Oxidation of Lipids Induced by Dioctadecyl Hyponitrite and Di-*t*-butyl Hyponitrite in Organic Solution and in Aqueous Dispersions. Effects of Reaction Medium and Size of Radicals on Efficiency of Chain Initiation

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The dynamics of the chain initiation of the oxidations of lipids by hyponitrites were studied in organic homogeneous solution and in aqueous dispersions of micelles and liposomal membranes. Di-*t*-butyl and distearyl hyponitrites were used in order to examine the effect of bulkiness of the alkoxyl radicals in different reaction media. Both hyponitrites induced the free radical-mediated chain oxidations of methyl linoleate in homogeneous solution and in aqueous emulsions and also of soybean phosphatidylcholine liposomal membranes. In aqueous dispersions, these lipophilic hyponitrites generated free radicals in the lipid compartment and aqueous radical-scavenging antioxidants could not suppress the oxidations induced by these hyponitrites. The efficiency of chain initiation decreased in the order of homogeneous solution > micelles > liposomal membranes in accordance with increasing viscosity of the medium. It was also dependent on the size of alkoxyl radical and distearyl hyponitrites was less efficient than di-*t*-butyl hyponitrite in initiating the chain oxidation in all of the above reaction media.

The free radical-mediated chain oxidation of lipids has received renewed attention recently in connection with its involvement as one of the important primary events in a variety of pathological, toxicological, and nutritional effects; that is, it is now generally accepted that the oxidation of lipids induces damage in biological molecules, membranes, and tissues, which eventually causes diseases, cancer, and even aging processes (for recent review see Ref. 1). It is also important in the oxidative deterioration of foods and oils.[2] The function and action of antioxidants against these oxidative damage have been also the subjects of numerous studies.[3] In order to study the mechanism and dynamics of the oxidation of lipids and its inhibition, it is essential to generate free radicals at a known and controlled rate and site. The azo compounds, especially 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), have been often used as a radical source for this purpose.[4] These azo compounds generate carbon centered radicals, which react with an oxygen molecule to give peroxyl radicals.

In the present study, alkyl hyponitrites have been used as a source of alkoxyl radical and the oxidations of methyl linoleate in homogeneous solution and in aqueous dispersions and of soybean phosphatidylcholine (PC) liposomal membranes have been studied aiming specifically at elucidating the action of small and bulky alkoxyl radicals in different reaction media. For this purpose, distearyl hyponitrite (dioctadecyl hyponitrite, abbrev. to SHN) has been synthesized and its action has been compared with that of di-*t*-butyl hyponitrite (BHN).

Experimental

The hyponitrites, BHN and SHN, were synthesized by a method described in the literatures.[5—8] The rates of decomposition of SHN and BHN in homogeneous solution and liposomal membranes were measured by following their concentrations either by ultraviolet absorption spectroscopy or with a high-performance liquid chromatography (HPLC). The appropriate amount of SHN or BHN was incubated at desired temperature in specific medium in air and an aliquot was taken out periodically to measure its absorption at 225 nm. The HPLC analyses were carried out using a silica-gel column with an eluent hexane/2-propanol (100/1 by v/v) at a flow rate of 1 ml min^{-1} for experiments in homogeneous solution and, for liposomal membrane system, with an LC-8 column and methanol/*t*-butyl alcohol (75/25 by vol) and methanol/water (70/30 by vol) as an eluent for SHN and BHN, respectively.

The oxidations of methyl linoleate were performed both in organic homogeneous solution and in aqueous dispersions. The micelles were prepared by adding an appropriate amount of methyl linoleate into 50 mM SDS aqueous solution (1 M=1 mol dm^{-3}) followed by vortex-mixing for 2 min. The multilamellar liposomal membranes of soybean PC were prepared as reported previously.[9] In short, soybean PC and, when required, lipid-soluble additives such as initiator and antioxidant were dissolved into chloroform and the solvent was removed by evacuation to obtain a thin film on a glass wall. The aqueous solution of 0.1 M NaCl was added and the mixture was shaken to peel off the film and finally subjected to vortex-mixing to obtain multilamellar liposomes.

The rate of oxidation was measured by several methods. The rate of oxygen uptake was followed by using either a pressure transducer (Model FMS-5M-2H, Toyoda Machine Co., Aichi, Japan) or an oxygen electrode (Model YSI 5300, Yellow Springs Instrument Co., Yellow Springs, OH, USA). The rate of lipid hydroperoxide formation was measured with an HPLC detected by absorption at 234 nm as reported before.[10]

The rate of consumption of α-tocopherol in hexane or benzene was measured with an HPLC using an LC-NH_2 column and hexane/2-propanol (100/1 by vol) as an eluent at a speed of 1.0 ml min^{-1}. It was detected either by absorption at 295 nm or with a fluorometric analyzer, excitation at

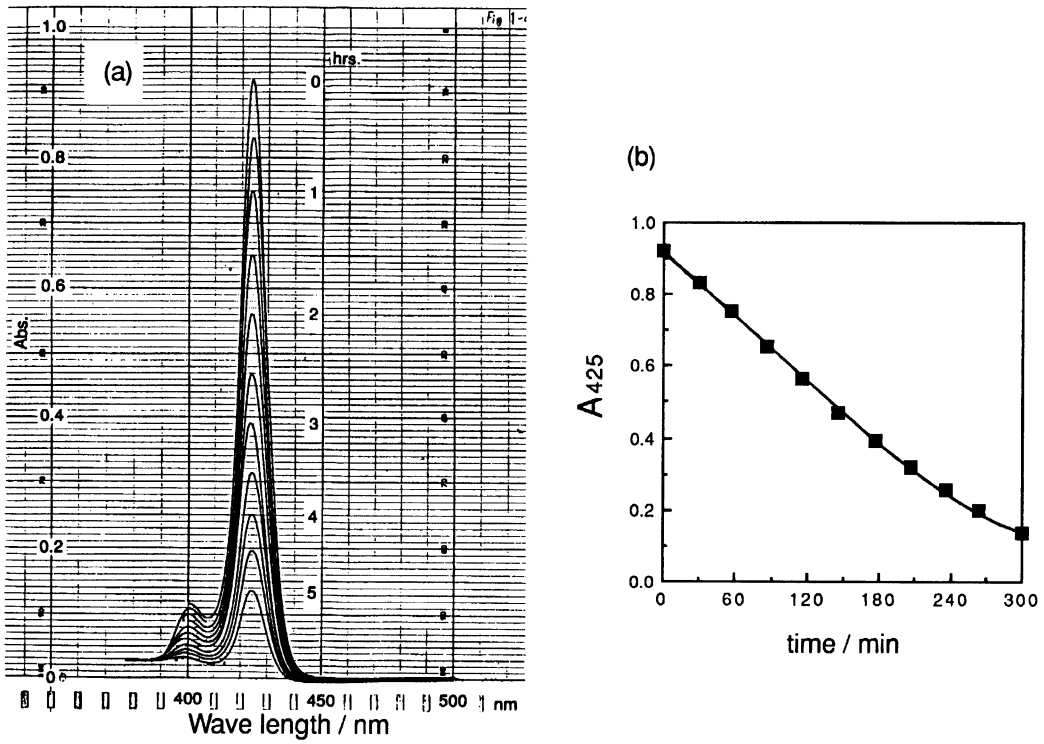

Fig. 1. (a) Decrease in the absorption of galvinoxyl radical ($\lambda_{max}=425$ nm) by the reaction with stearyloxyl radical formed from SHN in hexane at 37 °C. [galvinoxyl]=5.30μM, [SHN]=10.7 mM. (b) Plot of absorption at 425 nm (A_{425}) as a function of time. The experimental conditions are the same as in Fig. 1(a).

295 nm and emission at 320 nm. The consumption of α-tocopherol in the liposomal membranes was also followed with an HPLC with LC-18 column and methanol/*t*-butyl alcohol (90/10 by vol) as an eluent at a speed of 1.0 ml min^{-1} using an electrochemical detector.

The viscosity or fluidity within methyl linoleate micelles and soybean PC liposomal membranes was measured by an ESR spin label method as reported previously.[11] 16-[2-(14-Carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy, or 16-[4,4-dimethyl-3-oxazolidinyloxy (abbrev. to doxyl)]-stearic acid (16-NS), was used as a spin probe, which was incorporated into methyl linoleate micelles or soybean PC liposomal membranes. The ESR spectra were recorded on an X-band JEOL FE1X spectrometer using a quartz flat cell.

Results and Discussion

Rate of Decomposition of SHN and BHN. The thermal decomposition of SHN and BHN generates alkoxyl radicals at a constant rate. The formation of corresponding alkoxyl radicals was confirmed by its spin trapping with *N*-ethylidenedurylamine *N*-oxide. The spin adduct of alkoxyl radical by this spin trap can be distinguished from that of peroxyl radical quite distinctly.[12,13] The hyperfine coupling constants were obtained as $a^N=1.375$ mT, $a^H=0.748$ mT and $a^N=1.377$ mT, $a^H=0.716$ mT for *t*-butoxyl radical and stearyloxyl radical, respectively. Furthermore, as shown in Fig. 1, the decomposition of SHN in the presence of galvinoxyl radical consumed galvinoxyl at a constant rate by a reaction with stearyloxyl radical formed.

The rate constants for the unimolecular decomposition of SHN and BHN were measured by following their concentrations either with an HPLC or a ultraviolet absorption spectroscopy. Figure 2 shows the examples of the decomposition of SHN in hexane and benzene at 37 °C. As shown, it decomposed unimolecularly and the rate constants for decomposition (k_d) were obtained as 1.43×10^{-5} s^{-1} and 1.20×10^{-5} s^{-1} for SHN at 37 °C in hexane and in benzene respectively. The decomposition rate constants for SHN were also measured in decane at 37, 50, and 60 °C and obtained as 1.56×10^{-5}, 9.41×10^{-5}, and 3.50×10^{-4} s^{-1} respectively, which gives the activation energy for decomposition of SHN as 1.1×10^5 J mol^{-1}.

In some experiments, the decomposition of SHN was also followed spectroscopically by its ultraviolet absorption at 225 nm. An example of the results is shown in Fig. 3, which gives $k_d=1.56\times10^{-5}$ s^{-1} at 37 °C in hexane, in good agreement with that measured by HPLC (1.43×10^{-5} s^{-1}).

The rate constants for decomposition of SHN were also measured in methyl linoleate micelles and in soybean PC liposomal membranes in aqueous dispersions. As shown in Fig. 4, the decomposition rate constant for SHN was found to be much smaller in liposomal membranes than in homogeneous solution.

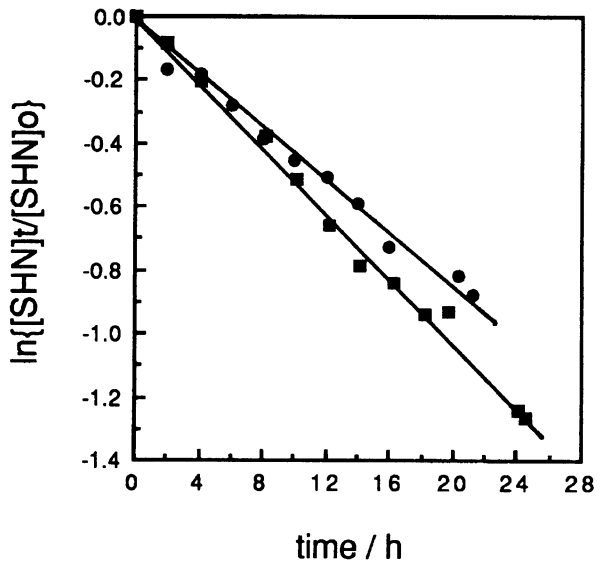

Fig. 2. Decomposition of SHN at 37 °C under air in hexane (■) and benzene (●).

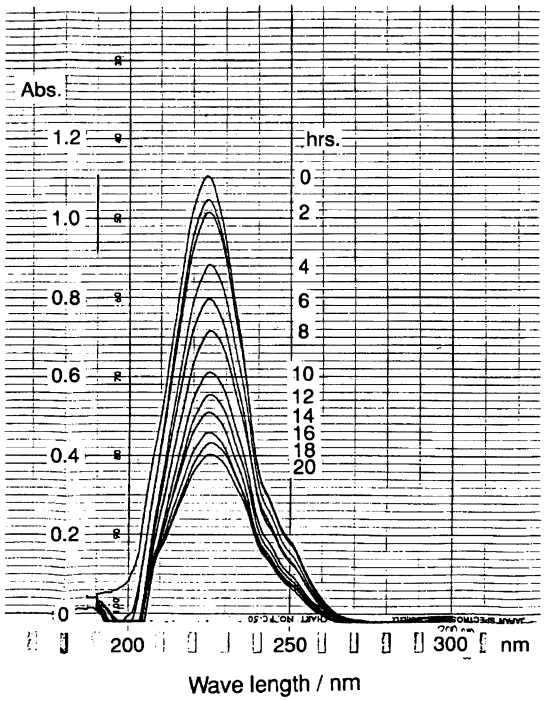

Fig. 3. Decomposition of SHN at 37 °C in hexane followed spectroscopically by its ultraviolet absorption at 225 nm.

The rates of decomposition of BHN were also measured similarly in homogeneous solution, micelles and liposomal membranes. The data are summarized in Table 1.

Oxidation of Methyl Linoleate Induced by SHN or BHN. The rate of free radical formation, that is, the rate of chain initiation is determined by the rate constant for the decomposition of hyponitrite, k_d, and also by the efficiency of free radical formation, e, or, in other words, by the extent of cage reaction.

$$\text{SON=NOS} \xrightarrow{k_d} [\text{SO}\cdot\text{N}_2\cdot\text{OS}]_{\text{cage}} \begin{cases} \nearrow 2\text{eSO}\cdot & (1) \\ \searrow (1\text{-e})\text{SOOS} & (2) \end{cases}$$

(SHN)

It has been well-established that the azo compounds induce the free radical-mediated chain oxidation of methyl linoleate in solution.[4] It was confirmed in the present study that SHN and BHN also induced the oxidation of methyl linoleate in solution. A radical-scavenging antioxidant such as α-tocopherol suppressed the oxidation efficiently and produced a clear induction period as shown in Fig. 5. α-Tocopherol was consumed at a constant rate and, when it was depleted, a fast oxidation took place. Similar results were observed when the rate of oxidation was followed with a pressure transducer, that is, the rate of oxygen uptake was quite small during the induction period, which was followed by a fast and constant rate of oxygen uptake after depletion of α-tocopherol (data not shown). Under these conditions, the oxidation proceeds by a following scheme:[14,15]

$$\text{chain initiation: SON=NOS} \longrightarrow 2\text{eSO}\cdot \quad (3)$$

$$\text{SO}\cdot + \text{LH} \xrightarrow{\text{O}_2} \text{SOH} + \text{LO}_2\cdot \quad (4)$$

$$\text{chain propagation: LO}_2\cdot + \text{LH} \xrightarrow{k_p} \text{LOOH} + \text{L}\cdot \quad (5)$$

$$\text{L}\cdot + \text{O}_2 \longrightarrow \text{LO}_2\cdot \quad (6)$$

$$\text{chain termination: LO}_2\cdot + \text{IH} \longrightarrow \text{LOOH} + \text{I}\cdot \quad (7)$$

$$\text{LO}_2\cdot + \text{I}\cdot \longrightarrow \text{LOOI} \quad (8)$$

where LH and IH are methyl linoleate and antioxidant respectively. It has been known that each molecule of α-tocopherol traps two molecules of peroxyl radicals[14,15] and the product LOOI has been identified as an adduct of peroxyl radical to the 8a position of α-tocopheryloxyl radical.[16—21] The rate of chain initiation, R_i, can be calculated from the induction period, t_{inh}, by Eq. 9.[14,15]

$$R_i = 2ek_d[\text{SHN}] = 2[\text{IH}]/t_{inh} \quad (9)$$

After the induction period or in the absence of antioxidant, the rate of oxidation, R_p, is given by Eq. 10,[15]

$$R_p = k_p[\text{LH}]\,(R_i/2k_t)^{1/2} \quad (10)$$

where k_t is the rate constant for bimolecular, terminating interaction of lipid peroxyl radicals.

The oxidation of methyl linoleate micelles in aqueous dispersions proceeds by substantially the same mechanism as in homogeneous solution. In fact, as shown in Figs. 6 and 7, the rate of oxidation in the absence of

Table 1. Rate Constants for Unimolecular Decomposition (k_d) and Efficiency of Free Radical Formation (e) for SHN and BHN in Homogeneous Solution, Micelles and Liposomal Membranes at 37 °C

	Medium	Homogeneous solution			Micelles	PC liposomes
		Hexane	Benzene	Decane		
	Viscosity, η/cp	0.27[0.24]	0.51	0.73[0.61]		
	τ_c/s	0.20[0.11]	0.32	0.41[0.36]	2.7[1.8]	4.3[2.8]
	$10^6\ ek_d/s^{-1}$	7.8	5.3	6.3[44]	2.7	0.23[0.98]
SHN	$10^6\ k_d/s^{-1}$	14.3	12.0	15.6[94.1]		6.4[9.72]
	e	0.54	0.44	0.40[0.46]	0.18[a)]	0.035[0.10]
	$10^6\ ek_d/s^{-1}$	6.7	5.7	6.0	3.4	0.57[42]
BHN	$10^6\ k_d/s^{-1}$	7.19		8.24		[149]
	e	0.93	0.79[a)]	0.72	0.47[a)]	0.08[a)][0.28]

a) The value of k_d was assumed to be the same as in hexane. b) Numbers in the brackets are those for 50 °C.

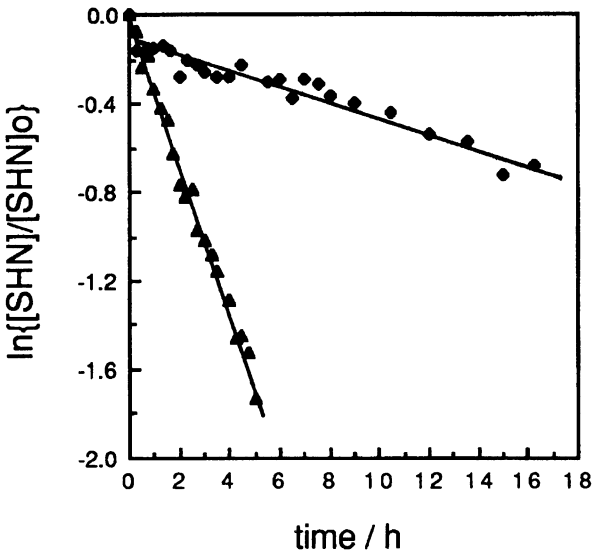

Fig. 4. Rates of decomposition of SHN in decane (▲) and in PC liposomal membrane (◆) at 50 °C.

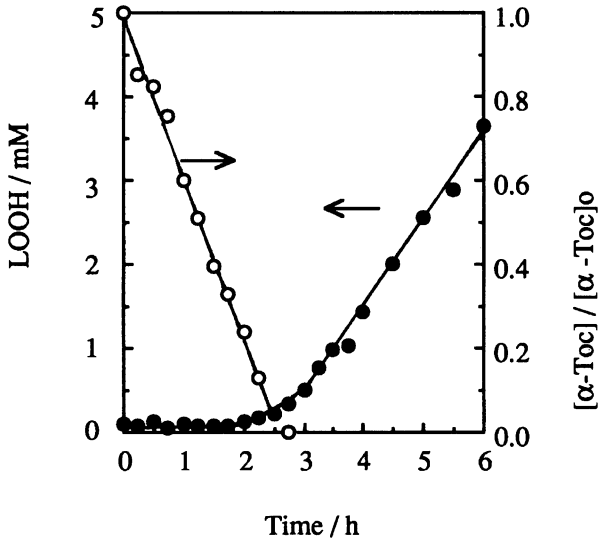

Fig. 5. Oxidation of methyl linoleate (LH) in benzene at 37 °C induced by BHN in the presence of α-tocopherol (α-Toc).

antioxidant was proportional to the first order of substrate concentration and to the half power of the initiator concentration, and the length of induction period was directly proportional to the antioxidant concentration. Thus, the values of ek_d can be also calculated from the Eq. 9. Figure 7 gave $R_i = 2.69\times10^{-9}$ M s^{-1} and ek_d was obtained as 2.69×10^{-6} s^{-1}.

It has been found that the water-soluble, radical-scavenging antioxidants such as ascorbic acid, uric acid, and Trolox suppress the oxidation of methyl linoleate micelles initiated with water-soluble radical initiator, but that it can not suppress the oxidation of methyl linoleate micelles initiated with lipid soluble radical initiator.[4)] In this study, it was found that these water-soluble antioxidants could not suppress the oxidation of methyl linoleate micelles induced by SHN and BHN, suggesting that SHN and BHN generated free radicals within the lipophilic region.

Oxidation of Soybean PC Liposomes Induced by SHN or BHN. SHN and BHN incorporated into soybean PC multilamellar liposomal membranes also induced the oxidation, which was suppressed by α-tocopherol incorporated into the same membranes. Figure 8 shows, as an example, the results of oxidation of soybean PC liposomes initiated with BHN and inhibited by α-tocopherol.

All the results described above show that SHN and BHN act as lipophilic radical initiator and induce the chain oxidation of methyl linoleate in homogeneous solution and in aqueous dispersions and also of soybean PC liposomal membranes. The values of ek_d, k_d, and e measured in different reaction media are summarized in Table 1. In Table 1 are also included the data for viscos-

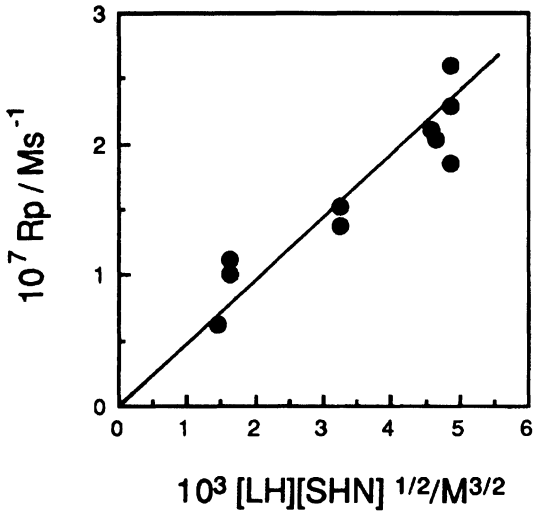

Fig. 6. Rate of oxidation of methyl linoleate micelles in aqueous dispersions at 37 °C induced by SHN in the absence of antioxidant. [LH]=72, 144, 216 mM; [SHN]=0.102, 0.512, 1.02, 1.05 mM.

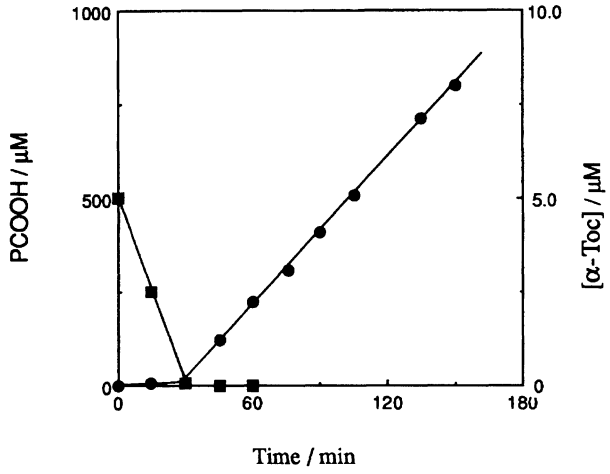

Fig. 8. Formation of PC hydroperoxide (PCOOH, ●) and consumption of α-tocopherol (α-Toc, ■) in the oxidation of soybean PC liposomal membranes initiated with BHN at 50 °C in air. Soybean PC (5.15 mM), BHN (73.4 μM) and α-tocopherol (5.0 μM) were incorporated into the same membranes.

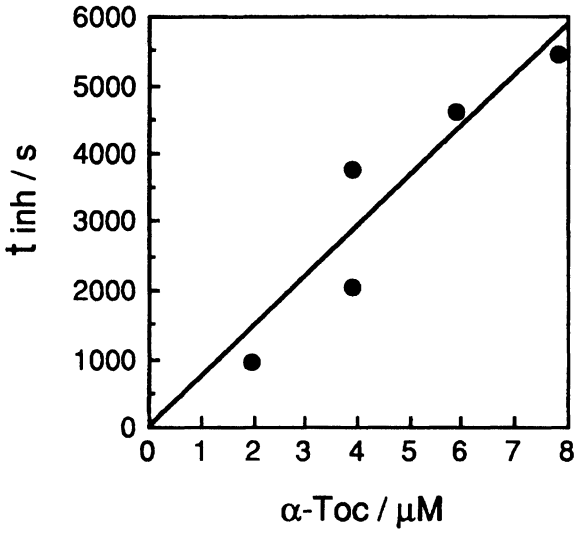

Fig. 7. Plot of the length of induction period as a function of α-tocopherol concentration in the oxidation of methyl linoleate micelles in aqueous dispersions induced by SHN at 37 °C.

ity of the solvent, and a correlation time, τ_c, measured with ESR using 16-NS as a spin probe.

Several points can be deduced from Table 1. Firstly, viscosity increases in the order of hexane < benzene < decane < micelles < liposomes. Secondly, SHN decomposes about twice as rapidly as BHN in homogeneous solution. The solvent effect on k_d is small, but the rate constant for decomposition of SHN in liposomal membranes was considerably smaller than that in solution. This may be due to the reversible one-bond scission[22] of SHN to give stearyloxyl radical and diazenyl radicals, SO· and ·N=N–OS, followed by recombination of these radicals to yield starting SHN in a viscous medium. Barclay et al.[23] also found that the rate of decomposition of di-α-cumyl hyponitrite was smaller in PC liposomes than in "isooctane" and they interpreted this by the one-bond scission of the hyponitrite, followed by recombination. Thirdly, the efficiency of radical production and ek_d or the rate of chain initiation decrease as the viscosity of medium increases. This effect is more profound for SHN than for BHN. It was also found that, when the oxidation was carried out in a mixture of hexane and viscous liquid paraffin, the value of ek_d decreased with increasing ratio of liquid paraffin.

That the efficiency of free radical production decreased in the phospholipid bilayer has been observed and reported previously.[16,23—27] Barclay et al.[23—25,27] found that the efficiency of chain initiation by di-*t*-butyl hyponitrite decreased in the phospholipid liposomal membranes and attributed this decrease to the high microviscosity of the liposomal membranes. Porter et al.[26] synthesized the amphipathic 1,2-disubstituted azo compounds and found a pronounced decrease in efficiency of free radial production from them in the dipalmitoyl PC liposomal membranes from the value obtained in chlorobenzene. Our data are in agreement with these results reported by other investigators. Our data show that the microviscosity in the fatty acid micelles is markedly larger than that in the organic solvent but smaller than that within the PC liposomal membranes.

Our data show that the efficiency of free radical production from SHN is always smaller than that from BHN in all the media studied, suggesting that the bulkiness of radical is also important in determining the efficiency of chain initiation. The microviscosity in the

membrane depends not only on the composition of fatty acids in phospholipid but also on the distance from the membrane surface.[11] It is not clear where SHN and BHN are incorporated in the membrane, but it may be safely concluded that the mobility of molecules is markedly reduced in the PC liposomal membranes and this decrease is more pronounced as the bulkiness of molecules becomes larger.

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