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Antioxidant Activity of Reduced Menadione in Solvent Solution and in Model Membranes

DIANA FIORENTINI, MARTA CIPOLLONE, MARIA CRISTINA GALLI and LAURA LANDI*

Dipartimento di Biochimica "G. Moruzzi", Università di Bologna, Italy

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The antioxidant activity of reduced menadione was investigated and compared with that of α-tocopherol both in solvent solution and in large unilamellar vesicles by using azocompounds as free radical generators. The results show that: i) reduced menadione behaves as a chain-breaking antioxidant; ii) its inhibition rate constant is similar to that of α-tocopherol in homogeneous solution, whereas it is 4 times larger in egg yolk lecithin vesicles; iii) the stoichiometric factor is found lower than 1 in both systems, since a substantial portion of menadiol is consumed by autoxidation and does not contribute to radical trapping; iv) when both α-tocopherol and menadiol are present in vesicles, reduced menadione can spare α-tocopherol. Data presented here suggest that the reduced form of vitamin K may protect, when present, cellular membranes from free radical damage.

Keywords: Antioxidants, lipid peroxidation, lipid vesicles, vitamin K, azocompounds, α-tocopherol

Abbreviations: α-T, α-tocopherol; K_1H_2, reduced vitamin K_1; K_3H_2, reduced menadione; $Q_{10}H_2$, ubiquinol-10; Q, ubiquinone; LUVET(s), large unilamellar vesicle(s) obtained by extrusion technique; PC, phosphatidylcholine; AAPH, 2,2′-azobis(2-amidinopropane hydrochloryde); AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); AIBN, 2,2′-azobis(isobutyro-nitrile); BHT, butylated hydroxytoluene.

INTRODUCTION

Vitamin K (K) is an indispensable cofactor for the microsomal enzyme γ-glutamate carboxilase,[1,2] a post-translational enzyme which converts glutamil residues in precursor proteins to γ-carboxyl glutamil (Gla) residues in mature proteins. This aminoacid is not only present in plasma proteins, but Gla residues were also discovered in proteins of other tissues, most notably bone[3,4] and kidney.[5] The carboxilase is described as a reduced vitamin K- and O_2-dependent enzyme and the formation of vitamin K-2,3-epoxide is an obligatory step in the carboxilation reaction. In two successive steps the epoxide may be reduced, *via* the quinone, to reduced vitamin K (KH_2), which may be reused several thousand folds. Furthermore, vitamin K_2, i.e. menaquinone, like ubiquinone (Q), serves as a redox mediator in bacterial electron transport between the membrane bound dehydrogenases and the enzymes catalyzing the

*Corresponding author. Fax: 051/351224. E-mail: landi@biocfarm.unibo.it.

reduction of the terminal electron acceptor, such as O_2, NO_3^- and fumarate.[6] Evidence supporting the antioxidant role of ubiquinol (QH_2) has been obtained in several systems, such as solutions, phospholipid vesicles, mitochondria, microsomes, LDL and *in vivo* systems.[7,8] Also the reduced form of vitamin K, being a substituted 1-4-naphthoquinol, would be expected to exhibit antioxidant activity. Mukai *et al.*,[9] in fact, recently measured the rate constant for the reaction of biologically important hydroquinones (reduced forms of ubiquinones and vitamin K) and α-tocopherol (α-T) with a substituted phenoxyl radical as a model of biological free radicals. They found that the approximate orders of magnitude of the scavenging rate are: K_1H_2 and $K_3H_2 > \alpha$ - T > $Q_{10}H_2$ in solvent solution. Furthermore, for each hydroquinone this rate constant increased by decreasing the solvent polarity. Quantitative data of lipoperoxyl-scavenging activity of KH_2 are not available as far as we know. This prompted us to investigate whether the high antioxidant activity of KH_2 measured in several solvents is also exhibited in model membranes, since it has been found that the rate of inhibition for α-tocopherol in the oxidation of vesicle membranes is considerably smaller than that in homogeneous solution.[10] In this work we examined the antioxidant effect of reduced menadione (K_3H_2) both in solvent solution and in large unilamellar vesicles (LUVETs) of egg yolk lecithin. Azocompounds were chosen as free radical generators, since this type of initiators, ensuring a constant radical flow, are very useful for kinetic analysis. The quantification of the antioxidant effectiveness of reduced menadione was then compared with that of α-T, the biological antioxidant of choice. The results strongly suggest that K_3H_2 is a potential antioxidant in peroxyl radical-mediated membrane peroxidation. Therefore, the vitamin may play, in addition to its well known function, an antioxidant role in membranes where it resides.

MATERIALS AND METHODS

Chemicals Egg yolk lecithin (PC) was purchased from Lipid Products (Redhill, U.K.) and stored at –20°C in chloroform/methanol 1:1 (v/v) under nitrogen. The thermolabile azocompounds 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azobis(isobutyronitrile) (AIBN) were obtained from Polysciences Inc. (Warrington, PA). Menadione (K_3), α-T, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), methyl linoleate and MES buffer were from Sigma Chemical Co. (St. Louis, MO). Fusinite was obtained from Illinois EPR Research Center. All other chemicals of the highest available purity were from Merck. All aqueous solutions were passed through Chelex-100.

Reduction of Menadione 10 mg of menadione in 10 ml of isopropanol were added to 5 ml of 0.1 M Tris/0.15 M NaCl, pH 9. A pinch of $NaBH_4$ was added and the mixture was shaken until it became colorless, then the pH was rapidly turned to acid, to allow the formation of the K_3H_2. The aqueous solution was extracted with petroleum benzine, and evaporated under vacuum. The residue was dissolved in absolute ethanol, acidified with 1 N HCl and stored at –20°C. K_3H_2 concentration was determined by recording the UV spectrum in the wavelength range 200–360 nm (λ_{max} 244 nm; $\varepsilon = 33{,}110\ M^{-1}\ cm^{-1}$).[11]

Autoxidation in Solvent Solution The rate of PC oxidation in solvent solution was determined by monitoring oxygen uptake at 40°C both with a Clark-type oxygen electrode (Yellow Spring, OH) and by EPR measurements. In the first case the reaction mixture (3 ml) in methanol contained 125 mM PC, 4 mM AMVN and, when present, either α-T (7 μM) or K_3H_2 (7–15 μM).

EPR Measurements Solutions of 0.25 M methyl linoleate in tert-butanol were air saturated at room temperature and introduced (ca. 100 μl) into a capillary tube (with an internal diameter of

about 1.85 mm) in the presence of 31 mM AIBN and 77 μM α-T or 196 μM K_3H_2. A small amount of fusinite, a very sensible oxygen probe,[12] was introduced at the bottom of the capillary tube in contact with the solution. A second capillary tube, with an external diameter of 1.65 mm, sealed at one end, was inserted into the sample tube so to leave very little dead volume space. The tube was then put into the EPR cavity, kept at 40°C and the first spectrum was recorded after 1 min to allow for the temperature equilibration time. The temperature was controlled with a standard variable temperature accessory and was measured before and after each experiment with a copper-constantan thermocouple. The EPR spectra were recorded on a Bruker ESP 300 spectrometer by using the following settings: microwave frequency 9.74 GHz, modulation amplitude 0.4 G, center field 3321 G, sweep time 81 sec and time constant 81 msec.

LUVET Preparation Multilamellar vesicles were prepared by adding in a round-bottom tube the appropriate amount of PC and, when necessary, α-T. After each addition the solvent was carefully removed with a stream of nitrogen to obtain a thin film, then 0.6 ml of 10 mM MES buffer, pH 6.5, containing 0.14 M NaCl and 1 mM Na_2EDTA were added. The film was vortex-stirred for 7 min. The milky suspension was then transferred into LiposoFast (produced by Avestin, Ottawa, Canada) and extruded 19 times back and forth through two polycarbonate filters (100 nm pore size, Nucleopore Corp., Pleasenton, CA). The total volume of LUVETs was then adjusted to give a final concentration of 15 mM PC, and, when present, 7 μM α-T. K_3H_2-containing vesicles were prepared by adding an ethanolic solution of K_3H_2 to preformed LUVETs to give a final concentration between 6.4 and 15 μM.

Vesicle Autoxidation Autoxidation experiments were carried out by monitoring oxygen consumption electrochemically. After thermal equilibration at 40°C, the appropriate amount of AAPH was added to the LUVET suspension (3 ml) in order to obtain a final AAPH concentration of 20 mM, suitable for initiation of LUVET peroxidation.[13] The reaction cell was always protected from room light to avoid initiator photodecomposition.

α-Tocopherol Determination LUVETs containing α-T were placed into a water-bath at 40°C in the presence of 20 mM AAPH. To determine the amount of α-T remaining during the course of oxidation, vesicle aliquots were withdrawn at different time intervals, added with BHT (final concentration 0.01%) and the same volume of isopropanol. The solution was injected into an HPLC system, equipped with a Spherisorb ODS2 5μm column (15 cm × 4.6 mm). The elution was isocratic at a flow rate of 1.5 ml/min with methanol, detection was at 290 nm.

RESULTS AND DISCUSSION

Quinones of metabolic significance, such as ubiquinone, plastoquinone and vitamin K, all possess a terpenoid side-chain indicating their preference for location in the lipid phase of biological membranes. Since the two electron reduction potential E_0' of vitamin K_1 is –60 mV vs. NHE,[14] it can be suggested that the reduced form of this molecule behaves as a very good electron donor. In fact, special care has to be taken during naphthoquinol preparation owing to its spontaneous oxidation under air. This behavior may constitute a serious drawback since to fully incorporate the natural lipophilic K_1H_2 into vesicles it is necessary to add the reduced form of the vitamin to phospholipid solution at the beginning of LUVET preparation (cfr. Materials and Methods). During this procedure, which needed about 1 hour, oxidation of K_1H_2 might have occurred. Since the final aim of this work was to study the antioxidant activity of the reduced vitamin K in lipid vesicles, we decided to use the non-physiological vitamin K_3 (E_0' = –5 mV vs. NHE),[14] which, lacking the side-chain, can be incorporated into pre-formed

vesicles. If we consider, in fact, log P of menadione as an operational definition of its hydrophobicity, the values obtained in the octanol/water and cyclohexane/water systems were 2.20 and 1.88, respectively.[15] Therefore, a preferable partition in the lipid phase of the membrane model should be expected. Furthermore, in the kinetic study of free radical scavenging action of biological hydroquinones cited above,[9] K_1H_2 and K_3H_2 exhibited similar reactivity with a substituted phenoxyl radical.

The efficiency of the reduced menadione as an antioxidant was tested by studying the autoxidation of egg lecithin both in solvent solution and in large unilamellar vesicles initiated by azocompounds. The mechanism applicable both in homogeneous solution and in model membranes is summarized in eqs. (1–5):

$$\underline{\text{Initiation}} \qquad R\cdot + O_2 \longrightarrow ROO\cdot \qquad (1)$$

$$ROO\cdot + LH \longrightarrow ROOH + L\cdot \qquad (2)$$

$$\underline{\text{Propagation}} \qquad L\cdot + O_2 \longrightarrow LOO\cdot \qquad (3)$$

$$LOO\cdot + LH \xrightarrow{k_p} LOOH + L\cdot \qquad (4)$$

$$\underline{\text{Termination}}\ 2\,LOO\cdot \xrightarrow{k_t} \text{non radical products} \qquad (5)$$

where R· is the azocompound radical, LH represents polyunsaturated residues of lecithin, L· and LOO· are alkyl and alkylperoxyl radicals. In these equations k_p and k_t are the rate constants for propagation and termination of the radical chain, respectively. Using the steady state approximation eq. (6) is obtained for oxygen uptake:

$$-\frac{d[O_2]}{dt} = \frac{k_p[LH](R_i)^{1/2}}{(2k_t)^{1/2}} + (R_i) \qquad (6)$$

where R_i represents the rate of chain initiation. For quantitative determination R_i has to be measured, and this can be experimentally calculated by eq. (7):

$$R_i = \frac{n[AH]}{\tau} \qquad (7)$$

i.e. by adding an efficient chain-breaking antioxidant, AH, such as α-tocopherol or Trolox, known to trap two peroxyl radicals, that is to say that their stoichiometric factor, n, equals 2, and by measuring the inhibition period (τ) during which oxidation is suppressed. The presence of a phenolic chain-breaking antioxidant prevents lipid radical chain reaction from occurring by scavenging LOO· according to eqs. (8) and (9), where k_{inh} is the rate constant for inhibition:

$$LOO\cdot + AH \xrightarrow{k_{inh}} LOOH + A\cdot \qquad (8)$$

$$LOO\cdot + A\cdot \longrightarrow \text{non radical products} \qquad (9)$$

During the inhibition period, oxygen uptake is given by the following rate expression:

$$-\frac{d[O_2]}{dt} = \frac{k_p R_i[LH]}{nk_{inh}[AH]} + R_i \qquad (10)$$

Inhibition of PC Oxidation in Homogeneous Solution by K_3H_2

The AMVN induced peroxidation of PC in methanol has been used as model to obtain a kinetic characterization of the antioxidant activity of K_3H_2 in homogeneous solution. Oxygen uptake traces for PC oxidation at 40°C in the absence and in the presence of 7.0 μM α-T or of different amounts of K_3H_2 are shown in Figure 1. The rate of oxygen consumption occurred at a constant rate without any induction period in the absence of antioxidants (trace 1), while in the presence of either K_3H_2 or α-T the oxidation was suppressed. When inhibitors were completely disappeared, the induction period was over and the oxidation proceeded at the same rate as in the absence of antioxidants. The length of inhibition periods, as expected, turned out to be dependent on the K_3H_2 amounts added, as shown in traces 2

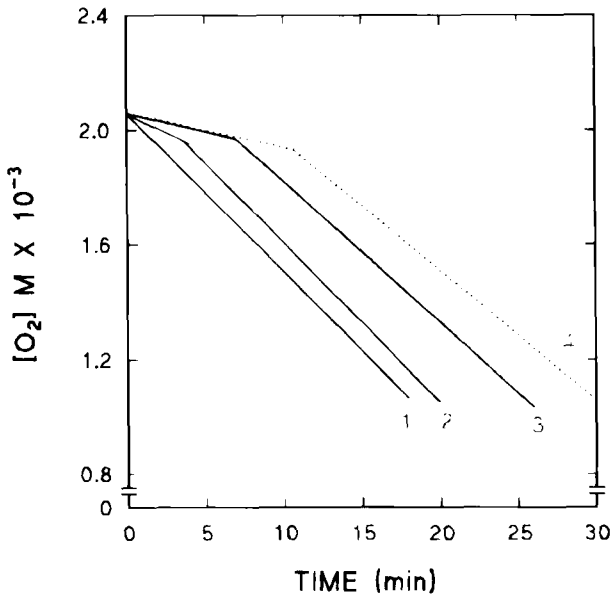

FIGURE 1 Rate of oxygen consumption during PC oxidation in methanol initiated by 4 mM AMVN at 40°C in the absence (trace 1) and in the presence of 7.0 μM α-T (trace 4, dotted line); 7.4 μM K_3H_2 (trace 2); 14.6 μM K_3H_2 (trace 3). PC concentration was 125 mM.

and 3. On the other hand, this length was very different when similar amounts of α-T and K_3H_2 were used (cfr. traces 2 and 4). The rate of oxygen uptake was slightly higher in the presence of the lowest concentration of K_3H_2, and it became superimposable to that obtained in the presence of α-T by increasing K_3H_2 concentration. The rate of initiation, R_i, was calculated by measuring the inhibition period, τ, obtained in the presence of a known amount of α-T.

The average length of the radical chain, $\nu = (-d[O_2]/dt)/R_i$, in the absence of inhibitors was calculated as 43 ± 10 (Table I). This value means that the peroxyl-driven chain reaction proceeds 43 times before the termination. The kinetic chain length was drastically lowered in the presence of K_3H_2, showing that the naphthoquinol acts as a chain-breaking antioxidant. By using eq. (7) and the previously calculated R_i value, the stoichiometric factor, n, for K_3H_2 was found to be 0.65 (see Table I), i.e. much smaller than the expected value of 2. This could be due to the spontaneous oxidation of the naphthoquinol under air, which reduces the effective amount of the available antioxidant. The ratio k_{inh}/k_p was derived by rearranging equation (10). The oxygen uptake was cal-

TABLE I PC autoxidation initiated by AMVN at 40°C in methanol in the absence and in the presence of α-T or K_3H_2.

Antioxidant	μM	τ (min)	$-d[O_2]/dt$ $(Ms^{-1}) \times 10^7$	ν^a	n	$k_{inh}(K_3H_2)/k_{inh}(\alpha\text{-T})$
none	—	—	9.2 ± 2.1	43.0 ± 10.0	—	—
α-T	7.0	11	1.8 ± 0.5	8.5 ± 2.4	2.0	1.0
K_3H_2	7.4	4	4.3 ± 0.4	20.0 ± 3.5	0.7 ± 0.2	1.2 ± 0.3
K_3H_2	14.6	7	1.9 ± 0.2	9.1 ± 1.0	0.6 ± 0.1	1.5 ± 0.4

The reported values are means of at least four independent measurements ± standard deviation. The AMVN and PC concentrations were 4 and 125 mM, respectively.
[a]$\nu = (-d[O_2]/dt)/R_i$

culated in the presence of either K_3H_2 or α-T; the value of *n* and the concentration of each antioxidant were substituted in the equation. From these data the relative efficiency of K_3H_2 as inhibitor with respect to α-T, $k_{inh}(K_3H_2)/k_{inh}$ (α-T), was found to be slightly higher than 1. This seems to indicate that K_3H_2 reacts with peroxyl radicals slightly faster than α-T. On the other hand, the rate constants for the reaction of K_1H_2 and K_3H_2 with 2,6-di-tert-butyl-4(4-methoxyphenyl) phenoxyl radical, measured by Mukai *et al.*[9] in ethanol with a stopped-flow technique, were found to be 31 and 21 times larger than that of α-T.

To corroborate our results we evaluated the same parameters by measuring oxygen consumption by means of EPR spectroscopy. This measurement relies on the fact that oxygen induces a broadening of EPR spectral lines of the other paramagnetic species present in solution and that the rate of oxygen uptake can be measured by monitoring the variation of the EPR line width of stable radicals added to the mixture where autoxidation takes place. The EPR method used to measure lipid autoxidation and activity of lipophylic antioxidants[16] is based on the use of fusinite, a paramagnetic probe derived from coal. This probe is totally insoluble both in water and in organic solvents and can report oxygen concentration that are lower than 0.1 μM.[17] Thus, the EPR method allows to measure more accurately and sensitively the oxygen concentration than oxygen electrodes, which are not very accurate below 15–20 μM.[18]

Autoxidation of 0.25 M methyl linoleate in tert-butanol induced by the thermal decomposition of 31 mM AIBN at 40°C was measured in the presence of 77 μM α-T and 196 μM K_3H_2. Table II shows the results obtained. The induction periods were 46 min and 39 min, respectively. The value of the stoichiometric factor for K_3H_2 was determined as 0.7, and the ratio $k_{inh}(K_3H_2)/k_{inh}$(α-T) was found to be 1.1. These parameters, which quantify the antioxidant activity of reduced menadione, are very similar to those determined electrochemically in the autoxidation of egg lecithin in methanol initiated by AMVN. Therefore, it can be concluded that menadiol is an efficient antioxidant in solvent solution and, as other phenols or poly-phenols, it behaves as a scavenger of lipid peroxyl radicals with an inhibition rate constant similar to that of α-T. It can be also suggested that the higher rate constant measured by Mukai *et al.*[9] in solution could be ascribed to the fact that substituted phenoxyl radicals can not be regarded as models of lipid peroxyl radicals LOO·, being much less reactive than the latter ones.[19]

Inhibition of Oxidation of PC Large Unilamellar Vesicles by K_3H_2

We recently characterized large unilamellar vesicles obtained by extrusion technique, LUVETs, as models for lipid peroxidation studies.[20] These vesicles were found suitable to undergo peroxidation reaction initiated by thermolabile azocompounds in the absence or presence of antioxidants. A quantitative analysis of the peroxyl radical scavenging activity of K_3H_2 in these model membranes was approached by using AAPH. This water-soluble radical generator produces peroxyl

TABLE II Autoxidation of methyl linoleate initiated by AIBN at 50°C in tert-butanol in the absence and in the presence of α-T or K_3H_2.

Antioxidant	μM	τ (min)	$-d[O_2]/dt$ $(Ms^{-1}) \times 10^7$	v	*n*	$k_{inh}(K_3H_2)/k_{inh}$(α-T)
none	—	—	9.2 ± 0.4	17.0 ± 0.2	—	—
α-T	77	46	1.5 ± 0.3	2.6 ± 0.3	2.0	1.0
K_3H_2	196	39	1.5 ± 0.5	2.6 ± 0.1	0.7 ± 0.4	1.1 ± 0.2

The reported values are means of at least four independent measurements ± standard deviation. The AIBN and methyl linoleate concentrations were 31 and 250 mM, respectively.

radicals in the aqueous phase and thereby attacks phospholipids at the membrane surface[21] according to the reaction scheme previously shown. The effect of increasing concentrations of K_3H_2 on the autoxidation of LUVETs was studied and compared with that of α-T. The results reported in Figure 2 show that the rate of lipid peroxidation, evaluated as oxygen uptake, was strongly inhibited by both antioxidants, but the inhibition time is strikingly different in the two cases, as previously shown for solvent solution. When inhibitors were consumed the oxidation proceeded at the same rate as in the absence of antioxidant in the case of α-T (trace 3), whereas oxygen uptake was somewhat retarded in the experiments with high K_3H_2 concentrations (trace 4). The latter result may be explained on the basis of a moderate inhibiting activity of the oxidized form of menadione. An antioxidant effect of various vitamin K homologues on ascorbic acid/Fe^{2+} induced peroxidation of lecithin vesicles has been reported.[22] The oxidized form of ubiquinone also exerts a protective action against peroxidation induced by iron salts[23] or ultrasonic irradiations.[24] However, quantification of Q antioxidant activity by means of azocompounds showed that its inhibition rate is about two order of magnitude lower than that of α-T.[25] Thus, high concentrations of ubiquinone were required to exhibit significant antioxidant activity, as well as in the paper cited above[22] only high concentrations of vitamin K homologues (50–200 μM) inhibited lipid peroxidation. From the traces of Figure 2 it can be observed that during the inhibition period the rate of oxygen uptake was higher in the presence of α-T (trace 3) than in the presence of K_3H_2. Table III summarizes the kinetic parameters for the oxidation of PC vesicles in the presence and in the absence of α-T or K_3H_2. In this case R_i was determined by adding a known amount of Trolox, a hydrophilic analogue of α-T. The average length of the radical chain, ν, in the absence of inhibitor was calculated as 9.0 ± 0.8. In the presence of each antioxidant the chain length was shortened, therefore K_3H_2 behaves as a chain-breaking antioxidant both in solvent solution and in lipid vesicles. As previously shown in organic solution, the inhibition period for K_3H_2 was always much lower than that measured with an equivalent amount of α-T and the stoichiometric factor turned out to be 0.85. This value, smaller than the expected value of 2, may be due to the fact that a portion of K_3H_2 is wasted by other reactions, e.g. by autoxidation and by direct scavenging of AAPH-derived radicals. As far as this last point is concerned, it has to be reminded that K_3H_2, lacking the side-chain, is probably faced at the membrane surface. Therefore, the K_3H_2 amount available for the antioxidant activity is considerably lower than that originally added to PC vesicles. The antioxidant effectiveness of reduced menadione is, however, very high, since in this case the ratio $k_{inh}(K_3H_2)/k_{inh}(\alpha\text{-T})$ turned out to be about 4. This higher ratio may be due to the smaller rate constant of inhibition for α-T in vesicles than in organic solvents, as cited above,[10] while the rate constant of inhibition for K_3H_2 is similar both in vesicles and in solution. Such an observation can be explained on the basis of a restricted mobility exhibited by α-T

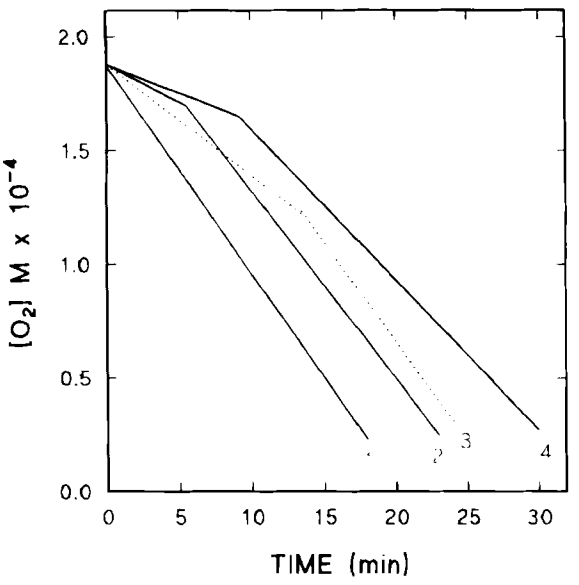

FIGURE 2 Rate of oxygen consumption during LUVET oxidation initiated by 20 mM AAPH at 40°C in the absence (trace 1) and in the presence of 7.1 μM α-T (trace 3, dotted line); 6.4 μM K_3H_2 (trace 2); 11.7 μM K_3H_2 (trace 4). PC concentration was 15 mM.

TABLE III LUVETs autoxidation initiated by AAPH at 40°C in the absence and in the presence of α-T or K_3H_2.

Antioxidant	μM	τ (min)	$-d[O_2]/dt$ $(Ms^{-1}) \times 10^8$	v	*n*	$k_{inh}(K_3H_2)/k_{inh}(\alpha\text{-T})$
none	—	—	15.3 ± 0.1	9.0 ± 0.8	—	—
α-T	7.1	14	7.5 ± 0.2	4.5 ± 0.1	2.0	1.0
K_3H_2	6.4	5	5.3 ± 0.8	3.1 ± 0.4	0.9 ± 0.2	4.3 ± 0.6
K_3H_2	11.7	9	4.1 ± 1.2	2.4 ± 0.7	0.8 ± 0.1	4.9 ± 0.8

The reported values are means of at least four independent measurements ± standard deviation. The AAPH and PC concentrations were 20 and 15 mM, respectively.

inside the vesicles, while K_3H_2, lacking the side chain, may move freely near the surface of the bilayer. Therefore these data indicate that menadiol reacts with lipid peroxyl radicals faster than α-T and that K_3H_2, due to its location, can trap some of the initiator radicals before they can attack LUVETs. This fact can lower the rate of chain initiation.

The Effect of The Simultaneous Addition of K_3H_2 and α-T

It has been previously reported that ubiquinol has a sparing effect on α-T during lecithin autoxidation induced by azocompounds both in vesicles and in homogeneous solution.[26-28] This sparing effect has been ascribed to a regeneration of α-T from the corresponding α-tocopheroxyl radical by ubiquinol and ubisemiquinone. To test whether K_3H_2 could spare α-T, we performed the experiments shown in Figure 3. When both α-T and reduced menadione were present simultaneously into the vesicles (trace 4), the induction period was lengthened to the sum of the induction periods observed when either α-T (trace 2) or K_3H_2 (trace 3) were used, and the slope of oxygen uptake trace during the inhibition period was intermediate between those obtained in the presence of each antioxidant. Figure 3B shows the amount of α-T remaining into lipid vesicles during the course of oxidation measured by HPLC; it can be seen that when only α-T was incorporated, it disappeared linearly with time. Nevertheless, when both α-T and K_3H_2 were present into the vesicles, the rate of α-T disappearance was very slow during the initial 10 min, corresponding to the induction period obtained with 11.7 μM K_3H_2 (cfr. Figure 3A and Table III). Afterwards α-T was consumed at the same rate as in the absence of K_3H_2. These results can be explained on the basis of the rate constant for inhibition of PC peroxidation due to K_3H_2, which is 4 times higher than that of α-T. This means that reduced menadione could scavenge peroxyl radicals more quickly than α-T. Therefore, the observation that α-T is initially consumed very slowly suggests that α-T, in these experimental conditions, is not regenerated from the corresponding radical by reaction with K_3H_2 but only spared for 10 min by the "sacrifice" of K_3H_2.

A stopped-flow kinetic study of the regeneration reaction of α-T with biological hydroquinones including K_1H_2 and K_3H_2 in solution was performed by Mukai *et al.*[29] The results indicate that both K_1H_2 and K_3H_2 can regenerate α-tocopherol from the corresponding radical in homogeneous solution. The different experimental results obtained in our membrane model might be explained by considering that many reactions can be taking place in heterogeneous systems. These competing reactions may be also influenced by the antioxidant localization in the lipid bilayer: it has to be expected, in fact, that K_3H_2 should be localized at the surface of vesicles. This location and the high reactivity towards peroxyl radicals may be prevalent on the regeneration of vitamin E from its radical.

CONCLUSIONS

The results here reported show that K_3H_2 behaves as a very effective chain-breaking antioxidant both in solvent solution and in vesicles. This can

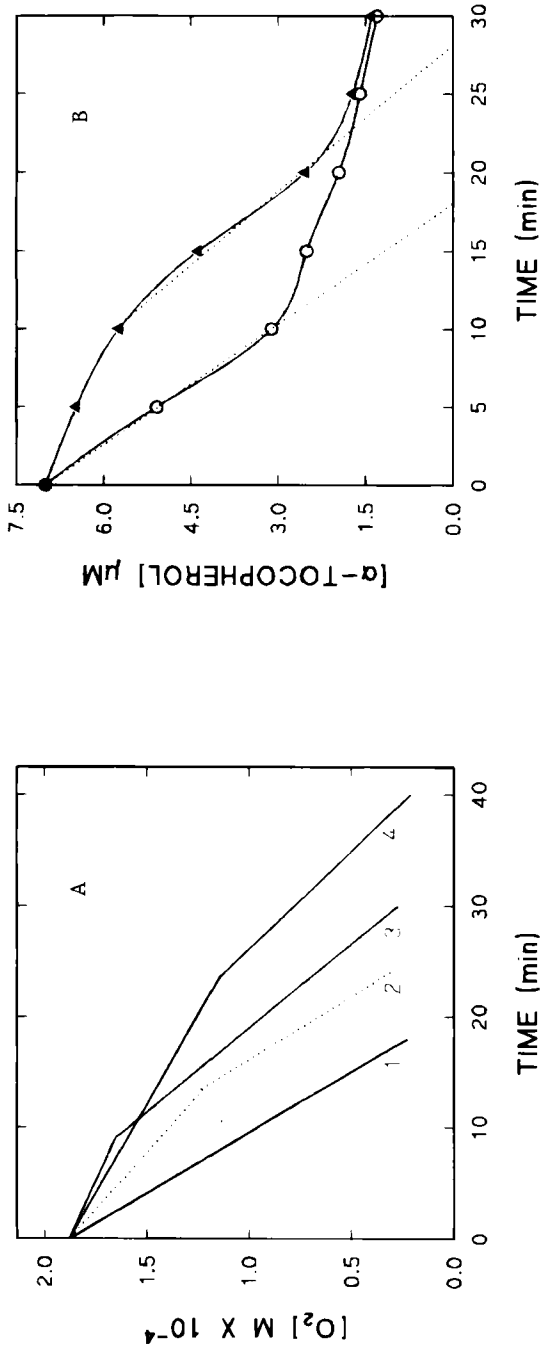

FIGURE 3 Rate of: (A) oxygen consumption and (B) α-T disappearance during LUVET oxidation initiated by 20 mM AAPH at 40°C. (A): trace 1: uninhibited reaction; trace 2, dotted line: in the presence of 7.1 μM α-T; trace 3: in the presence of 11.7 μM K_3H_2; trace 4: simultaneous addition of both 7.1 μM α-T and 11.7 μM K_3H_2. (B): ○ vesicles containing 7.1 μM α-T, dotted line; τ extrapolated; ▲ vesicles containing 7.1 μM α-T and 11.7 μM K_3H_2, dotted line: τ extrapolated.

be inferred from its inhibition rate constant, which is similar to that of α-T in homogeneous solution and 4 times larger in LUVETs, but not as high as those found by Mukai *et al.*[9] in different solvents. The stoichiometric factor of the reduced form of menadione is lower than 1 in both systems, because a substantial portion of the molecule is consumed by autoxidation and does not contribute to radical trapping. It is likely that *in vivo*, where the oxygen partial pressure is considerably lower than under our experimental conditions, K_3H_2 undergoes much less autoxidation, thus having a higher value of *n*; furthermore, and most importantly, vitamin K can be recycled by the reductase(s) in different tissues.

The results of these experiments support the possibility that the reduced vitamin K, like ubiquinol, has an additional role besides its function as a carboxylase cofactor, providing an antioxidant protection to cellular membranes.

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