# Phosphate-dependent Incorporation of Tritium into a Naphthoquinone during Oxidative Phosphorylation*

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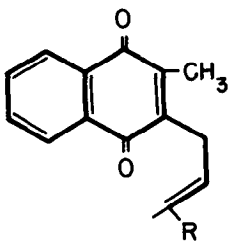
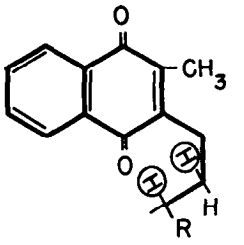
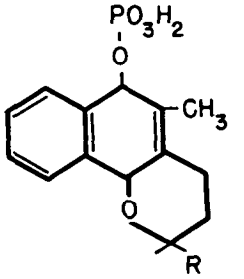
A role for a naphthoquinone in respiratory chain phosphorylation has been demonstrated with a system from *Mycobacterium phlei* (1, 2). Irradiation of the bacterial system with light at 360 mμ resulted in the destruction of the endogenous naphthoquinone (vitamin $K_9H$) and in a loss of coupled phosphorylation (1, 3). Restoration of both oxidation and phosphorylation following irradiation required the addition of the natural naphthoquinone, vitamin $K_1$ (Compound I), or closely related homologues (1, 2). The properties of the $K_1$-restored system were shown to be similar to those observed with the natural quinone, and the rate of oxidation and reduction of the naphthoquinone was compatible with the over-all rate of substrate oxidation (4).



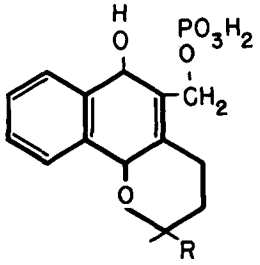
(I)



(II)



(III)



(IV)

The quinones which restore coupled phosphorylation, in contrast to those which restore only oxidation, all contain an unsaturated bond at the β,γ position of their isoprenoid side chain. Quinones containing a saturated β,γ position, such as dihydrophytyl vitamin $K_1$ (Compound II), restore oxidation by the same pathway as vitamin $K_1$ but fail to restore phosphorylation (2). These results have suggested the formation of a cyclic 6-membered chromanyl ring during quinone reduction. Both the 6-chromanyl phosphate (Compound III) (5–7) and the 5-phosphomethylchromanyl (Compound IV) (8, 9) derivatives of the quinone have been postulated as intermediates in coupling the reduction of the naphthoquinone to the synthesis of ATP. Although the 6-acetylchromanol derivative of vitamin $K_1$ has been isolated from incubation mixtures following acetylation with acetyl chloride, these results are inconclusive since the acetylating agent can give rise to this compound nonenzymatically if traces of water are present (10).

Protonation of the quinone would be expected during cyclization to the chromanyl structure or following quinone methine formation. Furthermore, identification of the protonated group could further elucidate the role of the quinone in oxidative phosphorylation. Thus, the incorporation of tritium into a naphthoquinone was studied with the system from *M. phlei* under conditions of oxidative phosphorylation.

The conditions for tritium incorporation into vitamin $K_1$ with the quinone-dependent system are given in Table I. The quinone was extracted from the reaction mixture following termination of the experiment and was purified by chromatography on Permutit until the specific activity remained constant. Similar results were obtained following purification of the quinone by reverse phase chromatography on Vaseline petroleum jelly-impregnated paper. Incorporation of tritium into vitamin $K_1$ required incubation of the extract with substrate, vitamin $K_1$, and inorganic phosphate. Malate and β-hydroxybutyrate were found to serve equally well as electron donors. Incorporation of tritium into the naphthoquinone did not occur in the absence of coupled phosphorylation, with controls in which the reaction was stopped before the addition of substrate, or with systems in which the quinone was omitted during the incubation period but added following termination of the reaction. The dependence on oxidative phosphorylation for the incorporation of tritium into the naphthoquinone did not appear to be an expression of a requirement for ATP since no appreciable incorporation was observed with ATP under anaerobic conditions (Table I). Incubation of tritium-labeled vitamin $K_1$ (obtained enzymatically) with the bacterial extract did not result in a lowering of the specific activity.

The dependence of tritium incorporation on the presence of inorganic phosphate was demonstrated with extracts which were rapidly dialyzed to remove endogenous phosphate (Table II). The extent of incorporation was dependent on phosphate concentration over a narrow range. The specific activity increased with increasing phosphate concentration and reached a plateau between 1 and 5 μmoles of inorganic phosphate. The addition of a phosphate acceptor system (glucose, hexokinase, and ADP) resulted in a requirement for higher concentration of inorganic phosphate for optimal incorporation. Tritium incorporation was found to occur when arsenate was substituted for inorganic phosphate. The reaction was found to be independent of ADP concentration. Incorporation of tritium into vitamin $K_1$ did not occur with preparations which were subjected to prolonged sonic oscillation. Preparations treated in this manner retain their oxidative capacity but are unable to couple phosphorylation to oxidation.

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TABLE I

*Incorporation of tritium into vitamin $K_1$ by extract from M. phlei*

The system consisted of 0.5 ml of irradiated (360 mμ) crude extract (33 mg of protein per ml), 0.6 μmole of vitamin $K_1$ in Asolectin micelles prepared according to the modification of Asano and Brodie (4), 15 μmoles of $MgCl_2$, 40 μmoles of L-malate, 2.5 μmoles of ADP or 10 μmoles of ATP as indicated, 15 μmoles of inorganic phosphate, 0.1 ml of tritiated water ($2.5 \times 10^5$ cpm), and water to a volume of 2.5 ml. The reaction mixture was incubated in a Warburg vessel with shaking for 15 min at 29°. The reaction was stopped by the addition of acetone (20 ml), and 2.5 mg of vitamin $K_1$ were added as carrier. The mixture was centrifuged to remove the precipitated protein, and the supernatant fluid was concentrated to dryness. The residue was suspended in 2 ml of petroleum ether and purified over washed Permutit as previously described (11). The fraction containing vitamin $K_1$ was collected, dried in a vacuum, rechromatographed over Permutit, and suspended in 5 ml of spectral grade isooctane. Aliquots were suspended in non-aqueous counting solution and counted in a liquid scintillation counter Quenching was determined by the channel ratio method. The amount of $K_1$ was determined spectrophotometrically. Coupled phosphorylation was measured manometrically for 13 min with similar systems lacking $T_2O$. The conditions in Experiment 2 were similar to those described above except that 0.5 ml of crude extract containing 25 mg of protein per ml was used.

| System | Coupled phosphorylation | | | Tritium incorporation (specific activity) | |
|---|---|---|---|---|---|
| | Oxidation | Phosphorylation | P:O | First purification | Second purification |
| | *μatoms* | *μmoles* | | *cpm/μmole $K_1$* | |
| Experiment 1 | | | | | |
| Complete | | | | 2918 | 2846 |
| − $P_i$ | 3.4 | 0.0 | 0.0 | 19 | 0 |
| − Malate | 0.4 | 0.0 | 0.0 | 58 | 0 |
| − $K_1$ | 0.84 | 0.0 | 0.0 | 0 | 0 |
| − Extract | | | | 0 | |
| + ADP | 3.2 | 2.3 | 0.72 | 2437 | 2223 |
| + ADP-$P_i$ | | | | 0 | |
| Experiment 2 | | | | | |
| Complete + ATP | | | | 1830 | |
| Complete + ATP (anaerobic) | | | | 50 | |

TABLE II

*Effect of phosphate concentration on tritium incorporated into vitamin $K_1$*

The conditions were similar to those described in Table I except that the crude extract (22 mg of protein per ml) was rapidly dialyzed by the procedure previously described (12) to remove endogenous phosphate and DPN (500 μg), and β-hydroxybutyrate (40 μmoles) was added as the electron donor. The reaction was carried out for 90 min.

| $P_i$ | $K_1$ recovered | Specific activity* |
|---|---|---|
| *μmole* | *μmoles* | *cpm/μmole $K_1$* |
| 0 | 3.1 | 0 |
| 0.1 | 2.4 | 1166 |
| 0.2 | 2.3 | 1406 |
| 0.5 | 3.6 | 2338 |
| 1.0 | 1.1 | 2539 |

* Corrected for dilution with carrier naphthoquinone.

A further indication that the incorporation of tritium into the naphthoquinone is associated with the coupling process was obtained from studies of the effects of uncoupling agents. Coupled phosphorylation with the system from *M. phlei* has been shown to be sensitive to low concentrations of 2,4-dinitrophenol (4, 12). The effects of this agent on the incorporation of tritium into vitamin $K_1$ are shown in Table III. The incorporation of tritium was completely abolished by $3 \times 10^{-4}$ M dinitrophenol. These results lend further support to the findings of Eisenhardt and Rosenthal (13) that dinitrophenol acts by inhibiting the formation of a high energy intermediate. Other uncoupling agents,

TABLE III

*Effect of dinitrophenol on phosphate-dependent tritium incorporation into vitamin $K_1$*

The conditions were similar to those described in Table II. The protein concentration of the dialyzed crude extract was 15 mg per ml.

| System | $K_1$ recovered | Specific activity of extracted $K_1$ |
|---|---|---|
| | *μmoles* | *cpm/μmole* |
| Complete | 2.8 | 1046 |
| + Dinitrophenol ($3 \times 10^{-4}$ M) | 1.1 | 3 |
| − $P_i$ + dinitrophenol | 3.4 | 0 |
| − $P_i$ | 2.2 | 0 |

such as hydroxylamine ($5 \times 10^{-4}$ M), pentachlorophenol ($10^{-4}$ M), and carbonyl cyanide *m*-chlorophenylhydrazone ($10^{-4}$ M), were found to inhibit the incorporation of tritium into vitamin $K_1$.

In contrast to vitamin $K_1$, which restores both oxidation and phosphorylation with the irradiated extracts, analogues of this naphthoquinone such as menadione, dihydrophytyl vitamin $K_1$, and lapachol restore only oxidation. The oxidation observed with menadione occurs by an electron transport bypass, whereas dihydrophytyl vitamin $K_1$ and lapachol were shown to restore oxidation by the same pathway observed with vitamin $K_1$ (2). Tritium incorporation did not occur following substitution of vitamin $K_1$ by these analogues. Incorporation of tritium into menadione or dihydrophytyl vitamin $K_1$ would have implicated the methyl group as the site of protonation; however, these results do not rule out the methyl group as the site of tritium incorporation.

The electron transport chains of *M. phlei* lack respiratory control. Reduction of the naphthoquinone has been shown to occur in the absence of phosphate. The fact that tritium incorporation requires the addition of phosphate and takes place only under conditions of oxidative phosphorylation suggests that two different reduced derivatives may be formed during electron transport: one in the phosphorylative chain and the other on a nonphosphorylative bypass pathway. This type of internal electron transport bypass may account for the lack of respiratory control in *M. phlei*. Identification of the position of the incorporated tritium atom is under investigation and may shed some light on the mechanism by which this lipid-soluble cofactor participates in respiratory chain phosphorylation.

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