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UBIQUINOL-3 AND UBIQUINOL-7 EXHIBIT SIMILAR ANTIOXIDANT ACTIVITY IN MODEL MEMBRANES

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This study was undertaken to compare, on a kinetic basis, the antioxidant efficiency of an ubiquinol homologue having a short isoprenoid side-chain length, such as ubiquinol-3, with that of the long chain ubiquinol-7, by determining their rate constants of inhibition with respect to α-tocopherol. To this purpose we incorporated ubiquinol-3, or ubiquinol-7, or α-tocopherol into liposomes of egg yolk lecithin, and triggered lipid peroxidation with the thermal decomposition of a lipophilic azocompound. The results show that: i) the rate constants of inhibition for the two quinols are similar and slightly lower than that of α-tocopherol; ii) the length of the radical chain obtained in the presence of the two quinols is almost the same. From these data we concluded that the two homologues tested behave as chain-breaking antioxidants with quite similar effectiveness.

KEY WORDS: Ubiquinol, ubiquinone, antioxidant, liposome, lipid autoxidation, azo-initiators.

INTRODUCTION

Ubiquinones are lipophilic compounds whose main function is to act as redox components of transmembrane electron transport systems, such as the respiratory chain of mitochondria. However, the presence of ubiquinone in most cellular membranes,[1] where its function is still unclear, has suggested that this molecule may have some physiological role other than mitochondrial energy production. In fact, much evidence is accumulating that ubiquinone, mainly in its reduced form, can act as an antioxidant both *in vitro* and *in vivo*.[2,3,4,5,6]

We recently reported the quantification of the antioxidant activity of an ubiquinone homologue having a short isoprenoid chain length, such as ubiquinone-3, both in its oxidized[7] and reduced[8] form. By studying the autoxidation of egg phosphatidylcholine both in solvent solution and in liposomes initiated by a lipid-soluble azocompound, we showed that both ubiquinone-3 (Q_3) and ubiquinol-3 (Q_3H_2) act as chain-breaking antioxidants. However, only Q_3H_2 is a very efficient antioxidant, having a rate constant of inhibition similar to that of α-tocopherol (α-T), while the value for Q_3 is two orders of magnitude lower than that of α-T. Therefore, high concentrations of Q_3 are required to exhibit significant antioxidant activity. Furthermore, we observed that Q_3H_2 exerts a sparing effect toward α-tocopherol.[9] Our reports are in accordance with the results obtained by Frei *et al.* on the antioxidant activity of ubiquinol-10

in liposomes.[10] However, the rate constant of inhibition of the physiological homologue was not determined by these authors. The antioxidant action of ubiquinol homologues with different isoprenoid chain length has been studied also by Kagan *et al.*[11] They found that, in natural membranes, ubiquinols with short isoprenoid chains, (Q_1–Q_4) are much more potent inhibitors of lipid peroxidation than the longer chain homologues, (Q_5–Q_{10}). The differences in the antioxidant power of ubiquinols in membranes are suggested to depend on differences in their partitioning into membranes, in their intramembrane mobility and in their non-uniform distribution.

The aim of the present study is to compare, on a kinetic basis, the antioxidant efficiency of the synthetic short-chain Q_3H_2 with that of a physiological long-chain homologue, such as Q_7H_2, by determining the rate constants of inhibition of the two ubiquinols with respect to α-T. To this purpose we incorporated into liposomes of egg yolk lecithin either Q_3H_2, or Q_7H_2, or α-T and triggered lipid peroxidation with the thermal decomposition of 2,2'-azobis-(2,4-dimethyl-valeronitrile) (AMVN). This lipid-soluble azocompound gives rise to radical chain initiation at a constant and reproducible rate within the lipid bilayer,[12] making possible quantitative studies of the antioxidant activity of any lipophilic chain-breaking antioxidant.

MATERIALS AND METHODS

Egg yolk lecithin (PC) was purchased from Lipid Products (Redhill, U.K.) and was stored at −20°C in chloroform under nitrogen. Q_3 and Q_7 were kindly supplied by Eisai Co. (Tokyo, Japan) and stored as a 10–20 mM solution in ethanol at −20°C. The AMVN was from Polysciences Inc. (Warrington, PA) and α-tocopherol (α-T) from Sigma Chemical Co. (St. Louis, MO). AMVN was stored as a 0.8 M solution in benzene at −20°C in the dark. All other chemicals of the highest available purity were from Merck. All aqueous solutions were passed through Chelex-100. Q_3 and Q_7 were quantitatively reduced according to Rieske[13] and kept in absolute ethanol under slight acidic conditions at concentrations between 5 and 10 mM.

Multilamellar liposomes were prepared as follows: AMVN, Q_3H_2 or Q_7H_2 or α-T, when present, and PC were added in a round-bottom tube. After each addition the solvent was removed with a stream of nitrogen keeping the tube in ice. The thin film obtained was vortex stirred for 7 min with an aliquot of 0.01 M Hepes buffer, pH 6.5, containing 10^{-5} M Na_2EDTA, in order to obtain 4 mM AMVN, 11–52.3 μM Q_3H_2, or 12–46.8 μM Q_7H_2, or 7–34 μM α-T and 15 mM PC dispersions. This procedure was always carried out in a cold room to prevent the start of oxidation. The reaction vessels were immersed in a water bath at 40°C and kept under air in the dark. Liposome autoxidation was measured spectrophotometrically by determining conjugated diene formation. Liposome aliquots were dissolved in ethanol and spectra in the wavelength range 200–300 nm were recorded. The increase in absorbance at 233 nm ($\varepsilon = 28000\ M^{-1}\ cm^{-1}$)[14] was taken as an indication of the appearance of conjugated dienes. The Q_3H_2, or Q_7H_2, or α-T remaining in liposomes during the course of oxidation were added with the same volume of isopropanol, and with BHT (final concentration 0.01%) and analyzed by HPLC using an Altex ODS 5 μm column (15 × 0.46 mm). The elution was isocratic at a flow rate of 1.5 ml/min with methanol for the determination of α-T and Q_7H_2, ad with methanol/water (82.5:17.5) for Q_3H_2. Detection was at 290 nm.

RESULTS

When a thermolabile azo-initiator such as AMVN is present, the autoxidation of PC liposomes proceeds by a free radical chain mechanism with a constant rate of oxidation. In this case the steady state treatment can be applied and the rate of oxidation is given by equation 1:

$$\frac{d[LOOH]}{dt} \cong \frac{-d[O_2]}{dt} = k_p(R_i/2k_t)^{1/2}[LH] \quad (1)$$

where LH and LOOH are unsaturated fatty acid residues of PC and lipid hydroperoxides, respectively; R_i represents the rate of free radical chain initiation, k_p is the rate constant for hydrogen atom abstraction by lipid peroxyl radical from the unsaturated lecithin and k_t is the rate constant of termination.

In the presence of a phenolic chain-breaking antioxidant, AH, the rate of oxidation, R_{inh}, is given by equation 2:

$$\frac{d[LOOH]}{dt} \cong \frac{-d[O_2]}{dt} = \frac{k_p R_i[LH]}{nk_{inh}[AH]} \quad (2)$$

where n represents the stoichiometric factor, i.e., the number of peroxyl radicals trapped by each antioxidant; k_{inh} is the rate constant for the hydrogen abstraction by lipid peroxyl radical from the antioxidant.

Since PC autoxidation is accompanied by both oxygen uptake and conjugated diene hydroperoxides formation, it is possible to follow this oxidation by measuring any of the above changes. It has been observed that the amounts of oxygen uptake and conjugated diene formation agree with each other when fatty acids contain only two double bonds.[15] In the case of PC, which contains fatty acids with three or more double bonds, other products besides conjugated hydroperoxides are formed during autoxidation. Therefore, the rate of LOOH formation is always proportional but lower than the rate of oxygen uptake. Since liposome autoxidation was measured in the experiments here reported by following spectrophotometrically the formation of conjugated dienes, a suitable correction factor was used in the calculation. By assuming that the average oxidizability of egg PC liposomes is the same as that reported by Barclay and Ingold,[16] a correction factor of 6.85 was used in this paper and in the previous one.[8]

In order to give a quantitative measure of the antioxidant activity, it is necessary to determine two principal parameters: n and the ratio k_{inh}/k_p.

The stoichiometric factor can be determined by measuring the length of the induction period, τ, obtained in the presence of the antioxidant, accordingly to equation 3:

$$n = \frac{\tau R_i}{[AH]} \quad (3)$$

In fact, when an antioxidant is present, the oxidation is suppressed and a clear induction period is observed. This period persists as long as the antioxidant is present in the system. When the induction period is over, the oxidation proceeds at the same

rate as that without antioxidant. The rate of initiation R_i was determined by measuring the induction period obtained in the presence of α-T, for which $n=2$ is assumed, and by using equation 3.

Equations 2 and 3 can be combined to give equation 4, from which the ratio k_{inh}/k_p can be calculated:

$$k_{inh}/k_p = \frac{[LH]}{R_{inh}\tau} \quad (4)$$

This ratio determines the efficacy of the antioxidant for scavenging the chain carrying peroxyl radicals before these radicals continue the chain propagation.

The formation of conjugated dienes as a function of time with and without the addition of α-T, Q_3H_2, and Q_7H_2 is reported in Figures 1A, 2A and 3A, respectively. Various amounts of Q_3H_2 and Q_7H_2 were incorporated into liposomes to investigate whether possible differences in quinol antioxidant ability were dependent on their concentration.

Diene formation vs. time is linear in control liposomes; in the presence of each antioxidant diene formation is strongly reduced and a clear inhibition period occurs. When all the antioxidant is consumed, the reaction proceeds at the same rate as that in the absence of the inhibitor.

When 8.7 or 34 μM α-T were incorporated into liposomes, the induction periods (τ) were 120 and 510 minutes respectively, as measured both by the inhibition of conjugated diene formation and by antioxidant consumption (Figure 1A and B). Figure 1A also shows that the value of τ is proportional to the α-T amounts present into liposomes and that autoxidation reaction is more inhibited at higher α-T concentration (see also Table 1). Liposomes containing 11.8, or 35.5, or 52.3 μM Q_3H_2 exhibited induction periods of about 70, 216 and 300 minutes respectively (Figure 2A). The length of the inhibition periods with Q_3H_2 is also confirmed by HPLC determination (Figure 2B) and appears proportional to the Q_3H_2 amount added and shorter than that measured with α-T. Liposomes containing similar amounts of the long-chain homologue Q_7H_2 exhibited induction times similar to those obtained with the short quinol (Figure 3A). By comparing the results shown in Figures 3A and B, a discrepancy can be observed between the induction periods measured by conjugated diene formation and those found by antioxidant consumption. This fact can mean that a small amount of Q_7H_2 is still present in the bilayer even though its antioxidant action is over. The slope of the straight lines, which represents the inhibited reaction in the presence of different amounts either of Q_3H_2 or Q_7H_2 can suggest that the antioxidant activity of the short-chain quinol is slightly higher than that of the long-chain one (cfr. R_{inh} in Table 1).

To put these observations on a more quantitative basis, the various parameters of the system under investigation were calculated and reported in Table 1. From the traces of control liposomes (see Figures 1A, 2A and 3A) the oxidizability, $k_p(2k_t)^{1/2}$, and the average length of radical chains, ν, were determined as $1.75 \times 10^{-2}\ M^{-1/2}\ s^{-1/2}$ and 43, respectively (see Table 1). In the presence of α-T the ν value became 7 and 2. When liposomes containing similar amounts of either Q_3H_2 or Q_7H_2 were used, about the same values of the chain length were obtained. These data confirm, as previously stated, that ubiquinols behave as chain-breaking antioxidants, and suggest that their effectiveness is comparable.

From the length of the induction periods and by using equation 3, the mean value

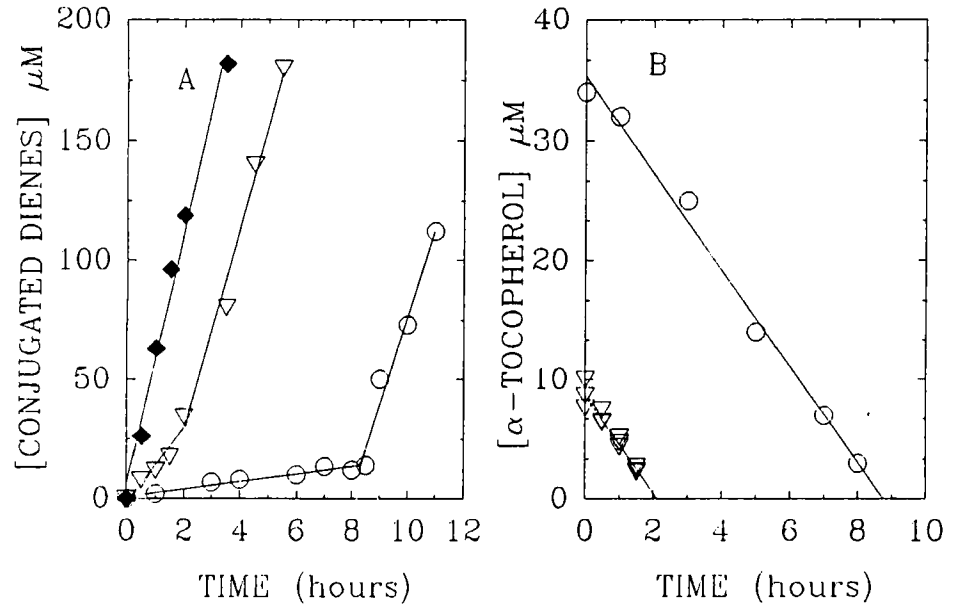

Figure 1 Rates of conjugated diene formation (A) and α-T consumption (B) during egg PC liposome autoxidation initiated by AMVN at 40°C. (◆) control liposomes; (▽) 7–11 μM α-T-containing liposomes; (○) 34 μM α-T-containing liposomes. The AMVN and PC concentrations were 4 and 15 mM, respectively.

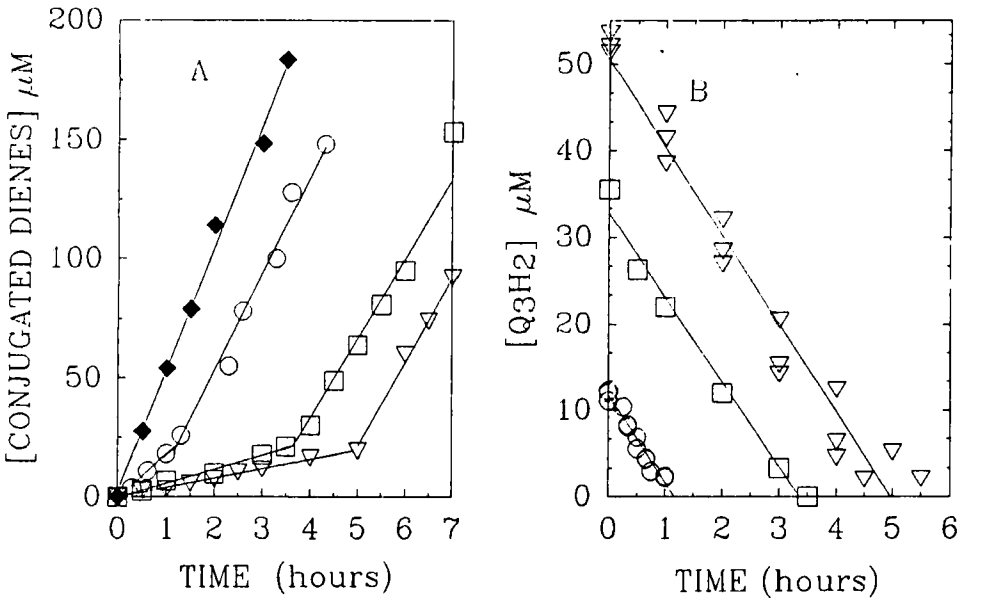

Figure 2 Rates of conjugated diene formation (A) and Q_3H_2 consumption (B) during egg PC liposome autoxidation initiated by AMVN at 40°C. (◆) control liposomes; (○) 11–13 μM Q_3H_2-containing liposomes; (□) 35.5 μM Q_3H_2-containing liposomes; (▽) 51–52.3 μM Q_3H_2-containing liposomes. The AMVN and PC concentrations were 4 and 15 mM, respectively.

Table 1 Autoxidation of egg PC multilamellar liposomes initiated by AMVN at 40°C in the absence and in the presence of Q_3H_2, or Q_7H_2, or α-T

Liposomes	Antioxidant μM	τ min	R_{inh}/M s^{-1a}	R_i/M s^{-1a}	ν	$k_p/(2k_t)^{1/2}$/$M^{-1/2}$ $s^{-1/2}$	n	k_{inh}/k_p	$k_{inh}(QH_2)/k_{inh}$(α-T)
Control	none	—	—	—	43	1.75×10^{-2}	—	—	—
+α-T	8.7	120	1.62×10^{-8}	1.62×10^{-7}	7	—	2.00	129	1.00
"	34.0	510	3.50×10^{-9}	1.47×10^{-7}	2	—	2.00	140	1.00
$+Q_3H_2$	11.8	70	3.90×10^{-8}	—	16	—	0.74	107	0.83
"	35.5	216	1.20×10^{-8}	—	5	—	0.88	97	0.69
"	52.3	300	0.70×10^{-8}	—	3	—	0.83	119	—
$+Q_7H_2$	13.4	90	4.03×10^{-8}	—	17	—	0.98	69	0.53
"	34.0	210	1.85×10^{-8}	—	7	—	0.89	64	0.45
"	46.8	270	1.18×10^{-8}	—	5	—	0.84	79	—

The reported values are means of at least four independent measurements.
[a]Expressed for the hydrophobic bilayer rather than for the total solution.

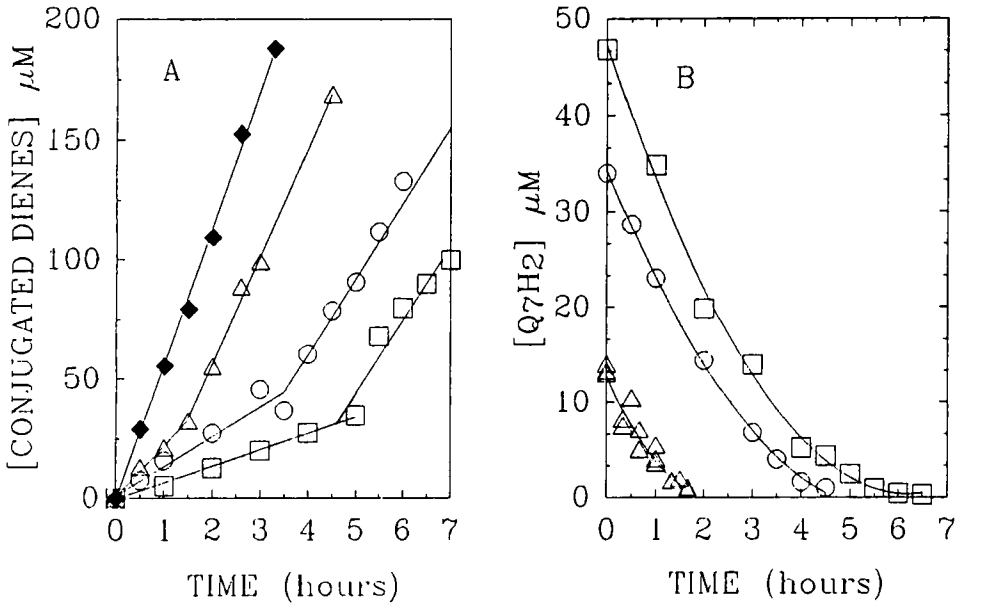

Figure 3 Rates of conjugated diene formation (A) and Q_7H_2 consumption (B) during egg PC liposome autoxidation initiated by AMVN at 40°C. (◆) control liposomes; (△) 12–15 μM Q_7H_2-containing liposomes; (○) 34 μM Q_7H_2-containing liposomes; (□) 46.8 μM Q_7H_2-containing liposomes. The AMVN and PC concentrations were 4 and 15 mM, respectively.

of the stoichiometric factor, n, for Q_3H_2 and Q_7H_2 was determined as 0.81 and 0.90 respectively. These values, smaller than the expected of 2, may be due to the fact that a portion of quinols is "wasted" by autoxidation (see discussion).

From the slopes of conjugated diene formation in the presence of each antioxidant and by using equation 4, the ratios k_{inh}/k_p were calculated and reported in Table 1 together with the ratio k_{inh} $(QH_2)/k_{inh}$ (α-T), which indicates the relative efficiency of the two homologues with respect to α-T. As shown in Table 1, the ratio k_{inh}/k_p for Q_3H_2 is very similar to that for α-T, but higher than that for Q_7H_2. On the other hand, the stoichiometric factor for Q_3H_2 is slightly lower than that for Q_7H_2.

DISCUSSION

The results reported here confirm that ubiquinols behave as chain-breaking antioxidants by trapping lipid peroxyl radicals generated in the autoxidation of PC liposomes initiated by the lipid-soluble AMVN. The antioxidant effectiveness of the two quinols is very high, since their inhibition rate constants are of the same order of that of α-T. Moreover, the antioxidant ability of the two homologues does not change by varying their concentration (cfr. Table 1). These data also show that the short- and long-chain ubiquinol homologues act as antioxidants with quite similar effectiveness, as suggested by the following considerations:

— in the presence of the two quinols the chain length is almost the same.
— the ratio k_{inh}/k_p is lower for Q_7H_2 than for Q_3H_2, while the stoichiometric factor of the long-chain homologue is slightly higher.

As far as the stoichiometric factor is concerned, in organic solvents this value was found approx. 2.[8] On the other hand in liposomes the value is drastically reduced (see Table 1). This effect has been previously reported by Frei *et al.*[10] and by us[8] as due to autoxidation reactions that can take place at the membrane surface. Therefore, the quinol amount available for the antioxidant activity is considerably lower than that originally incorporated into liposomes.

Under our experimental conditions a small amount of Q_7H_2, is still present at the end of the induction period (cfr. Figures 3A and B). This finding can reflect the fact that part of the quinol added to the system occurs in the bilayer in a nonmonomeric form, and may not contribute to radical trapping, thus partially decreasing Q_7H_2 effectiveness. A similar consideration was reported by Fato *et al.*[17] in a study on the determination of partition coefficients of ubiquinones by fluorescence quenching in phospholipid vesicles. They observed that these coefficients are lower for long-chain homologues than those expected from their solubility and suggested that part of the quinone is not accessible for quenching.

Data here reported are in partial disagreement with those obtained by Kagan *et al.*,[11] who studied the efficiency of ubiquinols with different isoprenoid chain length in preventing lipid peroxidation in rat liver microsomes, rat brain synaptosomes and mitochondria. They found that ubiquinols with long isoprenoid chains are much less efficient in preventing membrane lipid peroxidation than the short-chain ones. It is to be pointed out that in the above cited paper, the incorporation of the ubiquinols into membranes was accomplished by preincubating the membrane suspensions with the complete series of ubiquinol homologues at 10^{-4} M concentration. The supernatant obtained after membrane sedimentation was then extracted with hexane to test for any residual unincorporated ubiquinol. Since the quinol amounts found in the solvent were very low, the authors claimed that rather complete incorporation of ubiquinols was achieved. However, this procedure does not allow a quantitative and comparable incorporation of the different homologues and a quantitative evaluation of unincorporated quinols.[17] It is known, in fact, that the water solubility of ubiquinone homologues decreases with the increase of the isoprenoid chain length.[18] Apart from Q_1, whose water solubility is up to 10^{-3} M, all other ubiquinones are water insoluble at concentration higher than 2×10^{-5} M.[18] Thus a portion of the QH_2 added existed either aggregating in water or adhering to the membrane surface and it was not evaluated by the used method. Therefore, the lack of incorporation particularly of the longer chain homologues in biomembranes, could, in our opinion, probably explain the great difference in the antioxidant action between short- and long-chain quinols found by Kagan *et al.*[11]

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