The Reconstitution of Oxidative Phosphorylation in *Mycobacterium phlei* with *cis*- and *trans*-Phylloquinone. Evidence against Isomerization*

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ABSTRACT: The interconversion of *cis*- and *trans*-phylloquinones during oxidative phosphorylation by cell-free extracts from *Mycobacterium phlei* was studied using phylloquinone-5,6,7,8-t_4. Both *cis*- and *trans*-phylloquinone restored oxidative phosphorylation to light-inactivated extracts, but the *cis* isomer did so to a distinctly lesser extent. Recovered quinone from test systems to which *trans*-phylloquinone had been added showed no evidence of isomerization. Test systems to which *cis*-phylloquinone had been added did lead to a small fraction of *trans* isomer in the recovered quinone, but this was due to experimental manipulations and not enzymic action. In particular, ordinary laboratory light caused *cis–trans* interconversions.

It has been reported recently (Gutnick *et al.*, 1967) that cell-free extracts prepared from *Mycobacterium phlei* are capable of interconverting the *cis* and *trans* isomers of phylloquinone during oxidative phosphorylation. When pure *trans*-phylloquinone was added to irradiated extracts, the recovered quinone contained 3% of the *cis* isomer; addition of pure *cis* isomer led to recovery of 5% of the *trans*. The interconversions were not observed in zero-time controls. Conversion of *trans* → *cis*-phylloquinone was reported to be dependent upon oxidative phosphorylation whereas the *cis* → *trans* isomerization may have been independent of this process. *trans*-Phylloquinone was effective in restoring both oxidation and phosphorylation to their light-treated systems whereas the *cis* isomer partially restored oxidation but not phosphorylation.

These interesting results stimulated us to undertake similar experiments, since in our experience (DiMari *et al.*, 1968) maintenance of isomeric integrity during purification of *cis*- and *trans*-phylloquinones required extraordinary precautions, and Gutnick *et al.* (1967) had not reported any. Initial experiments involving incubation of the isomerically pure phylloquinones with light-treated *M. phlei* extracts showed that quinone isomerization had indeed occurred. Under standard procedures, isomer mixtures were recovered in each case. However, unlike the observations of Gutnick *et al.* (1967), zero-time controls, both ethanol and cyanide treated, were also found to give isomer mixtures.

Of the many factors influencing phylloquinone isomerization, light is the major one. Pure *cis*-phylloquinone, dissolved in pentane and allowed to stand at room temperature under ordinary laboratory fluorescent light, was converted into a 1:1 mixture of *cis* and *trans* isomers within 1 hr; *trans*-phylloquinone, under the same conditions, was converted into a 1:9 *cis*:*trans* mixture. Therefore the oxidative phosphorylation experiments were repeated with the precautions noted and particularly with exclusion of direct light from the moment of quinone addition to its final isolation.

Experimental Methods[1]

Phylloquinone-5,6,7,8-t_4 was separated into *cis* and *trans* isomers by preparative thin-layer chromatography on Camag Kiesel gel. Quinone was applied to plates illuminated indirectly only by a 40-W bulb covered with a Kodak Safelight filter (Wratten Series 1A); plates were developed in the dark with 8% *n*-butyl ether–hexane (Jackman *et al.*, 1965). Bands containing the isomers were removed separately from plates and quinone was eluted by washing with carbon tetrachloride. This chromatography was repeated until the fractions were shown to be isomerically pure by chromatography on Kiesel gel plates containing 0.05% Rhodamine 6G. After complete removal of solvent *in vacuo*, the isomeric phylloquinones were stored at −16° until use.

All of the additives necessary to maintain oxidative phosphorylation, in quantities previously described (DiMari *et al.*, 1968), were introduced into Warburg flasks in the light. When this was completed, overhead lighting was turned off and *all* of the remaining operations were conducted under the red light filter system described above. Each quinone isomer (4.8 mg) was sonicated at 4° into 0.5 ml of light-treated bacterial extract (prepared from *M. phlei* as described by DiMari *et al.*, 1968); the resulting emulsions were added to 4.5 ml of fresh light-treated extract and, after gentle mixing, the bulk reconstituted systems formed were divided into test and zero-time control samples for each isomer. After

* From the Department of Chemistry, University of California, Berkeley, California. *Received March 25, 1968.* This research was supported in part by Grant AI-04888 from the National Institutes of Health, U. S. Public Health Service.

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[1] We are indebted to Linda Skory for skillful technical assistance.

TABLE I: Oxidative Phosphorylation with Light-Inactivated Extracts of *M. phlei* Reconstituted with *cis*- and *trans*-Phylloquinone-5,6,7,8-t_4.

System[a]	Quinone Added[b]	ΔP_i (μmoles)	O (μatoms)	P/O
Standard[c]	None	26.0	23.6	1.1
Inactivated[d]	None	6.7	6.7	1.0
Inactivated	*trans*-Phylloquinone-5,6,7,8-t_4	23.7	18.3	1.3
Inactivated	*cis*-Phylloquinone-5,6,7,8-t_4	13.1	8.8	1.5

[a] Substrate, pyruvate; duration of experiment, 20 min. [b] Reconstituted systems contained 2.4 mg of quinone. [c] Protein concentration, 18 mg/ml of extract. [d] Treated with two 15-W General Electric black lights at 0° for 35 min.

TABLE II: *cis* and *trans* Isomer Distribution in Phylloquinones-5,6,7,8-t_4 Recovered from Reconstituted *M. phle* Extracts.

Quinone Sample	Isolation Procedure Used	Act. (dpm) of Tlc[c]-Separated Isomers		Isomer Distribution	
		cis	*trans*	% *cis*	% *trans*
trans					
Standard[a]		1,971	327,651	0.6	99.4
Zero time	Dark	712	142,064	0.5	99.5
	Light	8,261	135,123	5.7	94.3
Test	Dark	629	96,781	0.7	99.3
	Light	4,446	132,338	3.4	96.6
cis					
Standard[b]		64,074	664	99.0	1.0
Zero time	Dark	51,593	2,179	95.9	4.1
	Light	46,452	6,592	87.6	12.4
Test	Dark	44,739	1,034	97.7	2.3
	Light	41,435	8,545	82.9	17.1

[a] Activity of quinone used, 1.9×10^5 dpm/mg. [b] Activity of quinone used, 9×10^4 dpm/mg. [c] Thin-layer chromatographic.

a 5-min equilibration at 30°, substrate was introduced into test systems while zero-time controls which had also been allowed to equilibrate at 30° were pipetted from their Warburg flasks into 50 ml of cold absolute ethanol and stored at −16°. At the conclusion of the Warburg experiment, after aliquots had been removed from each system for phosphate analyses, the remainder was added to cold absolute ethanol and stored, along with their zero-time controls, at −16° overnight. The protein–lipid precipitate which had formed was removed by centrifugation, and each supernatant was diluted to twice its original volume with a saturated NaCl solution and was then extracted with a total volume of 250 ml of pentane. After the pentane extracts had been washed with water and saturated salt solution and the solvent had been removed *in vacuo* at room temperature, nonisotopically labeled carrier phylloquinone (*cis*:*trans* ratio in carrier, 3:7) was added to the residue and the resulting isomer mixtures were resolved on 0.05% Rhodamine–Kiesel gel (8% *n*-butyl ether–hexane). Concomitantly, carrier was also added to a sample of standard quinone from which the reconstituting phylloquinone aliquots had been removed initially, and this mixture was also separated by thin-layer chromatography as described above. After removal of the carbon tetrachloride and addition of scintillation solution, samples were counted at 3° in a Nuclear-Chicago Mark I scintillation counter equipped with an external probe.

Results

The data are presented in Tables I and II. Several experiments were conducted in each case, and the results were reproducible within experimental error. From Table I it can be seen that *cis*-phylloquinone is distinctly less active in supporting oxidative phosphorylation than is the *trans* isomer, restoring light-inactivated extracts to only approximately 50% of their original value. The level of reconstitution of phosphorylation by *cis*-phylloquinone in our system is approximately the same as

that observed for oxidation. The data presented in Table I are the Warburg portions of the experiments shown in Table II.

Table II presents the data from experiments demonstrating the effect of light upon quinone stability during isolation procedures. All systems were initially derived from the same bulk extract, the experiments were conducted on the same day, and during isolation procedures quinone samples were at room temperature for the same period of time. The procedure for these particular experiments followed the general procedure given above except that, after removal of precipitated protein–lipid from ethanol-treated test systems and zero-time controls which had stood at −16° overnight, the supernatant obtained from each was divided into two equal portions. Quinone was isolated from one portion in the dark whereas quinone isolation from the other portion was conducted under ordinary laboratory lighting. As seen in Table II, no isomerization of quinone (outside of experimental error) is observed if light is excluded both during testing and isolation procedures; however, if quinone is isolated from the bacterial system under laboratory lighting, isomerization is observed with both *cis*- and *trans*-phylloquinone.

Discussion

If no *in vivo* isomerization has occurred, the isomer ratio in the reisolated test phylloquinones should be the same as that in the standard, starting quinone. This is indeed the case for *trans*-phylloquinone. Reconstitution with *trans*-phylloquinone containing 0.6% of the *cis* isomer leads to restoration of oxidative phosphorylation and no isomerization when the necessary experimental precautions are taken. Without these precautions, the amount of *cis* isomer increases to over 3%. It is not possible to determine the per cent of added quinone which is involved in oxidative phosphorylation (DiMari *et al.*, 1968). However, if only as little as 0.3% of the added *trans*-phylloquinone had been converted into the *cis* isomer as a result of involvement in respiration, this change would have been detected.

The *cis*-phylloquinone case is more complicated because of the extreme facility with which isomerization occurs to the more stable *trans* isomer. But here also the data (Table II) indicate no oxidative phosphorylation-dependent isomerization. Zero-time controls showed a greater increase in *trans*-phylloquinone (1.0→4.3%) than did the *M. pheli* extract system (1.0 → 2.3%). The extent of this isomerization of *cis*- to *trans*-quinone for the test system is well within the experimental limitations of the operations. For example, *cis*-phylloquinone containing 1.0% of the *trans* isomer was added to a simulated biological system consisting of 1 ml of isooctane and 2 ml of water. This two-phase system was carried through exactly the same procedure followed for a Warburg experiment done in the dark, and the recovered quinone contained 2.8% *trans* isomer. This value is in good agreement with the 2.3% found with the *cis* test quinone in Table II. Therefore, the isomerization observed with *cis*-phylloquinone systems must be ascribed to the lability of the *cis*-quinone to the operations of the experiments, and this is reinforced by the fact that the zero-time dark sample contained more *trans* isomer than did the test system.

The data clearly demonstrate that in our system restoration of oxidative phosphorylation to light-inactivated extracts of *M. phlei* by addition of essentially isomerically pure phylloquinones is not accompanied by isomerization.

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