possibly reflect the deuterium content of the original molecule. Upon pyrolysis, phylloquinone was found to produce carbon monoxide, hydrogen, carbon, water, methane, and assorted alkanes. Methane was chosen for this analysis since it could be easily purified by freezing the mixture obtained in liquid nitrogen, methane, carbon monoxide, and hydrogen then being the only compounds of sufficient vapor pressure for mass spectrometric analysis. Unfortunately, the fragmentation of methane cannot be completely prevented even at low voltages; therefore, in order to calculate the exact deuterium content of the methane produced, correction must be made for this fragmentation. Using the reported (American Petroleum Institute, 1950) fragmentation patterns for CD_4, CHD_3, CH_2D_2, and CH_3D and correcting for the abundance of ^{13}C, eq 3 was derived. In this equation,

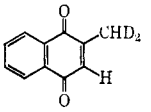
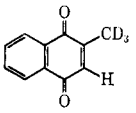
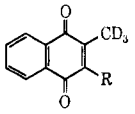
$$\begin{aligned}
[CD_4] &= [20] - 0.011[19] \\
[CHD_3] &= [19] - 0.011[18] \\
[CH_2D_2] &= [18] - 0.83[CD_4] - 0.28[CHD_3] - 0.011[17] \\
[CH_3D] &= [17] - 0.51[CHD_3] - 0.62[CH_2D_2] - 0.011[16] \\
[CH_4] &= [16] - 0.13[CD_4] - 0.13[CHD_3] - 0.31[CH_2D_2] - 0.77[CH_3D] - 0.011[15]
\end{aligned} \tag{3}$$

[20], [19], . . . are the relative intensities of *m/e* 20, *m/e* 19, . . . and then

$$\%D = \frac{4[CD_4] + 3[CHD_3] + 2[CH_2D_2] + [CH_3D]}{4([CD_4] + [CHD_3] + [CH_2D_2] + [CH_3D] + [CH_4])} \times 100$$

When this method was applied to the determination of

TABLE II: Isotope Contents of Various Deuterium-Labeled Naphthoquinones.

Compound	% D[a] Theor	Construction of Pyrolysis Tube	Temp (°C) (duration = 12 hr)	% D Found
	24.5	Glass	500	19.4
		Quartz	500	29.6
		Quartz	650	24.2
	36.4	Quartz	650	35.9 ± 0.1
 R = phytyl	6.33	Quartz	650	6.41 ± 0.04

[a] Calculated assuming each position is 99% D.

isotope in various deuterium-labeled quinones, the results in Table II were obtained.

It was found that pyrolysis of the sample in a glass tube gave low deuterium values; this phenomenon was ascribed to the loss of isotope *via* proton–deuteron exchange between the sample and labile protons contained in the wall of the glass tube. When quartz tubes were used and the sample was pyrolyzed at 500°, the deuterium values became greater than theoretical, indicating the probable incomplete equilibration of the aromatic protons with labeled fragments at this temperature. Upon increasing the pyrolysis temperature to 650°, results were obtained which were very close to the theoretical for all of the deuterated quinones examined. It is interesting to note that complete randomization of the deuterium label in methane had occurred, indicating facile CH_4–H exchange in this system. With careful control of all possible variables, the analysis was perfected to a point where the accuracy of the determination was limited only by the reproducibility of the mass spectrum recorder (<1% relative scatter). The advantages of this analysis relative to other published deuterium determinations are (1) the small sample size required (samples of 0.5 mg were assayed accurately), (2) ease of operation (simple pyrolysis involving no additional transfers of material), and (3) the accuracy obtainable (limited only by instrument capabilities). The applicability of this method to any deuterium compound appears feasible.

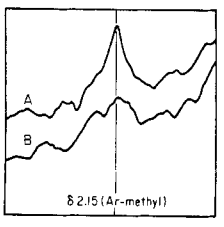


FIGURE 1: Nuclear magnetic resonance spectra of deuterated phylloquinones, using a Varian DP-60 spectrometer and an NMR Specialties SD-60 heteronuclear decoupler. Curve A: 10% 2-methyl-d_2-3-phytyl-1,4-naphthoquinone in 90% 2-methyl-d_3-3-phytyl-1,4-naphthoquinone (decoupler on); Curve B: 10% 2-methyl-d_2-3-phytyl-1,4-naphthoquinone in 90% 2-methyl-d_3-3-phytyl-1,4-naphthoquinone (decoupler off).

This technique was applied to the determination of the deuterium content of quinones reisolated from the bacterial system in the following manner. A standard sample of the quinone being analyzed was carried through the same column chromatographic procedures used in the purification of biologically exposed material. An aliquot of this sample (same weight as the recovered quinone) and an aliquot of test quinone were pyrolyzed and the methanes produced were analyzed by mass spectrometry. Using the above formula, the % D in the standard and in the recovered quinone were determined. The retention of deuterium in the test sample was then calculated *via* eq 4.

$$\% \text{ retention of deuterium} = \frac{\% \text{ D in recovered quinone}}{\% \text{ D in standard quinone}} \times 100 \quad (4)$$

E. Biological Test System. The biological test system used was the *M. phlei* extract prepared in the manner described (Brodie and Gray, 1956). Warburg systems, run at 30° for 12 min under standard Warburg procedure, contained the following: 40 μmoles of inorganic phosphate, 15 μmoles of $MgCl_2$, 1 mg of hexokinase,[7] 40 μmoles of substrate, 2.5 μmoles of AMP, 20 μmoles of mannose, 25 μmoles of KF, and 2.4 ml of extract all diluted to a final volume of 2.8 ml. The extracts used had protein contents ranging from 15 to 22 mg/ml (deter-

[7] The purified enzyme was obtained from Pabst Laboratories

TABLE III: Oxidative Phosphorylation with Intact and Light-Inactivated Reconstituted *M. phlei* Extracts[a] Containing T_2O.

Expt	System[b]	Quinone Added[c]	Substrate	ΔP_i (μmoles)	O_2 (μatoms)	P/O
1	Warburg (duration, 12 min)					
	Standard	None	Malate	14.5	12.6	1.2
	Inactivated	None	Malate	0.8	1.9	0.4
	Inactivated	Phylloquinone	Malate	10.1	10.3	1.0
	Macro (duration, 150 min)					
	Inactivated	Phylloquinone (49 mg/56 ml)	Malate	460		
2	Warburg (duration, 12 min)					
	Standard	None	Pyruvate	17.1	14.1	1.2
	Inactivated	None	Pyruvate	1.7	7.0	0.2
	Inactivated	Phylloquinone	Pyruvate	10.7	14.5	0.7
	Macro (duration, 120 min)					
	Inactivated	Phylloquinone (104 mg/101 ml)	Pyruvate	900		
3	Warburg (duration, 12 min)					
	Standard	None	Malate	10.9	12.7	0.9
	Macro (duration, 90 min)					
	Standard, 51 ml	None	Malate	350		
4	Warburg (duration, 12 min)					
	Standard	None	Pyruvate	13.7	14.1	1.0
	Macro (duration, 90 min)					
	Standard, 45 ml	None	Pyruvate	510		

[a] Protein concentration, 20–28 mg/ml. [b] All experiments in this series were aerobic. [c] Quinone was added to a final concentration of 1 mg/ml of extract.

mined turbimetrically by the method of Stadtman *et al.*, 1951).

Extracts were exposed to ultraviolet light for varying periods of time; test quinones were introduced by sonication in an aliquot of light-treated extract to a stable emulsion which was then added to the remaining treated system.

Warburg experiments contained the following systems: standard, nonlight-treated extract; standard, light-treated extract; light-treated extract reconstituted with unlabeled phylloquinone; light-treated extract reconstituted with isotopically labeled phylloquinone or unlabeled quinone and T_2O; zero-time phosphate controls for both light-treated and nonlight-treated extracts.

To provide the substantial amounts of quinone needed for purification and analysis *via* the previously described pyrolysis–mass spectral method, an alternate system, referred to as the "macro-Warburg," was devised. This consisted of all the components contained in the normal Warburg flask in amounts proportional to accommodate 60–80 ml of reconstituted extract. This system was contained in 125-ml erlenmeyer flasks (30–40 ml of system/flask) and shaken at 30° in a New Brunswick rotary shaker. The progress of these reactions was measured by periodic phosphate analyses (Fiske and Subbarow, 1928); the degree of reconstitution obtained was determined by removing an aliquot of the reconstituted macrosystem and using it as the "reconstituted system" in a concommitantly run Warburg experiment.

The "anaerobic macrosystems" were prepared in the same manner used for the normal large-scale system. At the outset of each experiment, these systems were maintained under a steady stream of nitrogen for specified periods of time to allow the accumulation of reduced intermediates and were then exposed to air to permit reoxidation of reduced quinone species.

The Warburg reactions were terminated after 12 min by addition of 0.1 ml of a 50% TCA solution and residual phosphate was determined. Macroreactions, both aerobic and anaerobic, were terminated by cooling the extracts to 4°, separating supernatant and particulate fractions by centrifugation at 104,000*g*, and denaturing each separate phase obtained by the addition of cold 95% ethanol. Quinone was isolated from these latter systems in the manner described previously.

Results

The values for phosphate and oxygen consumption observed with bacterial systems used in the tritium (Table III) and in the various deuterium experiments (Tables V–VII), together with the changes in isotope

TABLE IV: Incorporation of Tritium into Quinone during Oxidative Phosphorylation with Extracts of *M. phlei*.

Expt	Quinone Isolated	Phase	Yield of Quinones (μmoles/ 50 ml)	Act. of Quinone[a] (cpm/mg)	Sp Act. Quinone[c] (dpm/ μmole)	Sp Act. T_2O (dpm/ μmole of H)	App Incor (%)[d]
1	Phylloquinone	Particles	15	3.4:3.2	2.8	5.2×10^3	0.05 ± 0.03
	Phylloquinone	Supernatant	77	6.4:4.4	4.3	5.2×10^3	0.08 ± 0.03
2	Phylloquinone	Particles	27	4.9:5.9	4.7	5.8×10^3	0.08 ± 0.03
	Phylloquinone	Supernatant	69	4.3:6.1	4.1	5.8×10^3	0.07 ± 0.03
3	Native quinone	Particles	2.5	0:2.8[b]	0	2.5×10^3	0
4	Native quinone	Particles	1.8	4.2:4.7	30	6.6×10^3	0.5 ± 0.2

[a] Samples were counted by liquid scintillation; standard deviation is ±2 cpm/sample. [b] Less than standard deviation. [c] Calculated considering efficiency of counting and dilution factors. Efficiency was determined by internal standard. In expt 3 and 4 carrier native quinone was added during the purification procedure such that the dilution factors are three and five, respectively. The carrier native quinone was isolated from *M. phlei* extracts in a yield of 25 μg (0.032 μmole)/ml. [d] Calculated assuming one atom of hydrogen exchangeable per molecule of quinone.

content found in each set of experiments (tritium, Table IV; deuterium, Table VIII), are presented in the respective tables.

The time discrepancy between completion of a Warburg reaction and completion of a macro system reaction is explained by physical differences between the two. In the Warburg flask, a small volume of extract is aerated rapidly, while in the macro system, a large volume of extract is aerated more slowly, thus leading to a slower uptake of inorganic phosphate.

The data presented for the respective macro systems show that during the anaerobic portion of the experiment inorganic phosphate disappeared from the solutions. The possibility that this loss represented the operation of an anaerobic, phosphate-requiring process was eliminated when kinetic studies showed that the uptake of phosphate was complete after the initial 20–30 min of the 2–2.5-hr portion of the anaerobic phase of the experiment. This loss probably represents the partial operation of the NAD–FAD–quinone portion of the electron-transport–oxidative phosphorylation cycle initiated by the presence of excess added quinone; when all of the quinone present in the system is in the reduced form and is no longer able to oxidize preceding cofactors, the action ceases. This is in agreement with the observation that 30 min was required for all exogenous quinone added to treated *M. phlei* extract systems to be reduced (Watanabe and Brodie, 1966).

Measurement of oxygen absorption was made at 2-min intervals during each 12-min Warburg experiment. In none of the experiments was the rate of oxygen absorption observed to differ from that found with the unlabeled quinone control system; oxygen absorption was regular and occurred in an almost linear fashion. Consequently, if an isotope effect were operating in these systems, it was not manifest in the rate of oxygen consumption. Also, the rate of phosphate consumption in these experiments must not have been greatly affected by an isotope effect since the P/O ratios obtained for systems in which hydrogen isotopes were used did not significantly differ from those found in nonisotope-containing systems. Therefore, some of the previously considered complications which these experiments could have introduced into the biological system may be dismissed.

The tritium experiments were planned to investigate possible different modes of quinone action with different substrates. Two substrates were used in deference to the possibility that the reported apparent compartmentilization of NAD in this system (Brodie and Adelson, 1965) might also entail a compartmentalization of quinone at different sites within the particle and possibly a different mode of quinone action at these sites. As seen from the incorporation data presented in Table V, the difference in substrate does not influence the incorporation of isotope into unlabeled phylloquinone nor into the native menaquinone.

Two different quinones, exogenous phylloquinone and the endogenous menaquinone, also were used in these experiments to test any possible differences between the reconstituted and the natural bacterial electron-transport systems. As shown in the analytical data, no differences in isotope incorporation could be detected in either the malate- or pyruvate-containing systems.

Thus neither with native quinone nor with added phylloquinone does incorporation of significant isotope occur during oxidative phosphorylation. Similar findings have also been reported by others (Scherrer *et al.*, 1967) and are in contrast to the reported incorporation of tritium in the *M. phlei* system (Gutnick and Brodie, 1965, 1966). It is of interest that when ubiquinone was tested in rat mitochondria in the presence of T_2O, no isotope incorporation into the quinone was found (Parson and Rudney, 1966), thus demonstrating a similarity between the bacterial and mitochondrial systems.

It is important to note that all the deuterium-labeled quinones used did reconstitute the light-treated extracts to approximately the same extent as did the unlabeled

TABLE V: Oxygen and Phosphate Consumption in Bacterial Systems Containing 2-Methyl-d_3-3-phytyl-1,4-naphthoquinone.

Expt	System[a]	Quinone Added[b]	ΔP_i (μmoles)	O_2 (μatoms)	P/O
1	Warburg,[c] aerobic (duration, 12 min)				
	Standard	None	9.3	14.6	0.6
	Inactivated[d]	None	0.8	3.4	0.2
	Inactivated	Phylloquinone	7.0	11.8	0.6
	Inactivated	2-Methyl-d_3-3-phytyl-1,4-naphthoquinone (1.4 mg/2.5 ml)	5.3	9.2	0.6
	Macro,[c] aerobic (duration, 210 min)				
	Inactivated	2-Methyl-d_3-3-phytyl-1,4-naphthoquinone (36.8 mg/52.6 ml)	399		
2	Warburg,[e] aerobic (duration, 12 min)				
	Standard	None	16.2	13.0	1.2
	Inactivated[d]	None	5.1	3.3	1.5
	Inactivated	Phylloquinone	16.0	10.8	1.5
	Inactivated	2-Methyl-d_3-3-phytyl-1,4-naphthoquinone	14.6	11.3	1.3
	Macro,[e] anaerobic (duration, 120 min)				
	Inactivated	2-Methyl-d_3-3-phytyl-1,4-naphthoquinone (69.3 mg/70 ml)	350		
	Macro,[e] aerobic[f] (duration, 90 min)				
	Inactivated	2-Methyl-d_3-3-phytyl-1,4-naphthoquinone	720		

[a] The substrate used for these experiments was pyruvate. [b] Unless otherwise stated, quinone was present in Warburg systems to the extent of ~1 mg/ml of extract. [c] Protein, 18 mg/ml. [d] Light treatment was 40 min at 0°. [e] Protein, 21 mg/ml. [f] This macro system is the aerobic continuation of the anaerobic system presented immediately above.

phylloquinone controls. The data presented in Table VI for 2-methyl-d_3-3-phytyl-1,4-naphthoquinone might suggest that this quinone did not reconstitute the extract system as efficiently as did the other analogs. However, the data in this table represent only those experiments from which quinone was isolated and analyzed; in other experiments, 2-methyl-d_3-phylloquinone reconstituted inactivated systems to the same extent as unlabeled quinone. None of the deuterium-labeled phylloquinones tested in the *M. phlei* system lost isotope to any significant extent since recovered quinones all retained 99% or more of the initial deuterium, well within the experimental error (1%) of the method of analysis.

Conclusions

The results of these experiments indicate that if deuterium is being lost from the test quinones during respiration, it is being lost to the extent of less than 1% since this is the lower limit of detection of the method used for analysis, and if tritium is being incorporated into the quinone, the incorporation is less than 0.1%. Therefore, under the experimental conditions used and within the limits set by the analytical methods, no exchange of hydrogen from the quinone molecule occurred during oxidative phosphorylation. However, as mentioned in the discussion, covalent carbon–hydrogen bond making and breaking could have occurred and gone undetected had the reaction at the side-chain double bond of 2-methyl-3-phytyl-β-d-1,4-naphthoquinone been stereospecific.

Our findings eliminate those mechanisms which involve the intermediacy of the quinone methide (Chmielewska and Cieslak, 1960; Vilkas and Lederer, 1962; Erickson *et al.*, 1963) or which functionalize the 2-methyl group with various oxygen substituents (Vilkas and Lederer, 1962; Erickson *et al.*, 1963). Also, no mechanism which requires formation of the dihydrofuran (Chmielewska and Cieslak, 1960) or dihydropyran ring systems can be considered valid unless a stereospecific addition and removal of hydrogen is invoked. Results from experiments using ^{18}O-labeled quinones to reconstitute light-treated *M. phlei* systems impose additional restrictions on allowable mechanisms, and these are discussed in the following paper (Snyder and Rapoport, 1968).

TABLE VI: Oxygen and Phosphate Consumption in Bacterial Systems Containing 2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone.

Expt	System[a]	Quinone Added[b]	ΔP_i (μmoles)	O_2 (μatoms)	P/O
1	Warburg,[c] aerobic (duration, 12 min)				
	Standard	None	16.3	13.7	1.2
	Inactivated[d]	None	4.6	5.1	0.9
	Inactivated	Phylloquinone	15.0	14.3	1.1
	Inactivated	2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone	15.0	15.1	1.1
	Macro,[c] aerobic (duration, 90 min)				
	Inactivated	2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone (67.2 mg/65 ml)	935		
2	Warburg,[e] aerobic (duration, 12 min)				
	Standard	None	18.8	13.7	1.4
	Inactivated[f]	None	6.6	6.0	1.1
	Inactivated	Phylloquinone	13.8	13.9	1.0
	Inactivated	2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone	13.0	13.8	0.9
	Macro,[e] anaerobic (duration, 150 min)				
	Inactivated	2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone (65.5 mg/70 ml)	266		
	Macro,[e] aerobic[g] (duration, 90 min)				
	Inactivated	2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone	780		

[a] The substrate used for these experiments was pyruvate. [b] In all cases, quinone was added to Warburg systems to the extent of ~1 mg/ml of extract. [c] Protein, 16 mg/ml. [d] Light treatment, 43 min at 0°. [e] Protein, 23 mg/ml. [f] Light treatment, 60 min at 0°. [g] This macro system is the aerobic continuation of the anaerobic system presented directly above.

Experimental Section

A. Bacterial Test System

1. Preparation of Extracts. M. phlei ATCC 354 was aerated on New Brunswick rotary shaker at 37° for 18–22 hr in a medium described by Brodie and Gray (1956). Cells were harvested at 0° by centrifugation in a Serval SS3 centrifuge at 5000–7000 rpm. Cellular packs were resuspended twice in fresh, cold distilled water to remove remaining medium and recovered by centrifugation at 7000 rpm. Cells were then resuspended in distilled water to provide a final volume of suspension 2.2 times the wet weight of cells used. The suspension was divided into 10-ml aliquots and then sonicated at 120 mA for 8 min using a Biosonic oscillator which had been modified by the addition of an ammeter and a voltage regulator. The different sonicates were combined; cell husks and unlysed cells were removed by centrifugation at 20,000*g* for 30 min at 0°, and supernatants from this centrifugation were combined and dialyzed against distilled water at 0° for 1 hr. After dialysis, a portion of the extract was removed and buffered with 0.1 M Tris–HCl buffer to pH 7.2–7.3 and sufficient 0.18 M Na_2HPO_4 was added to ensure at least 40 μmoles of inorganic phosphate/2.4 ml of buffered extract; the remainder of the extract was placed into petri dish bottoms (9-cm i.d.) to a depth of 2–4 mm and irradiated under two 15-W General Electric black lights (distance from dish, 7 in.) for periods ranging from 40 to 60 min. At the end of the irradiation, the extracts were buffered and phosphate was added (all "inactivated" and "inactivated + quinone" systems reported were derived from this same bulk light-treated system).

2. Reconstitution of Light-Treated Extracts. WARBURG CONTROL SYSTEMS. Vitamin $K_{1(20)}$ (2.4 mg) and light-treated extract (0.5 ml) were sonicated at 120–130 mA for 3–4 min until a stable emulsion was obtained. This emulsion was added to 2.2 ml of fresh, treated extract, the suspension was evenly dispersed by gentle mixing, and the reconstituted extract (2.4 ml) was removed to the appropriate Warburg flask.

MACRO SYSTEMS. The appropriate weight of quinone

TABLE VII: Oxygen and Phosphate Consumption in Bacterial Systems Containing 2-Methyl-3-phytyl-β-*d*-1,4-naphthoquinone.

Expt	System[a]	Quinone Added[b]	ΔP_i (μmoles)	O_2 (μatoms)	P/O
1	Warburg,[c] aerobic (duration, 12 min)				
	Standard	None	11.4	16.0	0.7
	Inactivated[d]	None	1.7	4.0	0.4
	Inactivated	Phylloquinone	6.9	14.4	0.5
	Inactivated	2-Methyl-3-phytyl-β-*d*-1,4-naphthoquinone	9.0	12.2	0.7
	Macro,[c] aerobic (duration, 135 min)				
	Inactivated	2-Methyl-3-phytyl-β-*d*-1,4-naphthoquinone (71.5 mg/80 ml)	1216		
2	Warburg,[c] aerobic (duration, 12 min)				
	Standard	None	19.0	14.3	1.3
	Inactivated[d]	None	5.4	3.8	1.4
	Inactivated	Phylloquinone	15.2	13.6	1.1
	Inactivated	2-Methyl-3-phytyl-β-*d*-1,4-naphthoquinone	16.5	13.3	1.2
	Macro,[c] anaerobic (duration, 120 min)				
	Inactivated	2-Methyl-3-phytyl-β-*d*-1,4-naphthoquinone (68.5 mg/70 ml)	140		
	Macro,[c] aerobic[e] (duration, 90 min)				
	Inactivated	2-Methyl-3-phytyl-β-*d*-1,4-naphthoquinone	760		

[a] The substrate used for these experiments was pyruvate. [b] In all cases, quinone was added to Warburg systems to the extent of ~1 mg/ml of extract. [c] Protein, 17 mg/ml. [d] Light treatment, 40 min at 0°. [e] This macro system is the aerobic continuation of the anaerobic system presented directly above.

was emulsified in 10–15 ml of light-treated, buffered extract. This emulsion was then added to the remaining treated extract and the resulting mixture was gently shaken to ensure an even distribution of quinone. From this, 2.4 ml was removed to serve as the appropriate Warburg control system; the remaining reconstituted extract was used as the "macrosystem" reported.

3. Tritium-Labeled Phylloquinone Studies. A. BINDING OF EXOGENOUS PHYLLOQUINONE WITH ELECTRON-TRANSPORT PARTICLES. Crude bacterial extract (25 mg of protein/ml) was prepared in the above manner; 11.5-, 5.0-, and 1.0-mg aliquots of tritiated phylloquinone were sonicated into 5-ml portions of extract to form the reported systems I, II, and III, respectively. The reconstituted systems were equilibrated for 20 min at 30° on a New Brunswick rotary shaker, then transferred to centrifuge tubes and centrifuged at 105,000*g* for 1 hr. The supernatants were decanted, treated with 95% ethanol to precipitate protein factors, and then extracted with toluene. The particulate fractions were resuspended in a small amount of water, treated with ethanol, and also extracted with toluene. The respective toluene extracts were then counted for the presence of isotope.

B. EQUILIBRIUM BETWEEN PARTICULATE AND SUPERNATANT QUINONE POOLS. Bacterial extract (50 ml) was divided into two portions. One portion (20 ml) was centrifuged at 100,000*g* to yield a supernatant fraction and the other (30 ml) was equilibrated for 1 hr at 30° with tritiated phylloquinone (30 mg). After equilibration, the latter system was also centrifuged at 100,000*g* to yield the particulate fraction. An aliquot of this fraction (one-third) was extracted with ethanol and counted to determine the amount of labeled quinone associated with the particles (0.225 mg of quinone/ml of extract). The remaining particles (two-thirds) were suspended in 20 ml of the prepared supernatant into which 20 mg of unlabeled phylloquinone had been dispersed by sonication. The system was allowed to equilibrate for 1.5 hr at 30°; at the end of this time, it was centrifuged at 100,000*g* and the quinone was extracted separately from the particles and the supernatant and purified. The supernatant quinone fraction contained 6.1% of the tritiated phylloquinone originally associated with the particles. The equilibrium percentage was given by eq 5. Assuming the

$$\frac{K(\mathrm{T})_{\text{particles}}}{K(\mathrm{T})_{\text{particles}} + K_{\text{supernatant}}} \times 100 = \frac{4.5}{4.5 + 20} \times 100 = 18.4\% \quad (5)$$

TABLE VIII: Deuterium Content of Quinone Reisolated from Bacterial Systems.

Quinone	Expt	Phase	Ultraviolet[a] Purity (%)	%[b] Isotope	App[c] Exchange (%)
2-Methyl-d_3-3-phytyl-1,4-naphthoquinone	Aerobic	Supernatant	99.8	99.3	0.5
		Particulate	99.4	99.6	0.0
	Anaerobic	Supernatant	100.1	100.0	0.0
		Particulate	99.0	98.4	0.6
2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone	Aerobic	Supernatant	101.0	100.0	0.0
		Particulate	98.5	98.3	0.2
	Anaerobic	Supernatant	100.4	98.8	1.2
		Particulate	99.1	98.8	0.3
2-Methyl-3-phytyl-β-d-1,4-naphthoquinone	Aerobic	Supernatant	99.0	99.5	0.0
		Particulate	99.3	98.6	0.7
	Anaerobic	Supernatant	99.4	99.5	0.0
		Particulate	98.5	99.3	0.0

[a] Done in triplicate; standard deviation, 0.5%. [b] Standard deviation, 0.3%. [c] Standard deviation, 0.8%.

supernatant pool size has not changed, one can then calculate a rate constant for exchange between the two

$$kt = \ln \frac{\%_{\text{equil}} - \%_{\text{initial}}}{\%_{\text{equil}} - \%_{\text{found}}} = \ln \frac{18.4 - 0}{18.4 - 6.1} = 0.403 \quad (6)$$

pools (eq 6) and, since $t = 1.5$ hr, then $k = 0.269\ \text{hr}^{-1}$.

4. Methodology of Biological Experiments. Macro Warburg systems composed of reconstituted extract and additives (exclusive of substrate and fluoride) and contained in 125-ml erlenmeyer flasks (30–40 ml of extract/flask) were equilibrated at 30° for 10 min on a New Brunswick rotary shaker. Substrate and fluoride were then introduced, an aliquot of the system was withdrawn to serve as zero-time phosphate control, and aeration was continued for 1.5 hr. The progress of the reaction was measured by periodic phosphate analyses. When 80–90% of the originally added phosphate had been consumed, reaction was terminated by cooling the system to 4° followed by centrifugation in a Spinco refrigerated ultracentrifuge at 104,000*g* to separate supernatant and particulate fractions.

Anaerobic macro systems contained all of the components present in their aerobic counterparts. During the 10-min equilibration period, nitrogen was bubbled through the extract systems *via* a syringe needle fitted through a serum stopper which closed off the flasks from the atmosphere; another needle through the stopper served as a gas exit tube. Substrate and fluoride were then introduced *via* syringe and an aliquot was withdrawn as a zero-time phosphate sample. The initial nitrogen flow was decreased and was maintained at a low level for a 2-hr period to avoid excess evaporation of the solution. During this time, aliquots were withdrawn *via* syringe for phosphate analyses. At the conclusion of the anaerobic portion of the experiment, the serum stopper containing the nitrogen-gassing tubes was removed and the system was aerated until most of the phosphate present had been consumed. Reaction was terminated in the manner described for the totally aerobic systems.

5. Isolation and Purification of Added Quinone. After separation of the two phases of the extract by centrifugation, the bulk of the quinone added (approximately 80%) was found floating, together with other lipid materials, on the surface of the supernatant liquid. This quinone was removed *via* pipet. Quinone adhering to the sides of the tube was washed free with supernatant fluid and removed by pipet. After all visible quinone had been removed in this manner, the remaining supernatant was carefully decanted from the particulate pack and saved. The particles were removed by resuspending them in a minimum amount of distilled water and then transferring them to an appropriate container (this operation was repeated until all particulate matter had been removed).

To both supernatant and particulate fractions isolated in this manner was added an equal volume of cold, 95% ethanol; the addition of the alcohol resulted in the precipitation of lipid and protein materials. Both systems were stored at 4° overnight to allow maximum precipitation. At the end of this time, each was centrifuged at 5000 rpm to remove solid material. The supernatants were decanted into separate containers, the solid pack in the bottom of the tubes was resuspended in fresh alcohol by sonication at 120 mA until evenly dispersed, and the resulting suspensions were recentrifuged. This procedure was repeated until the ethanol supernatants were colorless.

The two sets of ethanol extracts were diluted to approximately four times their original volumes with distilled water and were then repeatedly extracted with *n*-pentane until the organic layer was no longer colored. The combined pentane extracts were washed with water

and finally with saturated NaCl solution, dried with magnesium sulfate, and concentrated *in vacuo*. The residue, consisting of an orange oil and a white solid, was triturated with pentane; this took up the oil but not the solid, thereby effecting a partial purification of the quinone. Evaporation of the pentane left a bright orange oil which was composed of test quinone, native quinone, bacterial carotenoids, and other lipid materials.

The residue was column chromatographed twice on Kiesel gel thin-layer adsorbant (pentane, 5% ethyl ether–pentane); the main effect of these chromatographies was to separate the carotenoids and a majority of the lipids present from the quinones. The quinone mixture was then chromatographed on Kiesel gel impregnated with 5% silver nitrate (5% ether–pentane); test quinones were eluted rapidly from these columns while native quinone and residual carotenes remained fixed at the top of the packing. Care was taken to fractionate the quinone band as closely as possible since a colorless lipid fraction usually preceded it. Phylloquinone purified in this manner was 98–100% pure by ultraviolet analysis. Following the above extraction and purification procedures, 75–85% of the initially added quinone could be recovered in pure form.

B. Synthesis of Isotopically Labeled Quinones.

2-Methyl-3-phytyl-α-d_2-1,4-naphthoquinone (IX). 6,10,14-Trimethylpentadecan-2-one (C_{18} Ketone XI). Commercial phytol (20 g, 68 mmoles) was dissolved in 1.4 l. of *t*-BuOH (refluxed over and distilled from $KMnO_4$) and 1.4 l. of distilled water was added, followed by 27.8 g (0.2 mole) of K_2CO_3. After the carbonate had dissolved, 116 g (0.54 mole) of sodium metaperiodate was added followed by 1.42 g (9 mmoles) of potassium permanganate. The mixture was stirred vigorously for 20 hr and the reaction was then terminated by the addition of either sodium bisulfite or sodium hydrosulfite until the dark color had just disappeared; at this point, two colorless layers had formed. After removal of the upper *t*-BuOH layer, the residual aqueous layer was extracted several times with pentane. The combined organic phase was washed with water and saturated NaCl solution, dried over $MgSO_4$, and concentrated *in vacuo* to yield a residual yellow oil. This was chromatographed on silica gel (0.05–0.2-mm mesh; pentane, 2–5% ethyl ether–pentane) to yield 13 g (70% yield) of pure ketone. The nuclear magnetic resonance spectrum of the product was identical with that reported for the same compound prepared in a different manner by Mayer *et al.* (1964).

Ethyl Phytenate (XII). A stirred suspension of 1.47 g (37 mmoles) of NaH (as a 50% oil emulsion) in 100 ml of dry THF was cooled in ice–water and 9.19 g (41 mmoles) of triethyl phosphonoacetate (XVI) in 5 ml of THF was slowly added during 20 min. After gas evolution had ceased, 10 g (37 mmoles) of C_{18} ketone dissolved in THF was added during 0.5 hr. Subsequent reaction conditions and product isolation were as reported by Jackman *et al.* (1965). The light yellow oil which resulted was chromatographed on silica gel (0.05–0.2-mm mesh; pentane, 2% ethyl ether–pentane), monitoring the chromatography by tlc on Kiesel gel (5% ethyl ether–pentane; R_F *cis* ester, 0.6; *trans* ester, 0.4; ketone, 0.2). Smaller quantities of material were chromatographed on the Kiesel gel–inorganic phosphor system described in the last section. After purification, 10 g (80% yield based on ketone used) of ester was obtained which was composed of 60% *trans* and 40% *cis* isomers. The product esters were characterized by their nmr spectra which were identical with those reported by Jackman *et al.* (1965).

Phytol-1-d_2. To a stirred suspension of 0.63 g (15 mmoles) of lithium aluminium deuteride and 0.448 g (3.3 mmoles) of aluminum chloride in 5 ml of THF maintained at −20° was added dropwise 2.24 g (6.7 mmoles) of ethyl phytenate (XII) dissolved in 2 ml of THF. The ester was added at such a rate that the temperature could be readily maintained at −20° by periodic immersion in the cold bath. When the addition of the ester was complete, the reaction mixture was transferred to a −20° cold room and stirred under dry nitrogen for 20 hr. Product was isolated in the manner described by Jackman *et al.* (1965). The resulting light yellow oil was purified by column chromatography on Kiesel gel (pentane, 1–10% ethyl ether–pentane), giving 1.6 g (80% yield) of pure phytol-1-d_2. The product was characterized by its nuclear magnetic resonance spectrum (Di Mari *et al.*, 1966) and by thin-layer comparison with purified commercial material.

2-Methyl-3-phytyl-α-d_2-1,4-napthoquinone (IX). To a stirred solution of 2.36 g (10.9 mmoles) of 2-methyl-1,4-naphthohydroquinone 1-monoacetate [prepared as described by Baker *et al.* (1942)] and 0.06 ml of 49% BF_3–etherate in ether dissolved in 5 ml of dry dioxane and maintained at 50° under nitrogen, 321 mg (1.0 mmole) of phytol-1-d_2 in 2 ml of dioxane was added slowly over a 15-min interval. Reaction conditions and subsequent product isolation were as reported (Hirschmann *et al.*, 1954).

Via this process, 215 mg (48% yield, based on the phytol used) of relatively pure phylloquinone, characterized by its nuclear magnetic resonance and mass spectra (Di Mari *et al.*, 1966), was obtained. The product quinone was separated into its component *cis* and *trans* isomers by preparative thin layer in the manner described above.

2-Methyl-3-phytyl-β-d-1,4-naphthoquinone(XV). Ethyl Phytenate-α-d (XIII) via Triethyl Phosphonoacetate-α-d_2 (XVII). In a nitrogen atmosphere, 20 g (88 mmoles) of triethyl phosphonoacetate (XVI), 20 ml (1.10 moles) of D_2O, and 200 mg (2 mmoles) of Na_2CO_3 were mixed and the solution was allowed to stand at room temperature for 0.5 hr (complete equilibration occurs almost instantaneously). The solution was heated at 50° for 2 hr, the D_2O was removed *in vacuo* (60 μ, 30°), fresh carbonate and 20 ml of D_2O were introduced, and the mixture was stirred until all the carbonate had dissolved. Stirring was continued for 0.5 hr and then the water was evaporated. Treatment in this manner was continued until the nmr showed no methylene absorption (δ 2.85, d, J = 21 cps) nor any increase in the intensity of the HDO peak (usually three to four treatments were necessary). As the amount of solid increased with each exchange, it was necessary to allow the solid to sediment and then to withdraw the clear supernatant

for further treatment. Approximately 70–80% of the phosphonate reagent was recovered at the end of four treatments. The resulting reagent had the following nuclear magnetic resonance spectrum: δ 4.2, m, 6 H, OCH_2CH_3; δ 1.35, m, 9 H, OCH_2CH_3.

To a stirred suspension of 0.255 g (5.6 mmoles) of sodium hydride (as a 40% oil emulsion) in 5 ml of dry THF, stirred and cooled to 0°, was added 19.5 μl (1.1 mmoles) of D_2O (99.8 mole % D). The suspension was allowed to warm to room temperature; 1.63 g (7.3 mmoles) of triethyl phosphonoacetate-α-d_2 in 5 ml of THF was added keeping the internal temperature at 25°. At the end of the addition, the solution was stirred at room temperature until hydrogen evolution had ceased and then 1 g (3.7 mmoles) of the C_{18} ketone dissolved in 3 ml of THF was added during 15–20 min. The solution was stirred under nitrogen at 60° for 2.5 hr. At the end of this time, 2–3 ml of D_2O was added and the solution was stirred at room temperature for 5 min. Water and pentane were added, the organic layer was removed, the aqueous layer was extracted several times with pentane, the combined pentane extracts were washed with water five times, then dried over $MgSO_4$, and the pentane was removed *in vacuo*.

After chromatography of the residue on silica gel (0.05–0.2 mesh, 2% ethyl ether–pentane) pure ester (875 mg, 70% yield) was obtained which contained 1–1.2 mole % protium in the vinylic position. The nuclear magnetic resonance spectrum of the product was identical with that of the unlabeled ester except for the absence of absorption at δ 5.70 for the vinylic proton and a singlet rather than a doublet for the *trans*-vinyl methyl group at δ 2.15.

Ethyl Phytenate-α-d (XIII) via Ethoxyacetylene-1-d. To a rapidly stirred solution of 51.2 ml (0.082 mole) of 1.6 N butyllithium (in hexane) in 50 ml of toluene was slowly added 5.0 ml (4 g, 0.057 mole) of ethoxyacetylene in 20 ml of toluene at such a rate that the temperature could be readily maintained between 25 and 30° by periodic immersion in an ice–water bath. When addition was complete, the mixture was rapidly stirred at room temperature for 1 hr and then purged with a rapid stream of dry nitrogen for 15 min to remove unreacted acetylene. The mixture was cooled to 0°, 12 ml of D_2O (99.8 mole % D) was slowly added, and the resulting mixture was stirred at room temperature for 1 hr. D_2O (10 ml) was then added and all the remaining solid went into solution as a two-layer system developed. The aqueous layer was removed (system still under nitrogen), the organic layer was washed with 5-ml portions of D_2O until the aqueous layer was neutral to litmus (six washes), and the organic layer was dried over $MgSO_4$ for 5 min. It was decanted into fresh $MgSO_4$ and the spent drying agent was washed several times with toluene, these washes being added to the original solution. After overnight drying at 0°, the solution was filtered and the filtrate was distilled. Five fractions were obtained over a boiling range of 30–50°; each contained a mixture of hexane and ethoxyacetylene-1-*d* (nuclear magnetic resonance). The amount of ethoxyacetylene-1-*d* recovered was 3 g (60% yield). The deuterium-labeled reagent had the following nuclear magnetic resonance spectrum: δ 4.05, q, J = 7 cps, OCH_2CH_3; δ 1.35, t, J = 7 cps, OCH_2CH_3. The singlet absorption for the acetylenic proton at δ 1.40 was absent. Since hexane would not adversely affect the condensation with the C_{18} ketone, the ethoxyacetylene–hexane mixture obtained was used directly to prepare ethyl phytenate-α-*d*.

C_{18} ketone, XI (5 g, 18.5 moles), dissolved in 10 ml of ether, was slowly added (under nitrogen) to a cooled (−10°) and stirred solution of 2.4 ml (18.5 mmoles) of BF_3–etherate in 25 ml of ether. The temperature was maintained at −10°, the resulting solution was stirred for 15 min, and ethoxyacetylene in hexane (11.8 ml of a 20 mole % solution of ethoxyacetylene-1-*d* in hexane; 1.3 g, 18.5 mmoles, of ethoxyacetylene-1-*d*) was added slowly over a 2-hr period after which the solution was stirred an additional 15 min, allowed to warm to 0°, and stirred for 1 hr. Ether and water were added, the acidic catalyst was removed by extraction with a 5% $NaHCO_3$ solution, and the ether layer was washed several times with water followed by saturated NaCl solution and then dried over $MgSO_4$. Removal of the ether *in vacuo* and chromatography of the residue on silica gel (0.05–0.2-mm mesh) produced 4.3 g (70% yield) of ethyl phytenate which was shown to contain 99.5 mole % *d* in the vinylic position (nuclear magnetic resonance).

Phytol-2-d (XIV). To a stirred, cooled (−30°) suspension of 1.21 g (33.2 mmoles) of $LiAlH_4$ and 0.86 g (6.3 mmoles) of $AlCl_3$ in ether was added dropwise 4.3 g (12.6 mmoles) of ethyl phytenate-α-*d* (XIII). After the addition, the mixture was stirred for 15 min and then product was isolated by the procedures described for phytol-1-d_2. In this manner, 3.3 g (90% yield) of phytol-2-*d* was obtained which was identical with commercial material in every manner except for the absence of vinylic proton absorption (δ 5.42), a crude singlet absorption at δ 4.13 for the hydroxy methyl protons rather than a doublet, and a sharp singlet at δ 1.68 for the *trans* $CH_3C{=}C$ in the nuclear magnetic resonance spectrum.

2-Methyl-3-phytyl-β-d-1,4-naphthoquinone (XV) was prepared in 72% yield from 24.3 g (0.112 mole) of menadiol monoacetate, 0.62 ml of 49% BF_3–etherate, and 3.3 g (10.3 mmoles) of phytol-2-*d* under the same reaction and isolation conditions as described above for the preparation of 2-methyl-3-phytyl-α-d_2-1,4-naphthoquinone. The product was characterized by its ultraviolet spectrum, by thin-layer comparison with commercial material, and by its nuclear magnetic resonance which was distinguished by the absence of vinylic absorption at δ 4.95, a crude singlet at δ 3.31 ($ArCH_2C{=}$), and a sharp singlet at δ 1.77 (*trans* $CH_3C{=}C$).

p-Nitrophenylazobenzoate (NABS) Esters of Phytol. Phytol (50 mg, 0.17 mmole) was dissolved in 10 ml of benzene, 98.6 mg (0.34 mmole) of *p*-nitrophenylazobenzoyl chloride and 107.4 mg (0.11 ml, 1.36 mmoles) of pyridine were added, and the resulting solution was stirred at room temperature for 17 hr. The pyridine hydrochloride which had formed was removed by centrifugation, and the red benzene solution was evaporated to a bright red-orange solid residue which was purified by thin-layer chromatography (Kiesel gel, 10% ethyl ether–pentane; R_F *cis*, 0.7; *trans*, 0.6). The products obtained were characterized only by their nuclear magnetic res-

onance spectra: *trans*-NABS-phytol: δ 8.4-7.7, 8 H, aromatic protons; δ 5.53, t, 1 H, methine proton; δ 4.92, d, 2 H, α-methylene protons; δ 2.17–1.90, t; δ 1.78, s, vinyl methyl group; and δ 1.25 and 0.87, side-chain alkyl protons. *cis*-NABS-phytol: δ 8.4-7.76, m, 8 H, aromatic protons; δ 5.60, t, 1 H, methine proton; δ 4.90, d, 2 H, α-methylene protons; δ 2.4–2.07, m; δ 1.83, s, vinyl methyl protons; δ 1.23 and 0.88, side-chain alkyl protons. No yields were measured in these preparations.

Hydrolysis of NABS-phytol Esters. To a solution of 66 mg (0.12 mmole) of NABS-phytol dissolved in 10 ml of benzene was added 51 mg (0.90 mmole) of pulverized KOH dissolved in 1.5 ml of methoxyethanol. The resulting solution was stirred at room temperature for 17 hr and centrifuged, and the solid obtained was washed three times with benzene. The pooled benzene solutions were washed with water until neutral and then with a saturated NaCl solution. After the extract had been dried over $MgSO_4$, the benzene was removed *in vacuo*. The resulting light yellow oil was shown to be phytol by thin-layer chromatography and by nuclear magnetic resonance. No trace of the starting NABS ester could be found. When pure *cis*- or *trans*-NABS-phytols were subjected to this treatment, isomerically pure phytol was obtained.

C. Deuterium Analysis. Pyrolysis of Quinone to Methane

A 7 × 0.9 cm quartz pyrolysis tube was constructed by drawing one end of a piece of 9-mm (o.d.) quartz tubing into a breakseal and fitting the other end with a ground-glass joint. Into this tube was placed 2 mg (5 μmoles) of purified deuterated phylloquinone; the tube was evacuated to <1 μ of mercury for 5 min, sealed *in vacuo* below the glass joint, and then heated at 650° for 12 hr. The cooled pyrolysis tube then was placed in the inlet tube of a Consolidated Electronics Corp. 130 mass spectrometer, the breakseal was opened, and the pyrolysis gases were cooled with liquid nitrogen. The uncondensed gases remaining were then admitted into the mass spectrometer and their fragmentation patterns were recorded.

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