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Characterisation of Novel Indenoindoles. Part II. Redox-Recycling with Ascorbate

Petter Björquist, Johanna Deinum, Kristina Taure, Christer Westerlund and Ann-Margret Östlund-Lindqvist*

ASTRA HÄSSLE PRECLINICAL RESEARCH LABORATORIES, S-431 83 MÖLNDAL, SWEDEN

ABSTRACT. In the accompanying paper it was shown that the new antioxidant, H 290/51 (cis-5,5a,6,10b-tetrahydro-9-methoxy-7-methylindeno[2,1-b]indole), is a powerful antioxidant in several pharmacological models of lipid peroxidation and could be useful as a therapeutic agent in pathophysiological situations where lipid peroxidation plays an important role. In the present study, we characterised H 290/51 as an inhibitor of peroxidation of pure methyl linoleate. H 290/51 almost completely inhibited peroxidation induced by a lipid-soluble initiator at 37°C during the induction period, both in an aqueous solution of micelles in the presence of detergents and in a homogeneous ethanol solution. In both systems, the time of the induction period was linearly related to the concentration of H 290/51. In the ethanol solution, ascorbic acid had a sparing effect on H 290/51, indicating effective interference with radical chain propagation. In aqueous solution with micelles of methyl linoleate made with the nonionic detergents Triton X-100 or Lubrol PX, ascorbic acid did not inhibit peroxidation. However, in these micelles, H 290/51 showed a concentration-dependent extension of the induction period by ascorbic acid, suggesting recycling. In the presence of the zwitterionic detergent CHAPS, although a clear induction period is seen with H 290/51, no recycling by ascorbic acid was found. The ability of H 290/51 to recycle in aqueous solutions, thus depends on the micellar composition. BIOCHEM PHARMACOL 51;10:1403–1410, 1996.

KEY WORDS. redox-recycling; antioxidant; micelle; lipid peroxidation; ascorbic acid; indenoindole

Chain-breaking antioxidants can scavenge chain-carrying oxygen radicals and suppress free radical chain oxidation during the oxidation of lipids. *In vivo*, a number of natural antioxidants are present both in tissues and in plasma, associated with proteins and lipoproteins. In plasma, ascorbic acid is an outstanding antioxidant [1, 2]. However, because ascorbic acid is not lipid-soluble, it may not be an optimal antioxidant for prevention of lipid peroxidation in lipoproteins and membranes. The most important chain-breaking lipid-soluble antioxidant present in human membranes *in vivo* is probably α-tocopherol. In several *in vitro* models, it has been shown that ascorbic acid in the water phase can reduce tocopheroxyl (the α-tocopherol radical) associated with the lipid phase; thus, maintaining the reducing potential of α-tocopherol [3–5]. This process is defined as recycling. This phenomenon was observed by pulse radiolysis and electron spin resonance spectroscopy [6–9]. In other studies, convincing evidence for the theory of recycling was presented in micelles [4], liposomes [3–5] and human plasma [10]. Despite its high lipid solubility, the antioxidant activity of α-tocopherol in micelles, with Triton X-100 as detergent, is poor compared to that in a homogenous system, perhaps due to the poor mobility of tocopherol between lipid particles dispersed in water [11]. Recently, the effects of different solvents on the antioxidant activity of α-tocopherol have been investigated [12]. However, in contrast to the conventional view of α-tocopherol as the major lipid-soluble antioxidant, Bowry *et al.* [13] have recently launched a new and interesting theory concerning a putative role of the α-tocopherol radical as a prooxidant agent under certain conditions.

The oxidation of methyl linoleate in organic solvents has been extensively studied [14, 15]. Niki *et al.* investigated the inhibition of the peroxidation of methyl linoleate by α-tocopherol in the presence of ascorbic acid in different organic solvents [16, 17]. In these studies, oxygen consumption was measured primarily, but the concentration of the antioxidants was followed with time.

* Corresponding author: Johanna Deinum, Biochemistry, Preclinical R & D, Astra Hässle AB, S-431 83 Mölndal, Sweden. Tel. +46 31 7761592; FAX +46 31 7763736.

† *Abbreviations:* AMVN, 2'azobis [2,4-dimethyl valeronitrile]; CHAPS, 3-[3-cholamid-propyl)-diethylammonio]-1-propane sulfonate; CMC, critical micelle concentration; H 290/51, cis-5,5a,6,10b-tetrahydro-9-methoxy-7-methylindeno[2,1-b]indole; n-dodecylmaltoside, n-dodecyl-β-D-glucopyranosyl(1-4)α-D-glucopyranoside. The induction period, t_i is defined as the period measured from the onset of inhibition, immediately after addition of the antioxidant, until the rate of oxygen uptake, R_p, has again obtained the same value as before addition of the inhibitor; R_p* rate after consumption of the antioxidant; R_i rate in the presence of antioxidant; t_i, extension of the induction period; t_i', extension of the induction period in the presence of ascorbic acid.

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In the accompanying paper [18] we have shown that the novel indenoindole H 290/51† is a potent antioxidant in a Fe/ascorbate system with soybean phospholipids, for the prevention of the peroxidation of low-density lipoproteins (LDL) and liver tissue. Moreover, the compound has been shown to have a protective effect on tissue transplants [19]. We have chosen a more basic assay to characterise H 290/51 as an inhibitor of the peroxidation of pure methyl linoleate, induced by a thermolabile free radical initiator. We monitored the uptake of soluble oxygen because oxygen consumption and the disappearance of methyl linoleate and peroxide and conjugated diene formation have been found to agree well with each other [20]. When a water-soluble initiator is used, radicals are formed initially in the water phase, whereas a lipid-soluble initiator generates free radicals within the lipid region. Different antioxidants can be ranked both in negatively and positively charged micelles [21], using a water-soluble initiator. We have chosen a lipid-soluble initiator because in a micellar system, this will prevent a direct antioxidant effect of ascorbic acid present in the aqueous phase. In a preliminary report, we have shown the effect of the micellar composition of the redox properties of different antioxidants [22]. Now, we show the ability of the potent novel antioxidant H 290/51 to recycle in ethanol and in aqueous solutions dependent on micellar composition.

MATERIALS AND METHODS

Chemicals

Triton X-100 and Lubrol PX (both 10% solution in water) were from Pierce (Rockford, IL, USA), CHAPS, 7-deoxycholic acid, SDS, and methyl linoleate from Sigma Chemicals Co. (St. Louis, MO, USA), n-dodecyl-maltoside from Boehringer Mannheim (Mannheim, Germany), AMVN from Polysciences (Warrington, PA, USA), and Chelex 100, 50–100 mesh, from Bio-Rad Laboratories (Richmond, CA, USA). Ethanol (99.5%) from Kemetyl (Stockholm, Sweden), NaOH (suprapur), HNO_3 (65%, suprapur), NaH_2PO_4 (suprapur), ascorbic acid (pro analysis) and all other organic solvents (pro analysis) were from Merck (Darmstadt, Germany). H 290/51 was synthesised [23, 24] at Astra Hässle AB (Mölndal, Sweden). All water was first deionised (MilliQ, Millipore Co., Bedford, MA, USA) and, then, further purified (Elgastadt UHP, Elga Ltd., High Wycombe Bucks, England).

Instruments

Sonication was carried out with a cell sonicator, Vibracell, model ASI from Sonics and Materials Inc. (Danbury, CT, USA). Oxidation was measured at the decrease in the soluble oxygen concentration with a Clark electrode, using a YSI 5331 standard oxygen probe, a YSI model 5300 biological oxygen monitor, and a sample chamber in a thermostatted bath assembly, all from Yellow Spring Inc. (Yellow Spring, OH, USA). Because traces of metal contaminants severely affect the system, the sample chambers were made metal-free by incubation overnight in 2.2 M HNO_3, followed by extensive washing with water [25]. Because the original Lucite holder was not resistant to ethanol and the original stirrer leaked metals, a homemade holder of Teflon and a magnetic stirrer covered with Teflon were used.

The HPLC equipment (LKB 2150-2152) was from Pharmacia LKB Biotechnology (Bromma, Sweden) and was equipped with a 20-μL loop. For analysis of H 290/51 a 125-mm Lichrospher Si-60 column (5 μm) from Merck (Darmstadt, Germany) with a 20-mm guard column was used with a mobile phase of 3.125% (v/v) 2-propanol in hexane and a flow rate of 1 mL/min.

Fluorescence emission spectra (bandwidth 3 nm) between 360 and 400 nm were recorded with a Shimadzu RF 5000 spectrofluorometer (Shimadzu Co., Kyoto, Japan) in the uncorrected spectra mode (λ_{ex} = 306 nm, bandwidth 5 nm). The 1-mL quartz cell (Hellma, Müllheim, Germany) was kept at 37°C in a thermostatted cell holder.

Oxygen Consumption Measurements

In the homogeneous assay, an optical clear solution of 0.14 M methyl linoleate in ethanol (50 μL methyl linoleate/mL ethanol) was used. The micellar solution contained a mixture of methyl linoleate, to a final concentration of 0.14 M, and detergent in 50 mM sodium phosphate buffer in water at pH 7.4, sonicated at 20°C for 1 min at 50% power output. The phosphate buffer had been passed through a column of Chelex 100 to remove traces of metal ions interfering with the assay [25–28]. With the suprapur quality of phosphate used, this treatment was sufficient to prevent autoxidation of ascorbate [25]. The concentration of Triton X-100 was 10 mM, and the other detergents were used at concentrations 10 times the critical micelle concentration [29, 30]. The suspension of methyl linoleate was kept in the refrigerator only for the day of the experiment. To start the measurement, 2 mL of the suspension was first equilibrated with air in the sample chamber at 37°C for 5 min by agitation with the magnetic stirrer, before the chamber was sealed with the oxygen probe. After a further 1-min equilibration, the oxygen concentration was recorded continuously and measured as a percentage of the value after saturation with air (0.2 mM O_2 in buffer, 1.7 mM in ethanol, respectively [31, 32].

Additions to the sealed sample chamber were made through the capillary on the side of the oxygen probe with an SGE gas-tight syringe, Scientific Glass Engineering Pty Ltd. (Ringwood, Victoria, Australia). To initiate radical formation, the lipid-soluble initiator AMVN (stock solutions 0.4 and 1.2 M, respectively, in methanol) was added to final concentrations of 1 mM in the micellar assay, and of 8.5 mM in the ethanol solution. In both assays, oxygen consumption was found to start immediately after addition of the initiator without any lag phase, at a rate independent of the oxygen concentration.

Quantitation of H 290/51 in the Ethanol Assay by HPLC and Fluorescence Spectroscopy

Radical initiation of the peroxidation assay in ethanol at 37°C was run simultaneously in an open sample chamber and in a sealed sample chamber to record oxygen uptake. Samples (30 μL) were removed from the open sample at 5-min intervals and immediately injected on the HPLC column. The absorbance of the eluate was monitored at 315 nm. The concentrations of H 290/51 were estimated from the amplitude of the peaks.

The peroxidation assay was also performed in a 1-mL optical quartz cell containing the ethanol/methyl linoleate solution at 37°C. After additions of the initiator and the antioxidants with a syringe, the cell was closed with a Teflon stopper. A fluorescence emission spectrum was recorded, before and after addition of the free radical initiator, then every 5 min after addition of the antioxidants. Because the fluorescence spectrum of H 290/51 disappeared upon illumination, the slits were kept closed in between. The solution was agitated immediately after the recording of a spectrum and 2.5 min and 15 sec before a new spectrum was recorded.

RESULTS

Effect of H 290/51 on Peroxidation of Methyl Linoleate in Aqueous Micellar Solution

The antioxidant H 290/51 was found to be an efficient inhibitor of lipid peroxidation (Fig. 1). The inhibition was dose-dependent (Fig. 2). Complete inhibition of oxygen consumption, $R_i = 0$, of methyl linoleate micelles in the presence of the detergent Triton X-100 was obtained in the initial phase by addition of 2.5 μM H 290/51 after peroxidation was induced by AMVN (not shown). This indicates complete inhibition of chain propagation. In the phase following the induction period, oxygen consumption gradually increased to the same rate as that found before addition of the drug. If AMVN was added to micellar solution already containing H 290/51, no oxygen consumption was initially found and, after the induction period, oxygen consumption again gradually increased to the same rate as that found in the absence of the drug. The time of the induction period, t_i, was completely comparable (not shown) to that found when the propagation phase was interrupted. The value for t_i increased with increasing amounts of H 290/51 (Fig. 2) in an apparently linear fashion, with a slope of 21 min/μM H 290/51. Ascorbic acid concentrations less than 1 μM had no measurable effect in themselves on oxygen uptake initiated by AMVN, but 10 μM ascorbic acid induced a slight decrease in the R_i/R_p ratio. To obtain a comparable effect with α-tocopherol, 50 times higher concentrations were needed (not shown).

Addition of ascorbic acid together with H 290/51 to the micellar solution of methyl linoleate in Triton X-100 micelles was found to potentiate the effect of H 290/51 (Fig. 1). A linear increase in t_i for 250 nM H 290/51 was found with different concentrations of ascorbic acid added (Fig. 3), with a 90% increase in t_i with each μM of ascorbic acid added. As expected, addition of ascorbic acid after termi-

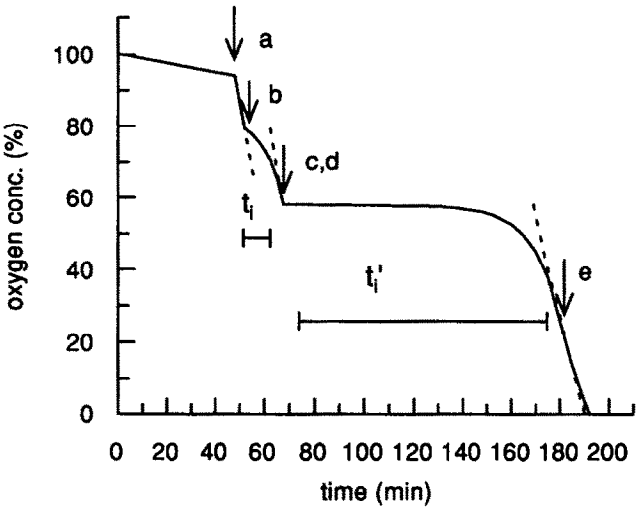

FIG. 1. Potentiation of the antioxidant effect of H 290/51 by ascorbic acid in a micellar solution. Oxidation of an aqueous micellar solution of methyl linoleate (0.14 M) in Triton X-100 (10 mM) and 0.05 M sodium phosphate buffer at pH 7.4, initiated (arrow a) by a lipid-soluble initiator AMVN (1 mM) at 37°C. The oxygen concentration was measured continuously with a Clark-type electrode in a 2 mL air-sealed assay chamber. H 290/51 was added to a final concentration (at arrow b) of 0.25 μM. At arrow c, ascorbic acid was added to 10 μM immediately before d, the next addition of H 290/51 to 0.25 μM. At arrow e, only ascorbic acid was added to 10 μM. T_i is the extension of the induction period in the absence and t_i' in the presence of ascorbic acid.

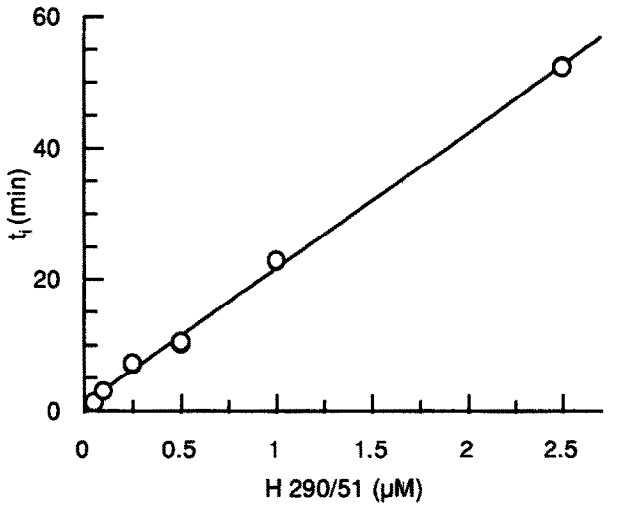

FIG. 2. Dose titration with H 290/51 in a micellar solution. The induction period, t_i for the inhibition of oxygen uptake versus added concentration of H 290/51. The line was drawn by linear regression (slope 20.6 ± 0.3). All measurements were made with two different batches of methyl linoleate. The experimental conditions were as described in the legend to Fig. 1.

nation of the induction period with 250 nM H 290/51 could not restore the inhibition phase (Fig. 1).

Other micellar solutions

The type of detergent used in the aqueous micellar solution of methyl linoleate altered the behaviour of the antioxidant. Only the classical nonionic detergents Triton X-100 and Lubrol PX gave rise to both a distinct t_i for H 290/51 and recycling by ascorbic acid (Table I). Addition of H 290/51 to an n-dodecyl-maltoside micellar solution of methyl linoleate gave a clear induction period, but no recycling effect was observed upon addition of ascorbic acid. In negatively charged micelles, made of SDS, no t_i could be detected with H 290/51. In another type of negatively charged micelles, made of deoxycholic acid, H 290/51 gave a clear t_i but, here, ascorbic acid acted as a prooxidant. In positively charged micelles, ascorbic acid itself was a strong inhibitor. Distinct induction periods could also be detected for H 290/51 in micelles made of the zwitterionic detergent CHAPS, although no recycling by ascorbic acid could be observed and ascorbate itself had no effect.

Effect of H 290/51 on Lipid Peroxidation of Methyl Linoleate in Ethanol Solution

The inhibition of oxygen consumption by H 290/51 in the ethanol assay presented a well-defined t_i. With high concentrations of the antioxidant, >10 μM, the oxidation rate during inhibition, R_i initially was zero (Fig. 4). A linear increase in t_i was found with increasing concentrations of H 290/51 (Fig. 5). In comparison to other antioxidants in ethanol, 5 μM of H 290/51 gave rise to a 7-fold lower R_i/R_p value and an approximately 1.5-fold longer t_i than found for 5 μM α-tocopherol (not shown). In contrast to the aqueous micellar system, in ethanol ascorbic acid is also able to interrupt free radical generation and inhibit oxygen consumption. However, with ascorbate, R_i never became zero and $t_i/\mu M$ was less than 50% of that for H 290/51. Addition of ascorbic acid together with H 290/51, after initiation of the peroxidation of methyl linoleate in ethanol with AMVN, resulted in a t_i that was approximately the sum of the t_i values caused by ascorbic acid and H 290/51 alone (Fig. 4). The initial oxidation rate during inhibition, R_i, was between the lower rate found with H 290/51 and the somewhat higher rate found with ascorbic acid. Normalisation, according to Eqn (2) (see appendix), did not seem reliable, suggesting other processes than simple chain propagation. Moreover, the t_i for ascorbic acid in the ethanol assay was apparently not linearly related to the concentration (not shown), similar to the previously reported effect of ascorbate on peroxidation of methyl linoleate in plasma induced by a water-soluble initiator [33].

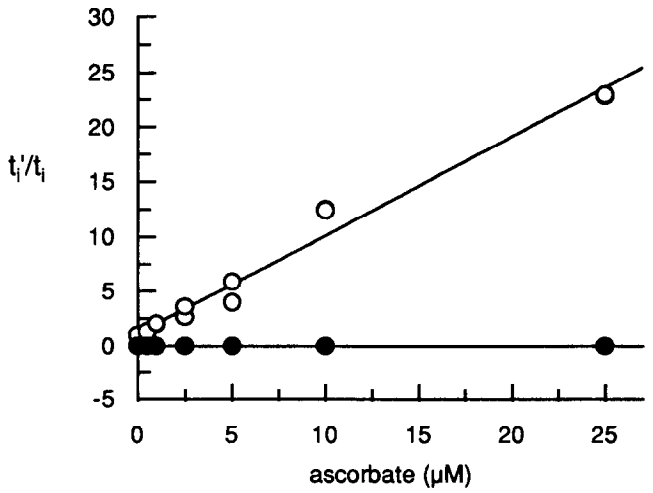

FIG. 3. Dose titration with ascorbic acid and 0.25 μM H 290/51 in a micellar solution. The experimental conditions were as described in the legend to Figs. 1 and 2: (○) with 250 nM H 290/51; (●) without H 290/51. The lines were drawn by linear regression, (slope 0.89 ± 0.04).

Quantitation by HPLC of H 290/51 During Peroxidation in Ethanol Solution

The concentration of H 290/51 in the homogeneous ethanol system was determined using straight phase HPLC (Fig. 6). The concentration of H 290/51 immediately started to decrease after being added to the peroxidation assay. During the period of inhibition of oxygen consumption, the concentration of H 290/51 was found to decrease gradually to zero after approximately 25 min. At this point, the oxygen consumption resumed the same rate as before addition of the antioxidant. In the presence of 50 μM ascorbic acid, the concentration of H 290/51 remained unchanged during the first 30 min, after which the concentration decreased to zero with the same rate as found in the absence of ascorbic acid. Because another batch of methyl linoleate was used, the value for t_i found in these experiments cannot be compared directly with those shown in Fig. 4. Preliminary results indicate that in the aqueous micellar assay, after extraction with n-propanol/hexane, ascorbate was also found to have a sparing effect on H 290/51.

Detection of Reaction Intermediates of H 290/51 by Fluorescence

H 290/51 exhibited hardly any fluorescence in buffer. However, in the less polar solvent ethanol, an appreciable fluorescence (λ_{ex} = 306 nm) was found. Addition of H 290/51 to the peroxidation assay, after initiation of free radical generation by AMVN, completely stopped oxygen consumption, and an immediate consumption of H 290/51 started according to the HPLC measurements (Fig. 6). During the same period, the intensity of the fluorescence emission of H 290/51 increased (Fig. 7), with an apparent shift in the position of the maximal intensity from 382 to 378 nm, suggesting the appearance of new species. After approximately 1 hr, when theoretically, according to the HPLC analysis, all H 290/51 should have been consumed

TABLE 1. The effect of the micellar composition on the action of H 290/51 on peroxidation of methyl linoleate in aqueous phosphate buffer

Micelles of 0.14 M methyl linoleate in buffer made with:*	Detergent type	Effect of 250 nM H 290/51
Triton X-100	nonionic	clear t_i† recycling‡
Lubrol PX	nonionic	clear t_i recycling
N-Dodecylmaltosid	nonionic	clear t_i no recycling
SDS	anionic	no t_i
Deoxycholic acid	anionic	clear t_i§
N-Cetyl-N-N-N-trimethyl ammoniumbromide	cationic	no effect
CHAPS	zwitterionic	clear t_i no recycling

Peroxidation of methyl linoleate (0.14 M) in 50 mM phosphate buffer at pH 7.4, initiated at 37°C by addition of 1 mM AMVN. H 290/51 was added at the propagation phase.

* the detergent concentration used was 10 × CMC.

† t_i, time of the induction period.

‡ recycling, extended inhibition time after addition of 10 μM ascorbic acid.

§ ascorbate is a prooxidant in micelles with deoxycholic acid.

and oxygen consumption should no longer have been inhibited, the intensity of the emission started to decline slowly to below the original intensity. After 2 hr, no distinct spectrum was found. The fluorescence emission spectrum of 20 μM H 290/51 in the presence of 50 μM ascorbic acid was unchanged during the first 40 min (Fig. 7). The intensity then increased, but reached only 75% of the intensity of H 290/51 alone, and declined in the next phase.

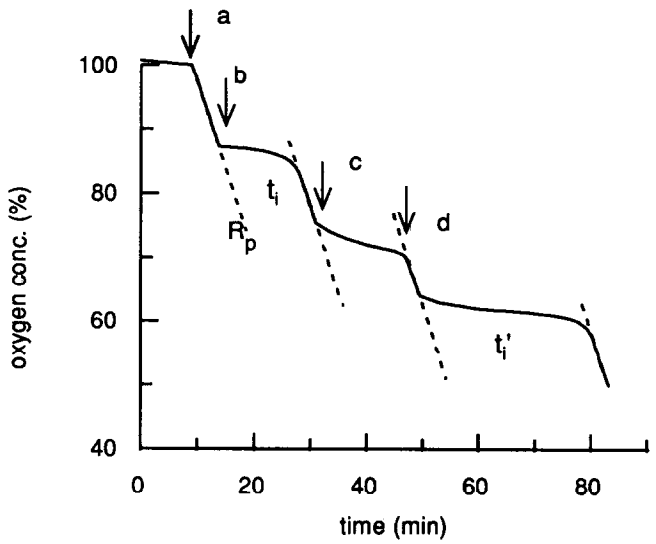

FIG. 4. Potentiation of the antioxidant effect of H 290/51 by ascorbic acid in ethanol solution. Oxidation of a homogeneous ethanol solution of methyl linoleate (0.14 M) was initiated (arrow a) by the lipid-soluble initiator AMVN (8.5 mM) at 37°C. The oxygen concentration was measured continuously with a Clark-type electrode in a 2 mL air-sealed assay chamber. H 290/51 was added from a stock solution in ethanol to a final concentration at arrow b of 10 μM, at arrow c ascorbic acid was added to 50 μM, and at arrow d ascorbic acid and H 290/51 were added simultaneously to 50 and 10 μM, respectively.

DISCUSSION

The new antioxidant H 290/51 seems to be an effective quencher of free radical propagation, because it was able both to interrupt oxygen uptake and inhibit the initiation of lipid peroxidation. The linear dependence of the extension of the induction period in both the micellar and the ethanol systems with increasing amounts of H 290/51 is consistent with the theoretically expected relation (Eqn

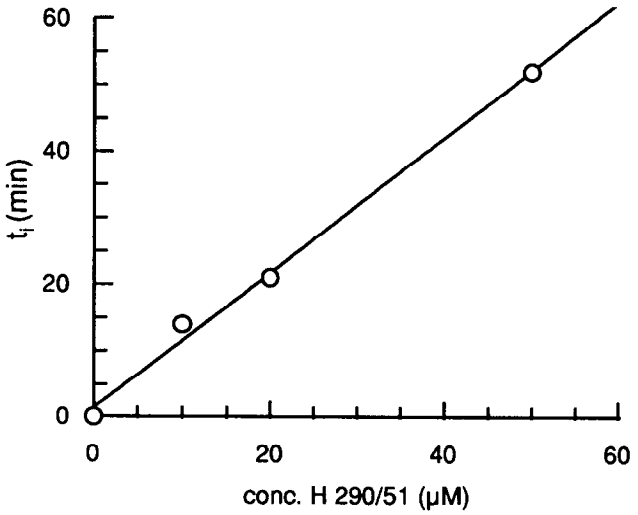

FIG. 5. Dose titration with H 290/51 in ethanol solution. The induction period, t_i, for the inhibition of oxygen uptake versus added concentration of H 290/51. The line was drawn by linear regression (slope 1.01 ± 0.06). The experimental conditions were as described in the legend to Fig. 5.

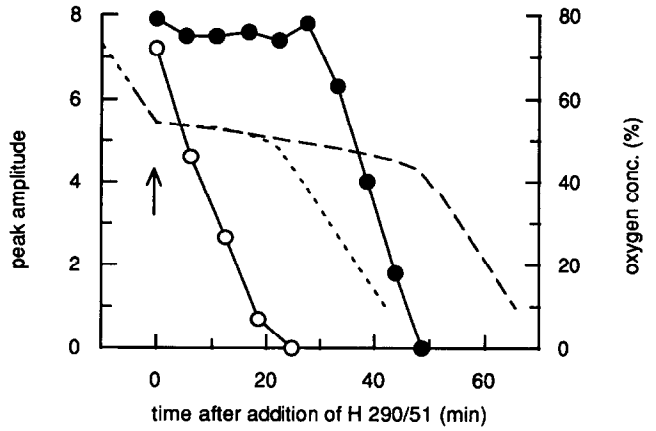

FIG. 6. Quantitation by HPLC of H 290/51 in ethanol solution. The concentration of H 290/51 was quantified by straight phase HPLC from the peak amplitude in samples from the open peroxidation assay at 37°C (5-min intervals). The oxygen concentration (broken lines) was monitored continuously in the parallel "sealed" assay, as described in the legend to Fig. 5. At t = 0, addition of 10 μM H 290/51 (○ and – – –) or 10 μM H 290/51 with 50 μM ascorbic acid (● and — — —).

(2), Appendix) for an antioxidant. According to Eqn (3) (see Appendix), the ratio between R_i and the propagation rate R_p is a measure of the potency of the antioxidant and is dependent on the ratio $k_i/(k_{inh}^2)$ and, thereby, both on R_i and t_i. For low concentrations of H 290/51 (0.25 μM) in the micellar system, the ratio R_i/R_p was approximately zero in the initial phase. For higher concentrations, R_i was zero

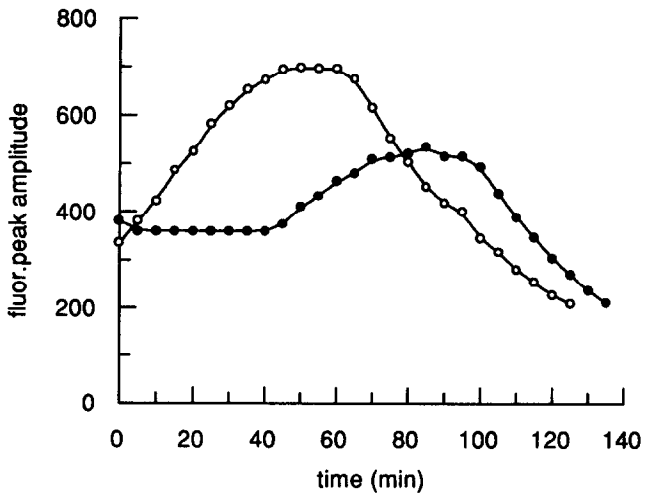

FIG. 7. Appearance of an intermediate of H 290/51 during lipid peroxidation in ethanol. The fluorescence emission spectra of the peroxidation assay in ethanol were recorded between 360 and 400 nm (λ_{ex} = 306 nm) at 37°C. At time = 0, 20 μM H 290/51 was added and the readings (○) continued at 5-min intervals. Alternatively, at t = 0, 20 μM H 290/51 was added together with 50 μM ascorbic acid (●). The maximal fluorescence intensity at 382 nm, corrected for the contributing fluorescence from the other constituents in the peroxidation assay, is given vs time.

during most of the induction period. In contrast, α-tocopherol had only a very weak effect in the same concentration range in the micellar assay. In comparison with other antioxidants, the k_{inh} of H 290/51 in ethanol can be estimated to be at least 10 times that of α-tocopherol, applying the differences in R_i/R_p and the concentration effect, according to Eqn (2) and (3) (see Appendix). The number of radicals scavenged by each molecule of H 290/51, n in Eqn (2), was 2 in the ethanol assay, similar to α-tocopherol.

In the concentration range used, the efficiency of ascorbic acid in recycling the free radical of H 290/51 in micelles with Triton X-100 correlated linearly with its concentration. The efficiency is not the optimally expected value. Ascorbic acid in water is a two-electron reducing agent. In our model system, 1.1 μM ascorbic acid can recycle 0.25 μM H 290/51 once. The ratio is, thus, 0.23, whereas one would expect a theoretical maximal value of 2. However, the efficiency is not only dependent on the lifetime of the intermediate radical, competing termination reactions (see Appendix) but also on the partition coefficient and localisation in the micelles. For the α-tocopherol as well, the maximal theoretical efficiency for recycling with ascorbate has never been reported for inhomogenous systems. Moreover, for α-tocopherol, it has been shown that, at far higher concentrations of ascorbic acid, efficiency also decreases as a result of loss by autoxidation [34]. Because the antioxidant radicals can also participate in other reactions, the rate of recycling should be crucial. It has been shown for tocopherol and ascorbic acid that cycling between the reduced and free radical forms occurs *via* the transfer of a single hydrogen atom rather than *via* separate electron transfer and protonation reactions [19].

In the homogeneous system, higher concentrations of AMVN, 8.5 mM compared to 1 mM, are needed to obtain relative rates of oxygen consumption comparable to that of the micellar system. However, the saturating concentration of oxygen in ethanol is nearly 8.5 times that in aqueous solution. Thus, consistent with Eqn (1) (see Appendix) higher concentrations of H 290/51 were needed in the homogeneous system to obtain the same relative effect as in the micellar system. This is in apparent contrast to α-tocopherol, which, despite its high lipid solubility, has a poorer antioxidant activity in micellar systems than in homogeneous systems, perhaps due to its poor mobility between lipid particles dispersed in water [11].

A limiting factor of the micellar system is the type of detergent used, crucial for the effectiveness of the antioxidant [21]. It has been shown that, in charged micelles made by SDS, the antioxidant capacity of different compounds can be ranked using a water-soluble initiator [21]. We have studied the effect of the micellar composition on recycling using a lipid-soluble initiator. Recycling with ascorbate was found for the classical nonionic detergents Triton X-100 and Lubrol PX, but not with n-dodecylmaltoside, which has the same aliphatic tail as Lubrol PX. The polar head of the maltoside is more hydrophilic than that of Lubrol PX, so that the still rather hydrophobic radical intermediate may

not be able to come near the water phase in which ascorbic acid is present. Alternatively, the similar polar head group of Triton X-100 or Lubrol PX may participate in the redox reaction. In that case, the detergent itself may give rise to free radicals. It is of interest that, in the presence of the zwitterion CHAPS, which most resembles a phospholipid, H 290/51 does not recycle, although a clear induction period is detected.

When both ascorbic acid and H 290/51 are present in ethanol, the t_i equals the sum of the individual t_is, as has been reported earlier for several other antioxidants, such as α-tocopherol. Thus, ascorbic acid and H 290/51 act either "synergistically" or consecutively, that is, one compound is consumed before the other because of the large difference in redox potential. Because the quantitation of the antioxidants either by HPLC (only H 290/51) or by fluorescence suggests a sparing effect by ascorbic acid for these compounds, the former theory seems the most valid. The initial decrease in fluorescence shown for H 290/51 during the first 10 min might imply the formation of a small amount of recycling intermediate. Moreover, an indication for "synergism" is the fact that the rate of oxygen consumption during inhibition is lower if both ascorbic acid and H 290/51 are present than if only ascorbic acid is present. In contrast, the "sparing time," (i.e., the initial period during which the fluorescence intensity did not change) was approximately 40 min with 50 μM ascorbic acid and 20 μM H 290/51. The intensity of the fluorescence emission of H 290/51 increased at a time when all the original compound should have been consumed, according to the HPLC measurements. During the continuous increase in the fluorescence intensity, the maximum emission of the spectrum shifted towards a lower wavelength. We, therefore, suggest that a new species with a higher oxidation state is responsible for this shift in peak position. If this new species is accumulated until all H 290/51 is consumed it will, then, hide the fluorescence of H 290/51 which, in fact, decreases as the drug is consumed. Preliminary electron spin resonance measurements have not been able to detect a new radical species in the homogenous peroxidation assay in the presence of H 290/51*, although the chromanoxyl radical has been detected under the same conditions but in the presence of α-tocopherol. The absence of an electron spin resonance signal may also mean that the concentration of the radical intermediate is very low, with a very short half-life and, thus, a higher reactivity than the chromanoxyl radical. Furthermore, no new species were detected with straight phase HPLC measurements during peroxidation of H 290/51; the reaction mechanism of H 290/51, thus, remains unknown. Because the inhibition of peroxidation by H 290/51 was also potentiated by the presence of ascorbic acid in an aqueous micellar assay, the sparing of H 290/51 by ascorbic acid in ethanol further supports the view that the substance can recycle. Moreover, preliminary results indicate that in the aqueous micellar assay, after extraction with n-propanol/hexane, ascorbate was also found to have a sparing effect on H 290/51.

It is important to realize that lack of recycling for a compound in one test system is not proof that recycling is impossible, because a statement is only valid for the specific test conditions used. On the other hand, recycling of a compound in the Triton X-100 model is not a guarantee for recycling *in vivo* because, since as we have shown here, the type of detergent used *in vitro* is crucial. Because the indenoindole H 290/51 has been shown [18, 19, 23] to have a powerful protective effect on tissue peroxidation *in vivo*, it is most probable that redox recycling of this compound contributes to its effects *in vivo*, as well.

APPENDIX

The rate of oxygen uptake is linearly dependent on the concentration of methyl linoleate, the substrate, but related to the square root of the initiator concentration: [14]

$$-dO_2/dt = R_p = k_p \cdot (R_c/2k_t)^{1/2}\,[LH]. \quad (1)$$

Where R_p is the rate of oxygen consumption, R_c is the rate of chain initiation (linearly dependent on initiator concentration), k_t the rate constant for radical chain termination and k_p the rate constant for chain propagation of methyl linoleate. The ratio $k_p/(2k_t)^{1/2}$ is generally referred to as the "oxidizability" of the substrate. In the presence of an antioxidant, IH, oxidation can be inhibited, presuming that the antioxidant radical formed is not a reactive chain initiator *per se*. Inhibitor efficiency is determined by the competition between chain propagation and direct chain termination in reaction with the antioxidant; that is, the ratio between the rate constants k_{inh}/k_p. Termination is also obtained by adduct formation, where n is the number of peroxy-radicals scavenged by one molecule of antioxidant. The strength of the antioxidant depends on the ratio of the inhibited oxidation rate to that of the uninhibited oxidation, R_i/R_p. The smaller this ratio, the higher the activity of the antioxidant. However, this ratio is, to a certain degree, dependent on the test system used [11, 19]. The duration of the induction period for the inhibition, t_i, depends on both n, the concentration of the antioxidant [IH], and the inverse of R_c. The t_i values obtained under conditions with different R_p values can be normalised because R_c is proportional to R_p^2 if we introduce a constant C dependent on k_t and [LH] and define the rate of oxidation [11] during the induction period as R_i:

$$t_i = n \cdot C \cdot [IH]/R_p^2 \quad (2)$$

and

$$R_i/R_p = (2k_tR_c)^{1/2}(n \cdot k_{inh} \cdot [IH]) \quad (3)$$

* Lagercrantz C. and Deinum J., unpublished results.

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