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# Incorporation of a Lipophilic Series, as Coenzyme Q Homologs, in Phospholipid Vesicles

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## ABSTRACT

The incorporation of Coenzyme Q† homologs into phospholipid vesicles to achieve a concentration close to the quinone content of mitochondria has been studied with different methods. The results obtained can be summarized as follows: (a) stirring does not lead to comparable incorporation of the various CoQs tested; (b) ultrasonic irradiation results in a homogeneous incorporation both of the naturally occurring homologs of Coenzyme Q (polyisoprenoid chain-length ranging from ten to six units) and of the nonphysiological shorter chain homologs; (c) ethanol injection, which is a simple, rapid, and inexpensive technique, gives results comparable to those obtained by ultrasonication.

**Index Entries:** Vesicles, incorporation of coenzyme Q homologs; liposomes, multilamellar vesicles; incorporation of coenzyme Q homologs, in liposomes; gel filtration of vesicles; phospholipid peroxidation, malondihaldeyde formation during; phospholipid vesicles, incorporation of coenzyme Q homologs in.

## INTRODUCTION

Interest in the aqueous dispersion of phospholipids by biological and medical scientists has increased considerably: lipid vesicles have

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†Abbreviation used: CoQs, coenzymes Q.

been widely used as membrane models and, more recently, as drug carriers. The system is endowed with versatility since liposomes, owing to their nature, can vary widely in size, composition, and surface characteristics, and also can be made to accommodate a great number of substances, including drugs, enzymes, hormones, and toxins (*1*). These compounds, if water soluble, can be trapped in the aqueous space, whereas lipophilic compounds can intercalate into the bilayer.

Coenzyme Q (CoQ) is an essential lipophilic component in electron transport chain of oxidative phosphorylation (*2–5*). Besides its role in mitochondrial bioenergetics, CoQ may also affect membrane fluidity (*6,7*), protect membrane phospholipids against peroxidation (*8,9*), and be an effective therapy for myocardial disease (*10*).

CoQ and its shorter isoprenoid chain-length homologs are often used in studies on mitochondrial membranes (*2,11–13*) and on lipid vesicles (*14,16*). However, the incorporation of CoQs into the membranes, which is necessary to study their effects on the many phenomena in which they are supposed to be involved, seems to depend both on the type of membranes and on the length of the isoprenoid side-chain. Degli Esposti et al. (*16*), studying the incorporation of CoQ homologs from an aqueous phase both into preformed lipid vesicles and mitochondrial membranes, showed a higher incorporation into the natural membranes. In both experimental models, however, the incorporation pattern of the CoQ series was similar: there was an increased degree of incorporation by increasing the isoprenoid chainlength of the quinone.

Landi et al. (*9*) found that the short-chain quinone, $CoQ_3$, in *n*-pentane, can be incorporated into mitochondria, previously depleted of endogenous CoQ, at the same extent as $CoQ_{10}$. The incorporation method, then, seems to determine whether qualitatively and quantitatively different incorporation of the CoQ homologs is obtained.

To establish controlled conditions for the incorporation of CoQ homologs, which is necessary to correctly understand the results of our research, we examined the extent of incorporation of the various homologs into vesicles by different methods: mixing, ultrasonication, and ethanol injection.

## MATERIALS AND METHODS

Egg lecithin was obtained from Lipid Product Ltd. (Redhill, UK) and used without further purification. Diarachidonyl-L-α-lecithin was supplied by P.L. Biochemicals GmbH (West Germany). CoQ homologs were generously given by Dr. G. Lenaz.

Stock solutions (10–30 m*M*) of CoQs in absolute ethanol were stored at −20°C. Concentrations were determined by measuring the absorbance decrease at 275 nm upon the addition of $KBH_4$ (5 mg/mL) and using an extinction coefficient of 12.25 $mM^{-1}$ $cm^{-1}$. Sephadex G-50 and Sepharose 4B were obtained from Pharmacia Fine Chemicals AB, Uppsala

(Sweden). All other chemicals of the highest available quality were supplied by Merck (Darmstadt) and the Sigma Chemical Co. (St. Louis, MO, USA).

## Preparation of Multilamellar Vesicles

CoQ-containing liposomes were prepared by mixing suitable amounts of CoQs, which had been redissolved in $CHCl_3$ after ethanol evaporation, and egg lecithin in $CHCl_3$. The solvent was removed by evaporation under nitrogen.

Aliquots of 50 m*M* Hepes (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.4, were added to the dried lipid to give a phospholipid concentration of 3.2 mg/mL and a CoQ concentration of 64–192 nmol/mL.

The lipid film was dispersed with a Vortex mixer for 30 min at room temperature to yield a milky-white suspension.

## Preparation of Unilamellar Vesicles

### Ultrasonication

The milky-white dispersion of phospholipids containing the CoQ homologs (see above) were subsequently sonicated with a probe sonicator (MSE) for 30 min at 4°C under a nitrogen atmosphere (*17*).

Sonication was carried out intermittently for 40 s, followed by a 20 s resting period.

The vesicle dispersions were centrifuged for 20 min at 100,000*g* to remove any probe particles and large aggregates.

### Ethanol Injection

CoQ-containing vesicles were also prepared by the ethanol injection method of Kremer (*18*), partially modified. An ethanol/water solution (2:1 v/v), containing 12 mg/mL egg lecithin and 20–60 nmol of the various CoQs/mg phospholipid, was slowly injected (40 μL/min) through a Hamilton syringe into 5 mL of a magnetically stirred 50 m*M* Hepes buffer, pH 7.4, to obtain a final concentration of 1 mg lecithin/mL.

In order to separate CoQs not incorporated into the lipid bilayer, 1.5 mL of liposome suspension prepared by the above mentioned methods was applied to a 20 cm column either of Sephadex G-50 or Sepharose 4B, equilibrated with 50 m*M* Hepes, pH 7.4, and eluted with the same buffer.

## Determination of Coenzyme Q Incorporation

CoQ concentration in the vesicles was determined by two methods:

(a) Potassium deoxycholate, pH 8.2, was added to liposomes to give a final concentration of 1% (w/v) and the absorbance decrease at 275 nm upon the addition of $KBH_4$ (5 mg/mL) was

measured. Recordings were performed in a Perkin-Elmer 559-UV/VIS spectrophotometer.

(b) CoQs were extracted from aqueous suspension of liposome preparations according to Kröger (*19*). The absorbance decrease at 280–289 nm caused by the addition of a solution of $KBH_4$ to the ethanolic solution resulting from the extraction procedure was recorded. The difference extinction coefficient is 8.8 $mM^{-1}$ $cm^{-1}$.

These measures were performed in a SIGMA-ZWS II dual wavelength spectrophotometer at room temperature. Similar results were obtained with both methods.

Lipid phosphorus determination was made according to Marinetti (*20*).

## RESULTS

We used different methods to prepare CoQ-containing vesicles with a quinone content close to that of mitochondrial (*21*) and chloroplast membranes (*22*) (a molar ratio of lipid/quinone of 100; i.e., 13 nmol/mg phospholipid). The results obtained are reported hereafter.

Multilamellar liposomes, obtained by mixing and fractionation on a Sephadex G-50 column, were eluted in the void volume. CoQ determination, performed in the peak fractions, showed no comparable incorporation patterns of the various quinones tested.

It can be seen in Fig. 1 that $CoQ_1$, the most hydrophilic homolog of the CoQ series, was incorporated in an undetectable amount, if at all. The other short and middle chain homologs showed higher degrees of incorporation than those obtained with the long-chain ones. A direct correlation between the incorporation and the isoprenoid chainlength of the quinone was found (*16*). We have also evaluated the CoQ incorporation into liposomes as a function of the amount of CoQ added. Unphysiological $CoQ_3$, as short-chain homolog, and physiological $CoQ_9$, as long-chain quinone, were used. The relationship between the amount of the quinone added and its concentration in the hydrophobic phase is linear for $CoQ_3$ and sigmoidal for $CoQ_9$ (Fig. 2).

In the study above cited (*16*), the uptake was linear in the range of 0.05–0.3 m*M* of the various CoQ added to the system and reached saturation at a molar ratio lipids/CoQ of about 5.

Ultrasonic irradiation induced a similar apparent incorporation of both the naturally occurring homologs of CoQ with polyisoprenoid chain length ranging from ten to six units as well as of synthetic shorter chain homologs (Fig. 3). To obtain good reproducibility, care must be taken to evaporate $CHCl_3$ completely and to adjust sonic power and sonication time.

To test whether the CoQ present in the elution peak was effectively associated with the phospholipid vesicles and not a CoQ aggregate, we

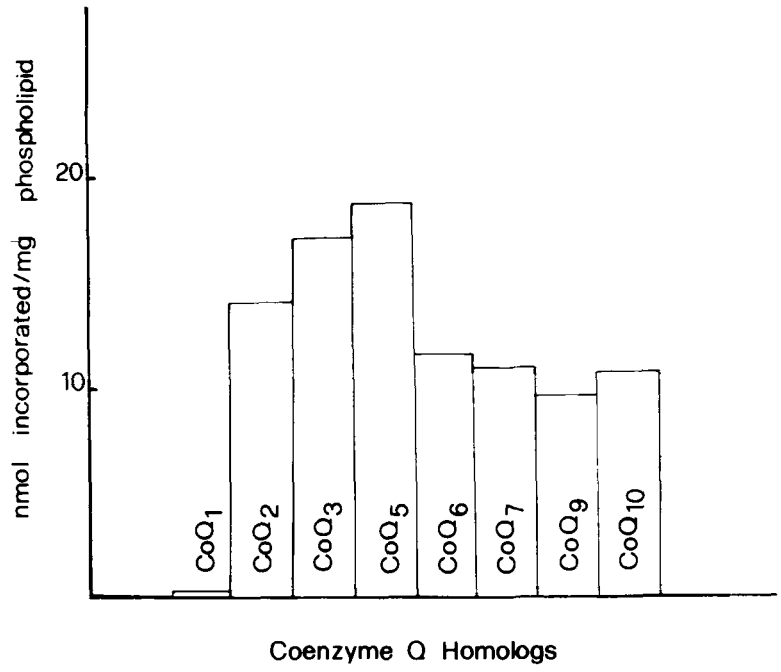


Fig. 1. Extent of incorporation of coenzyme Q homologs in multilamellar vesicles obtained by mixing. For determination of CoQ and total lipid concentrations, *see* the Materials and Methods section. CoQ added was 75 μ*M*.

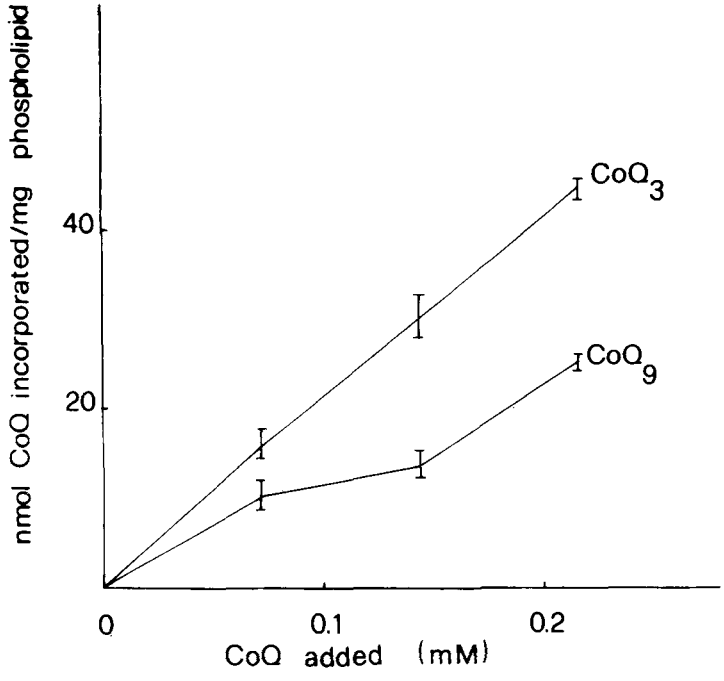


Fig. 2. Effect of the amount of CoQ added on the extent of incorporation in egg lecithin multilamellar vesicles. For determination of CoQ and total lipid concentrations, *see* the Materials and Methods section.

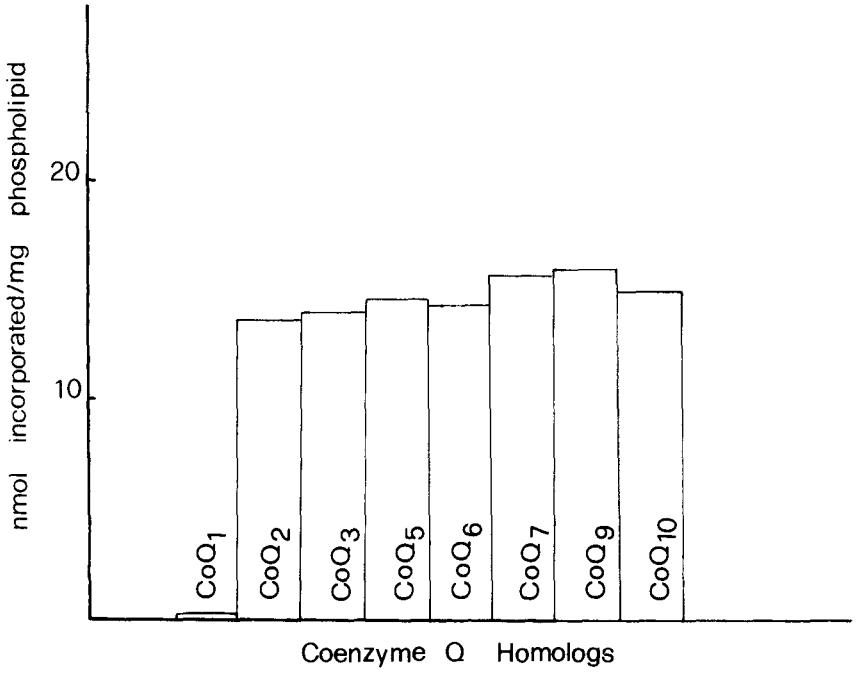


Fig. 3. Extent of incorporation of Coenzyme Q homologs in monolamellar vesicles obtained by sonication. For determination of CoQ and total lipid concentrations, *see* the Materials and Methods section.

have chromatographed the sonication product on a Sepharose 4B column that has been used to fractionate vesicless according to their sizes (*17*).

We found that for all CoQs tested, the ratio of CoQ and phospholipids through the elution peaks up to fraction 17 was constant (Fig. 4). Apparently, the CoQs with different side-chain length were then incorporated at the same extent both in large and in small vesicles. In the last chromatographic fractions (18–22), the content of the short side-chain CoQs is higher with respect to phospholipids, whereas a constant ratio was still present for the long chain homologs.

A similar pattern was obtained for $CoQ_{10}$ by Kingsley and Feigenson (*23*). The anomalous result obtained with $CoQ_3$ may be caused by a self-association of this quinone into micelles or small aggregates. A high ratio of $CoQ_3$ to phospholipids was also found in the first retention fractions from the Sephadex G-50. Since this molecular sieve chromatography is not time-consuming and does not excessively dilute the samples, Sephadex G-50 was used routinely to separate CoQ-containing vesicles from unincorporated CoQs.

To ascertain whether the degree of saturation of the fatty acyl residues affected the extent of CoQ incorporation, we also prepared egg lecithin vesicles at two proportions of arachidonic acid residues (6 and 12%) by sonication. These vesicles, because of their high content of polyunsaturated fatty acids, may be more prone to free-radical autoxidation in the presence of oxygen during sonication (*24*). The occurrence of

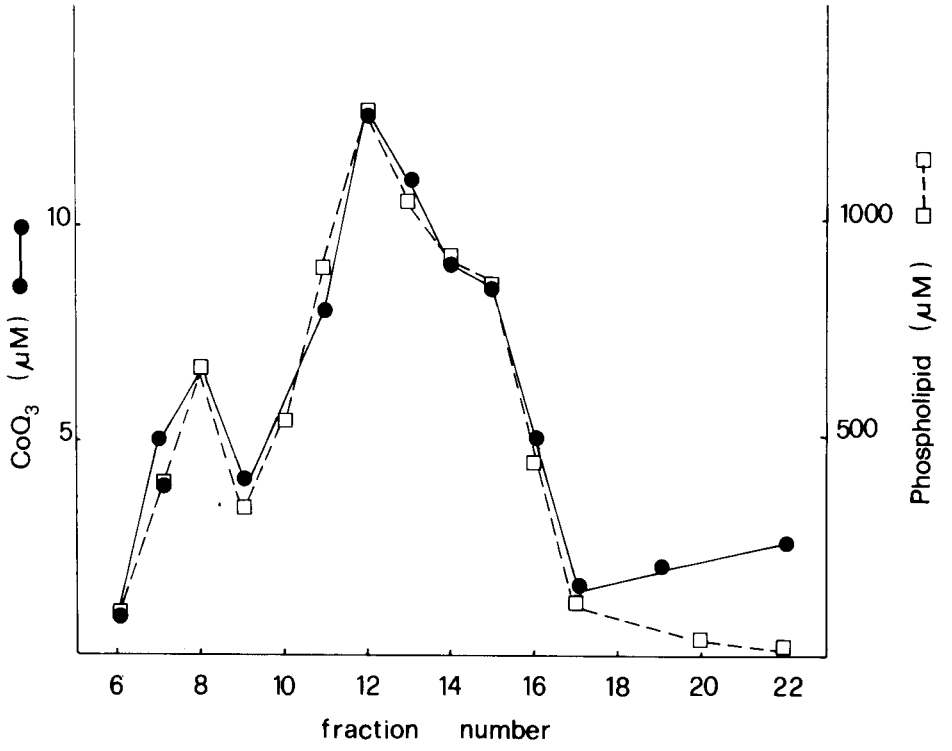


Fig. 4. $CoQ_3$ and phospholipid contents of fractions eluted from a Sepharose 4B column. A sonicated suspension of 75 μ*M* $CoQ_3$ and 4.3 m*M* egg lecithin in 1.5 mL of 50 m*M* Hepes, pH 7.4, was loaded onto a 1 × 20 cm Sepharose 4B column and eluted with 50 m*M* Hepes, pH 7.4. The total lipid in each 0.8 mL fraction was measured according to Marinetti (*20*) and the $CoQ_3$ concentration was determined from the absorbance decrease at 275 nm upon the addition of $KBH_4$ in 0.8% K-deoxycholate, pH 8.2.

this probable drawback has been evaluated determining the extent of peroxidation caused by sonication by the thiobarbiturate estimation of malondialdehyde (Table 1) (*25*). The eventual damage was minimized by the carefully controlled conditions used during sonication. This determination gave very low levels both in control vesicles and in CoQ-containing vesicles.

The lower levels of malondialdehyde found in CoQ-containing vesicles must be ascribed to the antioxidant properties of these coenzymes (*9*).

Ethanol injection, first described by Batzry and Korn (*26*) and slightly modified by Kremer et al. (*18*), was utilized to prepare CoQ-containing vesicles. The amounts of the various CoQs incorporated by this procedure in egg-lecithin monolamellar vesicles are similar, do not depend on the chain-length, and are close to the quinone content of natural membranes. The incorporation of the various CoQs tested was dependent on the CoQ concentration up to 60 nmol/mg phospholipid, and

TABLE 1
Malondialdehyde Formation During Sonication in CoQ-Containing Vesicles

| Vesicles | CoQ incorporated,[a] nmol/mg phospholipid | Malondialdehyde formation,[b] nmol/mg phospholipid | |
|---|---|---|---|
| | | A | B |
| Egg lecithin | — | 1.02 | 1.91 |
| Egg lecithin + $CoQ_3$ | 15.9 | 0.10 | 0.28 |
| Egg lecithin + $CoQ_7$ | 15.3 | 0.35 | 0.40 |
| Egg lecithin + $CoQ_9$ | 16.0 | 0.35 | 0.48 |

[a]CoQs added were always 25 nmol/mg phospholipid. For determination of CoQ incorporation, *see* the Materials and Methods section.
[b]Malondialdehyde formation was determined according to Beuge and Aust (25): A = egg lecithin containing 6% arachidonic acid residues; B = egg lecithin containing 12% arachidonic acid residues.

TABLE 2
Extent of CoQ Homologs Incorporation in Egg Lecithin Vesicles Obtained by Ethanol Injection

| Vesicles | CoQ incorporated/mg phospholipid,[a] nmol | |
|---|---|---|
| | A | B |
| Egg lecithin + $CoQ_3$ | 14.2 | 30.0 |
| Egg lecithin + $CoQ_4$ | 13.5 | 35.2 |
| Egg lecithin + $CoQ_7$ | 13.1 | 32.4 |
| Egg lecithin + $CoQ_9$ | 15.0 | 31.7 |

[a]A = CoQs added were always 20 nmol/mg phospholipid. B = CoQs added were always 50 nmol/mg phospholipid. For determination of CoQ incorporation, see the Materials and Methods section.

the amount of the CoQ incorporated was similar for all the homologs (Table 2).

Higher amounts of long-chain homologs had a conspicuous part remaining in an aggregate state in the ethanol/water mixture.

## DISCUSSION

The major objective of our research was to prepare vesicles with a CoQ content close to that of mitochondria to be used as models in the study of quinone role in these membranes. To achieve these results, different methods were utilized.

One of the attractions of using multilamellar vesicles as membrane models is that they are exceedingly easy to prepare. When these membrane models were used to incorporate the homolog series of CoQs, the amount of quinone associated with the vesicles was dependent on the

length of the isoprenoid side chain. Apparently, vigorous mixing was not able to disrupt the quinone aggregates that were insoluble in water. The model membranes are thus not suitable for comparative studies of the function of CoQs. These preparations, originally introduced by Bangham et al. (*27*) and employed to measure permeability parameters of various ions and molecules across lipid bilayer (*28*), seem most appropriate for drug delivery, especially because of their higher aqueous space-to-lipid ratio.

Monolamellar vesicles may be produced in two ways: ultrasonication of multilamellar smectic mesophase and ethanol injection. The vesicles prepared by both these methods incorporated comparable amounts of CoQs independently on the length of the isoprenoid side chain. In the case of monolamellar vesicles prepared by ultrasonication, the reason for similar incorporation of the various CoQ homologs can be found in the high energy of sonic oscillation that is able to fragment the aggregated forms of long-chain CoQs in water. The similar incorporation of the CoQ homologs in monolamellar vesicles prepared by ethanol injection is probably related to the species formed by CoQs and phospholipids in ethanol/water.

These two types of compounds probably form spontaneously (in this solvent) mixed micelles that, injected into a buffered solution, form unilamellar vesicles of compositions similar to those of the original micelles. The naturally occurring homologs that are insoluble in the ethanol/water mixture are solubilized by the phospholipid micelles, which probably act as a detergent, though only up to a critical concentration, beyond which aggregates are formed.

Monolamellar vesicles, then, whatever the preparation method employed, may accommodate comparable amounts of CoQs homologs and may be utilized in experimental systems in which a similar content of the different CoQs is an essential requisite.

The peroxidative damage that may occur when CoQs are incorporated into polyunsaturated lipid vesicles by sonication proved to be totally unimportant because of the antioxidant activity of these quinones.

The choice between the two methods then may be directed by other practical considerations.

Ethanol injection, being simple, rapid, gentle, and free of any need for expensive apparatus, seems to be the method of choice. On the other hand, to obtain dilute preparations of liposomes by this technique may render ultrasonication more advantageous in some cases.

## ACKNOWLEDGMENTS

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