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The efficiency of antioxidants delivered by liposomal transfer

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Abstract

Phenolic antioxidants of the hydroxychroman class, α-tocopherol (α-TOC) and 2,2,5,6,7-pentamethyl-6-hydroxychroman (PMHC), and the hindered phenols 2,3-dihydro-5-hydroxy-2,2,4-trimethylnaphtho[1,2-*b*]furan (NFUR), 2,6-di-*tert*-butyl-4-methoxyphenol (DBHA), and 2,6-di-*tert*-butyl-4-methyl phenol (BHT), were delivered into oxidizable (ACCEPTOR) liposomes of dilinoleoylphosphatidylcholine (DLPC) or 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC) from saturated DONOR liposomes of dimyristoylphosphatidylcholine (DMPC) by liposomal transfer. The antioxidant activities, k_{inh}, by the inhibited oxygen uptake method were compared with the k^s_{inh} determined when the antioxidants were introduced into the liposomes by coevaporation from organic solvents. The peroxidations were initiated using either thermal initiators, water-soluble azo-*bis*-amidinopropane hydrochloride (ABAP), lipid-soluble azo-*bis*-2,4-dimethylvaleronitrile (ADVN) and di-*tert*-butylhyponitrite (DBHN), or the photoinitiator benzophenone. The antioxidants PMHC, NFUR, DBHA, and BHT transferred rapidly between liposomes, but several hours of incubation were needed to transfer α-TOC. The average k^s_{inh} in liposomes, in the relative order NFUR $\simeq$ DBHA > PMHC > BHT $\simeq$ α-TOC, were markedly lower than known values in organic solvent. k_{inh} values in liposomes appear to be controlled by effects of hydrogen bonding with water and by restricted diffusion of antioxidants, especially in the case of α-TOC. Product studies of the hydroperoxides formed during inhibited oxygen consumption were carried out. The cis,trans/trans,trans (c,t/t,t) product ratios of the 9- and 13-hydroperoxides formed from PLPC during inhibited peroxidation by PMHC were similar for both the coevaporated and liposomal transfer procedures. The c,t/t,t ratio for the same concentration of α-TOC, 1.52, compares to a value of 1.69 for PMHC at the start of the inhibition period. The higher c,t/t,t ratio observed for NFUR in DLPC, which varied between values of 7.0 at the start of the inhibition to about 1.8 after the break in the induction period, is a reflection of the increased hydrogen atom donating ability of the antioxidant plus the increased concentration of oxidizable lipid provided by DLPC. © 1997 Elsevier Science B.V.

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1. Introduction

Antioxidants are of continuing interest for their ability to protect the unsaturated lipids of biological

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membranes from oxidative damage, and thus have been the subject of many scientific papers and reviews (e.g., [1–9]). Without protection, such oxidative damage can lead to tissue damage and various pathological events. Model membrane systems are commonly used to study antioxidant behaviour preliminary to monitoring their behaviour in a native membrane, where the membrane proteins and the variety of lipid types can complicate the study. It has been shown that the classical kinetic methods of autoxidation and antioxidant action in solution are applicable to studies in model phospholipid membranes [10,11]. Uninhibited autoxidation of phospholipid membranes follows the free-radical mechanism presented in Eqs. (1)–(4), initiated in this example by an azo-initiator.

Initiation:

$$\mathrm{R{-}N{=}N{-}R} \xrightarrow{k_i} 2\mathrm{R}\cdot + \mathrm{N_2}, \quad \text{rate} = R_i \tag{1}$$

Propagation:

$$\mathrm{R}\cdot + \mathrm{O_2} \xrightarrow{\text{fast}} \mathrm{ROO}\cdot \tag{2}$$

$$\mathrm{ROO}\cdot + \mathrm{RH} \xrightarrow{k_p} \mathrm{ROOH} + \mathrm{R}\cdot \tag{3}$$

Termination:

$$\mathrm{ROO}\cdot + \mathrm{ROO}\cdot \xrightarrow{2k_t} \text{non-radical products} + \mathrm{O_2} \tag{4}$$

The rate law for this mechanism is presented in Eq. (5):

$$-\mathrm{d}[\mathrm{O_2}]/\mathrm{d}t = k_p/\left(2k_t^{1/2}\right)[\mathrm{RH}]\,R_i^{1/2} \tag{5}$$

where k_p represents the propagation rate constant, $2k_t$ the termination rate constant, [RH] the substrate concentration, and R_i, the rate of chain initiation. By using an azo-initiator with known properties, it is possible to control the R_i (Eq. (6)), which is very important for quantitative studies.

$$R_i = 2ek_i[\mathrm{R{-}N{=}N{-}R}] \tag{6}$$

The quantity e is the efficiency of the initiator, and k_i is its decomposition rate constant.

When a phenolic antioxidant is added to a system undergoing autoxidation, the antioxidant molecules can trap peroxyl radicals formed during the propagation step, as represented in Eqs. (7) and (8):

$$\mathrm{ArOH} + \mathrm{ROO}\cdot \xrightarrow{k_{\text{inhibitor}}} \mathrm{ArO}\cdot + \mathrm{ROOH} \tag{7}$$

$$\mathrm{ArO}\cdot + \mathrm{ROO}\cdot \xrightarrow{\text{fast}} \mathrm{ArOROO} \tag{8}$$

As a result, oxygen uptake is suppressed until the antioxidant is consumed. This inhibition period, τ, has a simple relationship to the R_i, and thus the R_i can be calculated using Eq. (9):

$$R_i = n[\mathrm{ArOH}]/\tau \tag{9}$$

where n is the stoichiometric factor for the antioxidant, the measure of how many peroxyl molecules are trapped per antioxidant molecule.

Using a steady-state approximation, the rate law for suppressed oxygen uptake during inhibition (Eq. (10)):

$$-\mathrm{d}[\mathrm{O_2}]/\mathrm{d}t = k_p/k_{inh}[\mathrm{RH}]\,R_i/(2[\mathrm{ArOH}]) \tag{10}$$

can be expressed in terms of the R_i, the substrate and antioxidant concentrations, and the rate constants for propagation, k_p, and inhibition, k_{inh}.

Studies on antioxidant activity in liposomes typically use the process of coevaporation to introduce lipid-soluble antioxidants into a model membrane, combining the desired antioxidant, phospholipids, and lipid-soluble initiator in an organic solvent to ensure mixing, followed by removal of the solvent and preparation of the liposome in a selected buffer. There are, however, certain limitations associated with the coevaporation technique, namely:

(1) When initiator and inhibitor are both present in a sample, there is usually some loss of both during sample preparation, hampering quantitative kinetic studies.

(2) Time is required for the sample to reach the temperature of the surrounding water bath and for the rate of initiator decomposition to establish itself, and this initial lag period must be taken into account [10].

(3) One cannot introduce a lipid-soluble antioxidant into a natural cell membrane for study if coevaporation is the only way to deliver it, because the normal organization and behaviour of the cell membrane's phospholipids and proteins would be destroyed by the coevaporation process.

(4) Using the coevaporation procedure, each sample preparation would provide only one inhibition period for kinetic study.

In a preliminary study, we found that the use of intermembrane transfer to deliver antioxidants insoluble or sparingly soluble in water avoided some of these limitations [12]. Although little has been reported on the application of this method to deliver

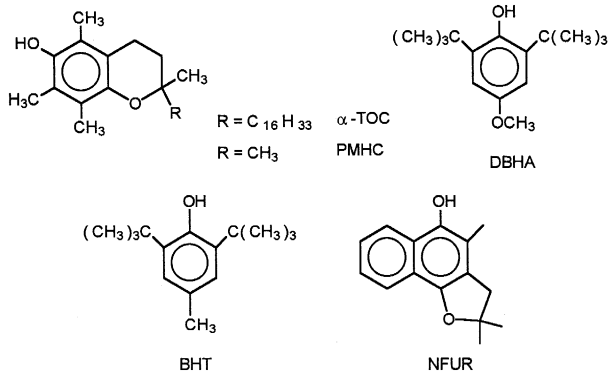

Fig. 1. Antioxidants used to study transfer of lipid-soluble antioxidants from DMPC to DLPC liposomes.

antioxidants [13–16], many report using liposomes as carriers for therapeutic drugs in animals and humans [17–25], a process which probably involves similar transfer phenomena between hydrophobic phases.

We now present a detailed quantitative study on the efficiency of intermembrane transfer of antioxidants. In order to study intermembrane transfer, known concentrations of the lipid soluble antioxidants α-tocopherol (α-TOC), 2,2,5,6,7-pentamethyl-6-hydroxychroman (PMHC), 2,6-di-*tert*-butyl-4-methoxyphenol (DBHA), 2,6-di-*tert*-butyl-4-methyl phenol (BHT) or the naphthofuran derivative, 2,3-dihydro-5-hydroxy-2,2,4-trimethylnaphtho[1,2-*b*]furan (NFUR) (Fig. 1) were prepared in saturated liposomes of dimyristoylphosphatidylcholine (DMPC, DONOR-liposomes). These DONOR-liposomes were used to inhibit peroxidation reactions in unsaturated liposomes of dilinoleoyl-phosphatidylcholine (DLPC, ACCEPTOR-liposomes), initiated by either the water-soluble azo-initiator azo-*bis*-amidinopropane hydrochloride (ABAP), the lipid-soluble ones azo-*bis*-2,4-dimethylvaleronitrile (ADVN) and di-*tert*-butyl-hyponitrite (DBHN), or the photoinitiator benzophenone.

The effect on the peroxidation of unsaturated bilayers of these two methods to deliver the antioxidants, liposomal transfer and coevaporation, are compared by: (1) the profiles of suppressed oxygen uptake; (2) the resulting antioxidant activities, k_{inh}; and (3) the effect on the linoleate hydroperoxides formed during peroxidation inhibited by PMHC and NFUR, compared to these products formed in the presence of α-TOC. In addition, a study is made of the time-profile to transfer α-TOC between DONOR and ACCEPTOR liposomes.

The antioxidant activities, k^{s}_{inh}, were determined by measuring the oxygen uptake during the course of inhibition periods. The results are applied to the integrated form of the inhibition equation, Eq. (11), as used before [10]:

$$\Delta[O_2]_t = -k_p/k_{inh}[RH]\ln(1 - t/\tau) \quad (11)$$

The k_{inh} is obtained from a plot of the linear equation, $\Delta[O_2]_t$ vs. $-\ln(1 - t/\tau)$, where the slope is equal to $k_p[RH]/k_{inh}$. The k_p value used for DLPC in bilayers is 36.1 mol^{-1} s^{-1} [10].

2. Materials and methods

2.1. Materials

The phospholipids DLPC, DMPC and 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC) were purchased from Avanti Polar Lipids and stored at −30°C in sealed vials.

Phosphate buffer was prepared using 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, and 0.1 mM Na_2EDTA in water obtained from a Millipore Milli-Q Plus Ultrapure Water System. The pH was adjusted to 7.0 using HCl. Traces of heavy metal were removed by passing the prepared buffer through a column of Chelex 100, 50–100 mesh (Bio Rad). PMHC was prepared using a known procedure [26]. α-TOC, DBHA, and BHT were purchased from Aldrich. Trolox C was a gift from Hoffman-Laroche (Nutley, NJ, USA). The NFUR was a gift from Dr. Kazuo Mukai (Ehime University, Matsuyama, Japan). ABAP was purchased from Polysciences Inc. ADVN was also purchased from Polysciences Inc. and was recrystallized from methanol before use, m.p. 70–72°C (lit. value 75–76°C [27]). DBHN was synthesized using a known procedure [28], and the purity of the product was checked using thin layer chromatography. Benzophenone was purchased from Anachemia.

2.2. Autoxidation procedure

A sensitive pressure transducer system, described previously [29], was used to monitor autoxidations at 37°C, under oxygen at 760 Torr. Antioxidants were introduced to the liposomes either using the coevapo-

ration method during liposome preparation or else via direct transfer from a DONOR liposome of DMPC. In the coevaporation method, antioxidant and initiator were added in solution to an oxidizable substrate (DLPC) in methanol. The solvent was then removed under reduced pressure, and the liposomes were prepared in buffer using freeze–thaw cycles in liquid nitrogen. For direct transfer, the antioxidant was coevaporated into an unoxidizable DONOR lipid (DMPC) and prepared as a separate liposome from the initiator/DLPC liposome. When oxidation of the DLPC was underway, a known volume of the DONOR antioxidant/DMPC was added to the DLPC liposome. Both multilamellar (MLV) and unilamellar (ULV) vesicles were used in these studies. In most experiments, unilamellar liposomes were prepared from multilamellar liposomes using an extruder [30]. In one series of experiments, unilamellar liposomes were also prepared by passing multilamellar liposomes through a French pressure cell [31]. Corrections for 'lag time' in the start of autoxidation experiments were made using a previously published procedure [10]. For one series of experiments, a film of dry PMHC was shaken with buffer at 37°C for 7 h to partially dissolve the PMHC. Concentration of the PMHC in the buffer was determined using ultraviolet (UV) analysis at 292 nm ($\epsilon = 3071\ \mathrm{mol}^{-1}\ \mathrm{cm}^{-1}$) to be 1.93×10^{-4} M. The solubility of DBHN in buffer was studied by adding buffer to a film of dry DBHN in a reaction cell and shaking it at 37°C for 30 min. The concentration of DBHN in buffer was determined using UV analysis at 227 nm ($\epsilon = 5954\ \mathrm{mol}^{-1}\ \mathrm{cm}^{-1}$) to be 2.13×10^{-4} M. For some experiments, DBHN was prepared in solutions of known concentration in *tert*-butyl alcohol and added in a small volume to prepared DLPC or antioxidant/DLPC liposomes in the autoxidation apparatus. No delay in initiation of the DLPC oxidation was seen when the DBHN was added in solution. Trolox was prepared in solutions of known concentration in buffer and stored at 5°C for up to 2 weeks. Solutions of other inhibitors, initiators, and substrates were kept in the dark at −10°C for up to 1 week.

2.3. Hydroperoxide analyses

To obtain a profile of hydroperoxide analysis during the course of inhibited peroxidation, two experiments were performed on the same liposome preparation. In the first experiment, the profile and length of the inhibition period (τ) were determined. In the parallel experiment, samples were withdrawn from the mixture at known intervals along the inhibition period using a precision evacuated syringe. Samples were immediately reduced in 1.0 ml of 10 mM triphenylphosphine in methanol and vortex mixed 20 seconds. A 1.5-ml volume of 0.45 M KOH in methanol was added and the sample was vortexed again for 20 s. The sample was left at room temperature for 15 min, then 1.0 ml 1.0 M NH_4Cl in water was added and the sample was vortexed for a final 20 s. Two 1.0-ml hexane extractions were carried out, and the hexane layers were combined and condensed under argon to approximately 200 μl.

The samples were analysed using an HP1050 HPLC with a Hibar LiChrosorb Si60, 5-μm, 4 mm × 25 cm column. The elution solvent contained 4:4:992 acetone/2-propanol/hexane. Peaks were detected using the HP1050 internal variable wavelength detector at 234 nm interfaced with a computer for calculations. Using a flow rate of 1.6 ml/min the product isomers eluted as follows: 13 c,t — 16.9 min; 13 t,t — 19.5 min; 9 c,t — 23.9 min; 9 t,t — 26.6 min. The peak areas were corrected for the known molar absorptivities of each isomer and the ratios calculated as before [31].

2.4. α-tocopherol analyses

Transfer of α-TOC from DONOR DMPC to ACCEPTOR DLPC liposomes required that the liposomes be shaken together at 37°C for prolonged periods under argon. Samples were removed from the mixture prior to initiation of DLPC oxidation to analyse the amount of α-TOC remaining in the total sample (DMPC + DLPC). To extract the α-TOC, 500 μl of sample were vortex mixed with 1.0 ml 0.5 M SDS for 30 s and then 1.0 ml methanol for 30 s to break up the liposomes. Two 2.0-ml hexane extractions were carried out to remove the α-TOC from the mixture, the extracts were separated by centrifugation in a benchtop centrifuge, and the hexane layers were combined and condensed to a known volume, to which was added an internal standard, benzophenone. The samples were analysed using an HP1050 HPLC with a Hibar LiChrosorb Si60, 5-μm, 4 mm × 25 cm

column. The elution solvent contained 1:99 2-propanol/hexane. Peaks were detected using the HP1050 internal variable wavelength detector at 290 nm. Using a flow rate of 0.5 ml/min, the benzophenone eluted at 7.7 min and the α-TOC at 9.9 min.

2.5. NMR *spectra of liposomes*

The nature of the liposome preparations was confirmed by ^{31}P-NMR spectra on samples prepared in THAM buffer, pH 7.0. Spectra were measured on a JEOL 270 MHZ Spectrometer using pulse sequences

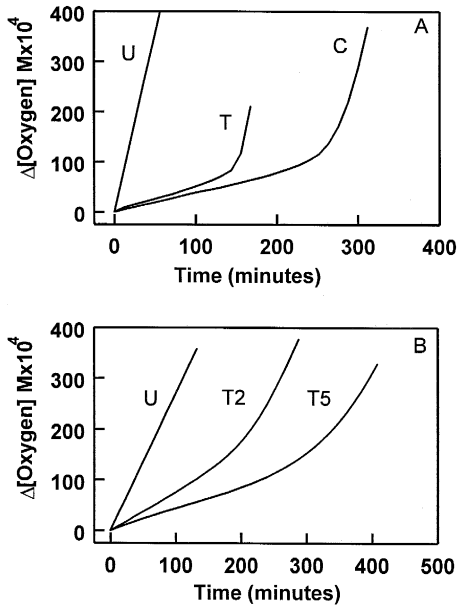

Fig. 2. A. Profiles of PMHC inhibited oxidation of DLPC liposomes comparing the liposomal transfer method (T) with the coevaporation method (C); initiation by ABAP added in buffer. U — uninhibited oxidation of DLPC; C — 17.05 nmol PMHC(c), 6.50×10^{-6} mol ABAP, 5.66×10^{-5} mol DLPC, the extended induction period is attributed to a lag time (see text); T — 21.11 nmol PMHC(t), 6.22×10^{-6} mol ABAP, 8.90×10^{-5} mol DLPC, $R_i = 4.56\times10^{-12}$ mol s^{-1}.
B. Profiles of α-TOC inhibited oxidation of DLPC liposomes to present qualitative differences when the antioxidant is incubated for 2 h (T2) or 5 h (T5) to allow transfer of the antioxidant from DMPC to DLPC liposomes before the initiation. Initial amount of α-TOC added in DMPC was 53.36 nmol. DLPC oxidation was initiated with ABAP added in buffer. U — uninhibited oxidation of DLPC; T2 — 28.70 nmol α-TOC (53.8% transferred after 2-h incubation), 3.70×10^{-6} mol ABAP, 4.29×10^{-5} mol DLPC, $R_i = 4.20\times10^{-12}$ mol s^{-1}; T5 — 52.76 nmol α − TOC (98.9% transferred after 5-h incubation), 3.71×10^{-6} mol ABAP, 4.29×10^{-5} mol DLPC, $R_i = 4.21\times10^{-12}$ mol s^{-1}.

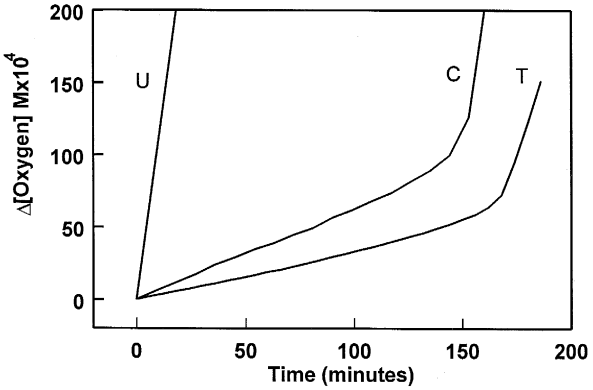

Fig. 3. Profiles of PMHC inhibited oxidation of DLPC liposomes comparing the liposomal transfer method (T) with the coevaporation method (C); initiation by coevaporated ADVN. U — uninhibited oxidation of DLPC; C — 15.00 nmol PMHC(c), 3.17 μmol ADVN, 5.76×10^{-5} mol DLPC, $R_i = 2.98\times10^{-12}$ mol s^{-1}; T — 9.44 nmol PMHC(t), 2.83 μmol ADVN, 7.43×10^{-5} mol DLPC, $R_i = 1.90\times10^{-12}$ mol s^{-1}.

reported before [31]. The MLV particles exhibited typical broad chemical shift anisotropies of 45–50 ppm, whereas the liposomes prepared using the extruder or French press exhibited single narrow isotropic lines characteristic of ULV liposomes [31].

3. Results

3.1. *Peroxidation initiated by the water-soluble initiator,* ABAP

An advantage of using a water-soluble initiator is that it can be introduced after liposome preparation. We observed, however, a delay in obtaining a constant rate of oxygen uptake after ABAP was added to the liposomes. When an antioxidant is already present (coevaporated), this delay becomes part of the inhibition period, resulting in large errors in determining the R_i (using Eq. (9)) and the k_{inh}. This effect is attributed to the inhomogeneity of the MLV system [12,32], whereby ABAP does not diffuse immediately through all layers of the membrane to initiate new peroxyl chain reactions in a uniform manner. Consequently, whenever feasible, the oxygen uptake was allowed to reach a constant rate, signifying a constant R_i, before an inhibitor was added. Under this condition, the R_i, calculated from the known e and k_i for

Table 1
Specific and average antioxidant activities of phenolic antioxidants in DLPC liposomes undergoing ABAP-initiated oxidation at 37°C — ABAP was added to the DLPC liposomes in buffer [a]

	ArOH (nmol)	C/T [b]	DLPC (10^5 mol)	ABAP (μmol)	R_i [c] (10^{12} mol s^{-1})	ν [d]	k_{inh} (10^{-4} M^{-1} s^{-1})	Average [e] k_{inh} (10^{-4} M^{-1} s^{-1})
1	PMHC (16.89)	T [f]	5.55	6.45	4.46	18–36	0.98	1.14 ± 0.30
2	PMHC (15.86)	T	5.66	6.26	4.80	19–39	0.98	1.04 ± 0.21
3	DBHA (11.45)	T	4.29	3.55	3.82	10–27	1.43	1.50 ± 0.18
4	α-TOC (26.60)	C	4.29	3.71	2.59 [g]	35–62	0.28	0.28–0.29
5	α-TOC (53.36) [h]	T	4.28	3.71	2.59 [g]	22–45	0.40	
6	α-TOC (53.36) [h]	T	4.29	3.71	2.59 [g]	13–26	0.34	

[a] Amounts are expressed as moles for comparison purposes between tables since the initiators ABAP and DBHN (Table 3) are introduced in the aqueous phase. The k_{inh} are reported as M^{-1} s^{-1} assuming that inhibition is entirely in the lipid phase of the bilayers.
[b] C = coevaporated in DLPC; T = transferred from DMPC liposomes.
[c] R_i was calculated based upon the induction period using $R_i = n$(moles inhibitor)$/\tau$, where $n = 2$ for DBHA and PMHC [10].
[d] The kinetic chain length during the inhibition period, calculated from the equation $\nu = ([O_2]_t - [O_2]_{t=0})/n[AH]_0(1 - t/\tau)\ln(1 - t/\tau)$ [10].
[e] Number of experiments averaged: 1 (3), 2 (10), 3 (10). Line 4 is from 2 experiments.
[f] PMHC was transferred from buffer.
[g] The R_i was calculated using the expression $R_i = 2ek_i[ABAP]$.
[h] Using the τ of the transfer runs, the R_i value (footnote [g]), and the expression $R_i = n$(moles inhibitor)$/\tau$, the moles of α-TOC transferred from DMPC to DLPC liposomes were calculated. In line 5, 28.7 nmol α-TOC (54%) transferred after 2-h incubation. In line 6, 52.8 nmol α-TOC (99%) transferred after 5-h incubation.

ABAP (Eq. (6), $e = 0.43$ and $k_i = 1.32 \times 10^{-6}$ mol s^{-1} [33]), agrees with the R_i calculated from the induction period (Eq. (9)).

Typical profiles of oxygen uptake for ABAP-initiated peroxidation of DLPC liposomes, inhibited by PMHC (coevaporated and transferred) and by α-TOC (transferred after incubation), are illustrated in Fig. 2. These profiles show that PMHC provides effective inhibition immediately upon injection of the DONOR DMPC liposomes. Similar results were observed for transfer of DBHA and NFUR from DONOR DMPC liposomes (not shown). Transfer of α-TOC, however, required rather long incubation times (2–5 h) before effective inhibition occurred (vide infra). Detailed data for reactions initiated by ABAP and inhibited by these antioxidants are given in Table 1. Satisfactory

Table 2
Specific and average antioxidant activities of phenolic antioxidants in DLPC liposomes undergoing ADVN-initiated oxidation at 37°C — ADVN was coevaporated into the DLPC liposomes [a]

	ArOH (nmol)	C/T [b]	DLPC (10^5 mol)	ADVN (μmol)	R_i [c] (10^{12} mol s^{-1})	ν [d]	k_{inh} (10^{-4} M^{-1} s^{-1})	Average [e] k_{inh} (10^{-4} M^{-1} s^{-1})
1	PMHC (15.00)	C	5.76	3.17	2.98	15–38	0.90	1.16 ± 0.28
2	PMHC (24.10)	T	14.90	2.88	2.97	17–35	1.22	1.49 ± 0.32
3	DBHA (16.6)	C	11.25	3.03	2.51	10–47	2.16	1.95 ± 0.46
4	DBHA (5.73)	T	15.00	3.21	2.51	35–94	1.92 [f]	1.87 ± 0.63
5	NFUR (16.83)	C	15.00	3.32	2.64 [g]	20–56	1.73	1.42 ± 0.24
6	NFUR (23.80)	T	14.88	2.61	2.48 [g]	12–42	2.24	2.04 ± 0.83

[a–d] See Table 1.
[e] Number if experiments averaged: 1 (5), 2 (13), 3 (8), 4 (3), 5 (3), 6 (8).
[f] In comparison, the k_{inh} value for transferred DBHA obtained using the lipid-soluble photo-initiator benzophenone was $(2.39 \pm 0.28) \times 10^4$ M^{-1} s^{-1} (9 experiments).
[g] R_i was measured using PMHC inhibition in the same experiment.

linear plots of $\Delta[O_2]_t$ vs. $-\ln(1-t/\tau)$ were obtained for these antioxidants and the k_{inh} values obtained are included in Table 1.

3.2. Peroxidation initiated by the lipid-soluble initiator, ADVN

The lipid-soluble azo-initiator ADVN has been frequently used to initiate peroxidation inside the lipid phase of bilayers [2,10,31,32]. In these experiments the initiator must be introduced by the usual coevaporation method along with the lipid (vide infra). The antioxidant activities of PMHC, DBHA, and NFUR, introduced by liposomal transfer, were compared to the values obtained when they were coevaporated along with the ADVN and DLPC. Fig. 3 shows typical profiles of suppressed oxygen uptake when introducing PMHC by the two methods. The PMHC appears as effective when introduced by liposomal transfer as by coevaporation. Similar results were obtained using DBHA and NFUR (not shown).

Summaries of the antioxidant activities, k_{inh}, determined for PMHC, DBHA, and NFUR introduced by the two methods are given in Table 2. The results show that the two delivery methods give similar values for the k^s_{inh} within experimental error, although the values using the liposomal transfer method appear marginally higher. Results when peroxidation was photochemically initiated with benzophenone and inhibited with transferred DBHA are included for comparison purposes (Table 2, footnote [e]).

Attempts were made to initiate peroxidation of DLPC by transferring ADVN from DONOR DLPC liposomes. Very long 'lag times' were observed before oxygen uptake was obtained for both MLV and ULV DLPC, thus this method seems unsatisfactory for quantitative studies.

3.3. Peroxidations initiated by DBHN — Initiator efficiency

Earlier studies reported advantages in the use of di-*tert*-butylhyponitrite (DBHN) for initiation of peroxidation in liposomes [34]. To avoid loss of DBHN, which occurs when it is introduced by coevaporation, it was added to prepared liposomes in buffer (where it was found to be soluble to 2.13×10^{-4} M concentrations) or in small volumes of *tert*-butyl alcohol. Both methods of introducing DBHN gave immediate and controlled uptake of oxygen. The experimental traces, including inhibition by BHT, shown in Fig. 4, are characteristic of these results. Summaries from inhibition studies employing PMHC, NFUR, and BHT under these conditions are given in Table 3.

The efficiency of initiation by azo-initiators is known to be diminished in solvents of high viscosity due to their effects on cage recombination. This is especially true for initiation in the hydrophobic phase of PC bilayers due to their high microviscosity. Thus the efficiency of DBHN when coevaporated into egg lecithin bilayers decreased to only 9.1% from 66% in chlorobenzene [34]. We found that the efficiency of DBHN when introduced to DLPC from *tert*-butyl alcohol was substantially greater than when it was coevaporated. The average value of e, determined using the induction period method with PMHC, DBHA, and BHT over the concentration range of DBHN used (Table 3), is $30 \pm 3\%$; more than three

Table 3
Specific and average antioxidant activities of phenolic antioxidants in DLPC liposomes undergoing DBHN-initiated oxidation at 37°C — DBHN was added in buffer or *tert*-butyl alcohol to the DLPC liposomes [a]

	ArOH (nmol)	C/T [b]	DLPC (10^5 mol)	DBHN (μmol)	R_i [c] (10^{12} mol s^{-1})	ν [d]	k_{inh} (10^{-4} M^{-1} s^{-1})	Average [e] k_{inh} (10^{-4} M^{-1} s^{-1})
1	PMHC (3.54)	T [f]	5.12	0.25	0.72	29–79	1.18	0.96 ± 0.30
2	NFUR (3.54)	T [f]	5.12	0.22	0.73 [g]	43–75	2.60	1.94 ± 0.64
3	BHT (23.39)	C	5.18	1.01	4.33	23–42	0.33	0.46 ± 0.16
4	BHT (23.38)	T	5.18	0.89	5.56	16–38	0.45	0.48 ± 0.04

[a–d] See Table 1.
[e] Number of experiments averaged: 1 (5), 2 (6), 3 (3), 4 (5).
[f] DBHN was added in buffer, otherwise the DBHN was added in *tert*-butyl alcohol.
[g] R_i was measured using DBHA inhibition in the same experiment.

times that found when DBHN is coevaporated into bilayers [34]. This value is similar to that found in 0.50 M SDS, which is 29% [35].

3.4. Transfer of α-TOC between DONOR (DMPC) and ACCEPTOR (DLPC) liposomes

To determine the antioxidant activity of α-TOC (Section 3.1), it was necessary to estimate the amount transferred between DMPC and DLPC liposomes. Previous studies have shown that the phytyl side chain of α-TOC affects its antioxidant activity in model systems [36], which could account for its slow transport from a protein to a lipid membrane [37] or between liposomes [12]. An estimate of the amount of α-TOC transferred to oxidizable DLPC liposomes was made by incubating samples under nitrogen of α-TOC/DMPC (DONOR) with DLPC (ACCEPTOR) liposomes for various periods. Samples (2.0 ml) were removed and oxidized by initiation with DBHN, using the procedure outlined in Section 3.3. The amount of α-TOC transferred to the DLPC liposomes was calculated using Eq. (9), $[\mathrm{ArOH}] = R_i/n\tau$, by measuring the inhibition period and calculating the R_i using Eq. (6) (using the known e and k_i for DBHN under these conditions). Results of this latter study are outlined in Fig. 5. This procedure was applied successfully to study transfer of α-TOC to ULV

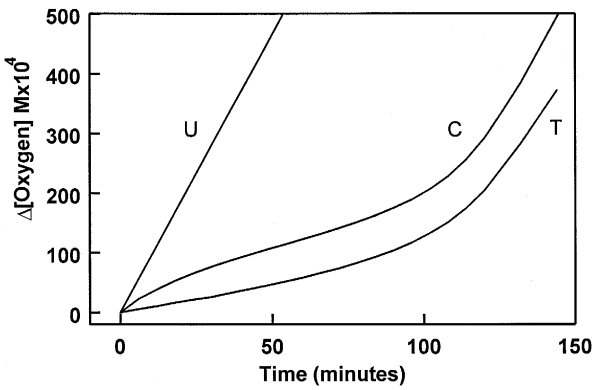

Fig. 4. Profiles of BHT inhibited oxidation of DLPC liposomes comparing the liposomal transfer method (T) with the coevaporation method (C); initiation by DBHN in *tert*-butyl alcohol. U — uninhibited oxidation of DLPC; C — 23.39 nmol BHT(c), 1.02 μmol DBHN, 5.18×10^{-5} mol DLPC, $R_i = 6.43\times10^{-12}$ mol s^{-1}; T — 23.38 nmol BHT(t), 0.93 μmol DBHN, 5.18×10^{-5} mol DLPC, $R_i = 6.48\times10^{-12}$ mol s^{-1}.

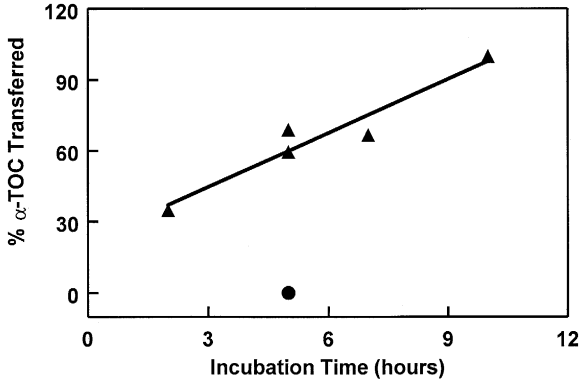

Fig. 5. Percent of total moles of α-TOC transferred from DMPC liposomes to unilamellar and multilamellar DLPC liposomes with shaking at 37°C under argon for various incubation periods. The percent transfer was calculated using the relationship $[\mathrm{ArOH}] = R_i/n\tau$, where the R_i was determined using the initiator DBHN delivered in *tert*-butyl alcohol. ▲ — DLPC liposomes were prepared with 0.05 M THAM buffer, pH 7.0, and made unilamellar by the extruder or French press method. The two measurements at 5 h represent typical error limits. ● — multilamellar DLPC liposomes were prepared with 0.1 M phosphate buffer, pH 7.0.

DLPC but satisfactory inhibition periods were not observed using MLV DLPC.

3.5. Profiles of product analyses of linoleate hydroperoxides during uninhibited and inhibited peroxidation

The hydroperoxides formed during controlled peroxidation of linoleate chains provide very important information on the mechanism of lipid peroxidation and the action of inhibitors. In particular, pioneering research by Porter et al. showed that the cis,trans to trans,trans (c,t/t,t) ratio of the geometrical isomers of the hydroperoxides formed at the 9- and 13- positions of the linoleate chain bear a simple linear relationship to the hydrogen atom donating ability of the medium [38,39]. The c,t/t,t ratio during inhibited oxidation of model membranes was earlier found to be affected (e.g., raised) by an antioxidant such as α-TOC [40]. We have found it useful to use this ratio to monitor the effect of antioxidants such as α-TOC and Trolox on DLPC liposomes under different methods of initiation [41].

The present work starts with examining peroxidation of bilayers of 1-palmitoyl-2-linoleoyl-

phosphatidylcholine (PLPC), which has only one unsaturated acyl chain. Hydroperoxide product results from PLPC are not expected to be complicated by a mixture of intramolecular or 'arm-to-arm' propagation [42] along with intermolecular propagation, which can affect the c,t/t,t ratios [43].

The hydroperoxides formed were reduced with triphenylphosphine to alcohols which were analysed as their corresponding methyl esters by HPLC. The c,t/t,t ratios formed during uninhibited, DBHN-initiated peroxidation of PLPC bilayers remained constant at 0.59 ± 0.01 from 1% to 5% oxidation, the maximum percent oxidation in our kinetic and product studies. On the other hand, the c,t/t,t ratio increases linearly as the concentration of α-TOC in the bilayer increases, as shown in Fig. 6. This serves as a useful 'standard plot' for comparison with the hydroperoxide ratios formed when peroxidation is inhibited by other antioxidants.

The variations in c,t/t,t ratios of the linoleate hydroperoxides formed during inhibition by PMHC, for the two methods of delivery, are illustrated in Fig. 7A (coevaporated) and 7B (liposomal transfer) for the DBHN-initiated peroxidation of PLPC.

Similar experiments were carried out to measure the profiles of c,t/t,t ratios for DLPC liposomes when liposomal transfer of an antioxidant was used to inhibit oxidation (again DBHN-initiated). For example, transfer into DLPC of 0.523 mM NFUR showed c,t/t,t ratios which varied from 7.26 at the start of the inhibition to 1.81 after the break in the induction period. At the same concentration of α-TOC in PLPC, the c,t/t,t ratio was 1.25 at the start of the inhibition (Fig. 6).

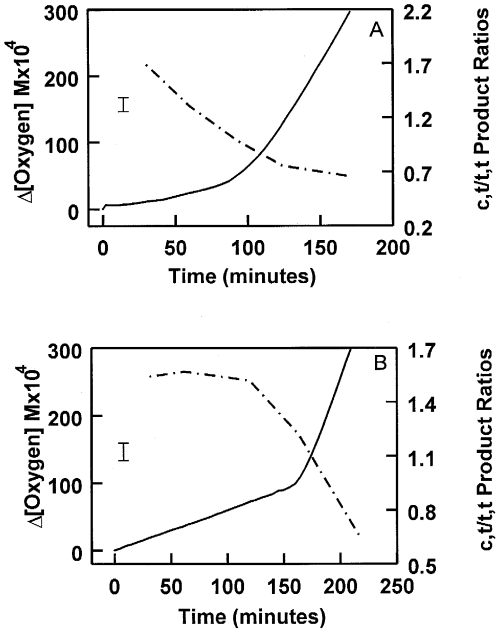

Fig. 7. A. Profile of inhibited oxygen uptake during PMHC inhibited oxidation of PLPC, showing changing c,t/t,t product ratios during the inhibition. PMHC (3.756×10^{-8} mol) was coevaporated into the PLPC liposomes (5.25×10^{-5} mol PLPC). Oxidation of PLPC was initiated by DBHN in *tert*-butyl alcohol (3.17×10^{-6} mol). The error bar represents average error.
B. Profile of inhibited oxygen uptake of PMHC inhibited oxidation of PLPC, showing changing c,t/t,t product ratios during the inhibition. PMHC (8.729×10^{-8} mol) was transferred from DMPC into the PLPC liposomes (5.25×10^{-5} mol PLPC). Oxidation of PLPC was initiated by DBHN in *tert*-butyl alcohol (2.99×10^{-6} mol). The error bar represents average error.

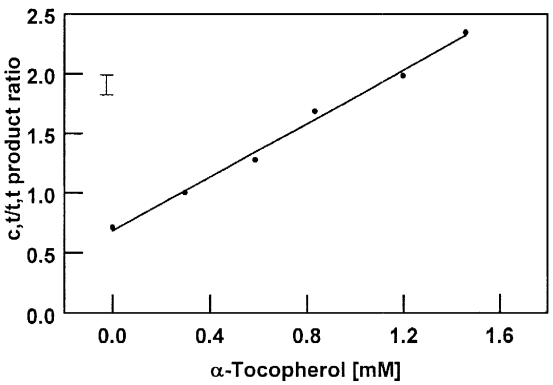

Fig. 6. Plot of changing c,t/t,t product ratios with increasing concentration of α-tocopherol in PLPC. PLPC amounts ranged from 2.98×10^{-5} to 3.40×10^{-5} mol. Oxidation was initiated with 1.14×10^{-6} mol DBHN in *tert*-butyl alcohol. The error bar represents average error.

4. Discussion

The profiles of inhibited oxygen uptake obtained by liposomal transfer demonstrate the remarkable usefulness of this method to deliver antioxidants to bilayers. The results show (Figs. 2–4) that liposomal transfer gives immediate inhibition of peroxidation without the disadvantages shown by the more time-

consuming coevaporation methods commonly used to deliver lipid-soluble antioxidants. This means that compounds like PMHC, DBHA, BHT and NFUR must pass the water–lipid barrier between liposomes and diffuse readily through each layer of the MLV system to provide efficient inhibition throughout the bilayer. This is not surprising since PMHC was found earlier to pass through a dialysis membrane and distribute between liposomes on both sides of this barrier [12]. It is anticipated that this method of delivery of antioxidants will prove very useful to explore antioxidant action in natural lipid systems of biological membranes.

The introduction of DBHN in small amounts of *tert*-butyl alcohol (10–15 μl in a 2.00-ml liposome volume) has been found to have some advantages over other methods of initiation, the main one being the absence of the delays or 'lag times' in reaching constant oxygen uptake found with water-soluble ABAP (added after liposome preparation) and with lipid-soluble ADVN (added to liposomes by coevaporation). The increased efficiency observed for DBHN (30% when introduced in *tert*-butyl alcohol vs. 9.1% when coevaporated into the bilayer phase) was unexpected. It implies that DBHN undergoes appreciable decomposition in the aqueous phase rather than in the bilayer. The resulting *tert*-butoxyl radicals, $(CH_3)_3C–O\cdot$, are expected to readily cross the water–lipid barrier and initiate reaction by H-atom abstraction. Methylperoxyl radicals could also form due to expected β-cleavage of *tert*-butoxyl radicals formed in the polar aqueous phase [44] and rapid oxidation of the derived methyl radicals. Such derived methylperoxyl radicals might also contribute to the initiation process, although this is less likely when DBHN is thermolyzed inside the bilayer [34].

The quantitative kinetic method, using a known and controlled R_i, is a very reliable one to determine antioxidant activities. The absolute inhibition rate constants, k^s_{inh}, based on measurements of suppressed oxygen uptake by phosphatidylcholine membranes are determined to within 30% error. All known methods to measure k^s_{inh} have uncertainties unless it is known that the radical derived from the antioxidant does not continue the oxidation chain either directly by H-atom abstraction [45] or addition to a multiple bond, or indirectly after addition to O_2 to form a new peroxyl radical. This 'prooxidant effect' may become significant in heterogeneous systems of isolated lipid particles compared to solution [46–55]. In addition, lipid particle size is recognized as important in the balance between pro- and antioxidant effects of α-TOC [47–49]. Our system of bilayers may be susceptible to such prooxidant effects so that comparisons with data in homogeneous solution are made with some reservation. Nevertheless it is significant to find remarkable differences in k^s_{inh} determined in liposomes compared to known, reliable values in homogeneous solution also determined using the sensitive inhibited oxygen uptake method [56]. In homogeneous solution the k_{inh} values obtained for α-TOC, PMHC and BHT were 320×10^4, 380×10^4 and 1.4×10^4 M^{-1} s^{-1}, respectively [56], and for NFUR 2870×10^4 M^{-1} s^{-1} [57]. Our current study in liposomes shows that the order of antioxidant activities of the hydroxychromans is NFUR > PMHC > α-TOC. Thus the superior activity of NFUR found in solution [57] and micelles [58] is carried over to lipid membranes. However, there are marked effects of these aqueous systems on the activities. The most pronounced effect is observed with α-TOC which gives an antioxidant activity about three orders of magnitude less in DLPC liposomes than in solution. The k_{inh} of PMHC also drops by a factor of about 300. On the other hand the 'effect' on BHT results in a decrease by a factor of only 3. In other words, we find the k_{inh} of BHT to be similar to α-TOC in bilayers, whereas α-TOC is at least 200 times more active in solution [56].

Quantitative explanations for the diminution of antioxidant k_{inh} in aqueous dispersions were given earlier in terms of hydrogen bonding at the phenolic hydroxyl and *para* ether oxygen by water [10,59,60]. Later it was shown that the latter effect is not significant in reducing the k_{inh} of α-TOC [61]. Recent important quantitative studies have been carried out on solvent effects for abstraction of the phenolic hydrogen from α-TOC [62] and analogues [63] which concluded that most of the diminution in antioxidant activity of α-TOC in bilayers is due to it being 'physically inaccessible' to the (lipid) peroxyl radicals, and only a small fraction of the k_{inh} was affected by hydrogen bonding by water [63]. The activities of antioxidants of the vitamin E class in aqueous dispersions of bilayers are thus controlled by a combination of hydrogen bonding and restricted

diffusion of lipid peroxyls and antioxidants in the bilayer region. We found the latter effect to be most pronounced for α-TOC as an extended mixing time, perhaps as long as 9 to 10 h, was required to transfer most of the α-TOC between liposomes (Fig. 5), whereas the smaller more mobile compounds like PMHC appear to transfer rapidly between bilayers under the same conditions. These differences must be kept in mind when designing experiments for testing the behaviour of different antioxidants which include α-TOC on natural systems.

Studies of the 9- and 13-hydroperoxides formed from linoleate chains during inhibited oxygen consumption provide further information useful for comparing the effectiveness of different antioxidants. For example, PLPC oxidation, when inhibited by coevaporated PMHC, shows a c,t/t,t ratio of 1.69 at the start of the inhibition period, compared to a ratio of 1.52 for the same concentration of α-TOC (Fig. 6). This is another way of showing the more efficient action of PMHC than α-TOC in this model system. The profile of the c,t/t,t ratio for transferred PMHC during the corresponding inhibition period (Fig. 7B) is much 'flatter' than that for the coevaporated case (Fig. 7A). This may simply reflect the higher initial concentration of PMHC, 1.62 M, in the transfer study compared to 0.75 M in the coevaporation study. The higher c,t/t,t ratios observed for NFUR in DLPC is a reflection of two factors contributing to increased hydrogen atom donating ability, the increased substrate concentration provided by the dual chain system and the higher antioxidant activity provided by NFUR. We plan to estimate antioxidant activities by employing quantitative studies of c,t/t,t ratios and will report on this separately.

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