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# Action of 21-Aminosteroid U74006F as an Antioxidant Against Lipid Peroxidation

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**ABSTRACT.** The dynamics of the action of the 21-aminosteroid U74006F as an antioxidant against lipid peroxidation were studied in organic solution and membranes. It was confirmed that the reactivities of this compound toward stable phenoxyl radical and peroxyl radical were quite low. In fact, U74006F did not exert appreciable antioxidant effect against the free radical-driven oxidation of methyl linoleate in acetonitrile solution. However, it suppressed the oxidation of phosphatidylcholine liposomal membranes into which it was incorporated in a concentration-dependent manner. The 21-aminosteroid U74006F did not exert any sparing effect on the rate of α-tocopherol consumption in the oxidation of methyl linoleate in solution, but when they were simultaneously incorporated into the membrane, U74006F spared α-tocopherol and exerted a synergistic effect against the oxidation of liposomal membranes. This suggests that lipophilic U74006F acts as an antioxidant against lipid peroxidation through a physicochemical and not a pure chemical mechanism, and that a physical interaction with the liposomal membrane may facilitate the inhibition of lipid peroxidation with U74006F. BIOCHEM PHARMACOL **55**;6:785–791, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** aminosteroid; antioxidant; lipid peroxidation; oxidative stress; vitamin E; liposomal membrane

Recent experimental, clinical and epidemiological studies have shown that active oxygen species and free radicals play an important causative role in the development of various diseases, cancer and aging. As a consequence, the function and protective effect of antioxidants against such oxidative stress have received renewed attention. The antioxidant effects of a variety of natural and synthetic antioxidants have been explored. The 21-aminosteroids are the products of an effort to develop an antioxidant against lipid peroxidation for the acute treatment of traumatic or ischemic central nervous system injury [1]. Among others, 21-aminosteroid U74006F (ASU)†, generic name Tirilazad mesylate (Fig. 1), has been selected as a novel antioxidant for clinical development. The antioxidant action of ASU has been studied in several systems and reported by other groups [2–10]. It exerted antioxidant effects in the oxidations of rat brain homogenate and purified brain synaptosomes induced by ferrous ion [2] and also of isolated liver microsomes induced by cumene hydroperoxide and by diquat [5]. The antioxidant activity of ASU in simpler model systems has also been studied, where it has been shown to scavenge peroxyl radicals [6]. Furthermore, the physical membrane-stabilizing effect of ASU has also been reported [7–10].

In spite of these studies, the dynamics of the antioxidant action of ASU are not yet well understood. This study was undertaken to further elucidate the kinetics and mechanisms of the inhibition of lipid peroxidation by ASU. The actions of this compound toward free radicals and against the oxidations of polyunsaturated lipids in homogeneous solution and in aqueous dispersions were studied. The azo radical initiator was used to initiate the free radical-mediated chain oxidations of lipids at a constant and readily controled and reproducible rate, thereby creating a steady-state kinetic system and enabling quantitative kinetic analysis [11].

## MATERIALS AND METHODS

### *Materials*

Methyl linoleate was purchased from Sigma Chemical Co. and purified with a silica-gel column before use. Soybean phosphatidylcholine (PC) was also obtained from Sigma and purified with silica-gel and alumina columns. ASU and α-tocopherol were a kind gift from Pharmacia & Upjohn Inc. and the Eisai Co, respectively. 2,2′-Azobis(2,4-dimethylvaleronitrite) (AMVN) and 2-2′-azobis(2-amidinopro-

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† *Abbreviations:* AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); ASU, 21-aminosteroid U74006F, 21-[4-2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methylpregna-1,4.9(11)-triene-3,20-dione monomethane sulfonate; αT, α-tocopherol; DPBQ, *N*,*N*′-diphenyl-1,4-benzoquinone diimine; DPPD, *N*,*N*′-diphenyl-1,4-phenylenediamine; ESR, electron spin resonance; MeLOOH, methyl linoleate hydroperoxides; PC, phosphatidylcholine; SIN-1, 3-morpholinosydnoimine hydrochloride.

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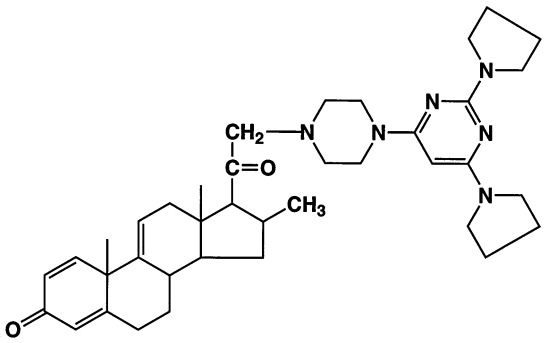


**FIG. 1. Structure of 21-aminosteroid U74006F (ASU).**

pane) dihydrochloride (AAPH) used as a lipophilic and hydrophilic radical initiator respectively were obtained from Wako Pure Chemical Co. 3-Morpholinosydnonimine hydrochloride (SIN-1) was obtained from Dojindo Laboratories and used as received. Galvinoxyl, *N*,*N*′-diphenyl-1,4-phenylenediamine (DPPD), and other chemicals were of the highest grade available commercially.

### *Interaction of Antioxidant with Galvinoxyl*

The interactions of ASU or α-tocopherol with galvinoxyl were measured with a spectrophotometer equipped with a rapid-mixing stopped-flow apparatus (RX-1000, Applied Photophysics) by following the decrease in maximum absorption of galvinoxyl at 429 nm [12]. The change in absorption spectrum was also followed at specific time intervals after mixing the acetonitrile solutions of ASU or α-tocopherol and galvinoxyl prepared separately beforehand.

### *Estimation of Reactivity of Antioxidant Toward Peroxyl Radical*

The reactivities of ASU and α-tocopherol toward peroxyl radical were estimated as follows. One molecule of DPPD reacts rapidly with two molecules of peroxyl radicals to give *N*,*N*′-diphenyl-1,4-benzoquinone diimine (DPBQ), which has a strong absorption at 440 nm [13]. Appropriate amounts of DPPD and AMVN were incubated in acetonitrile at 37° under air in the absence or presence of antioxidant, and the formation of DPBQ was followed spectrophotometrically at 440 nm. The reactivity of the antioxidant toward peroxyl radical was assessed from the extent of reduction of formation of DPBQ by the test antioxidant.

### *Inhibition of Oxidation of Lipids by ASU*

The oxidations of methyl linoleate and soybean PC liposomes were carried out at 37° in air. Methyl linoleate was incubated in acetonitrile in the presence of AMVN and an appropriate amount of antioxidant, and the formation of methyl linoleate hydroperoxides was measured with an HPLC equipped with a reversed-phase LC18 column (particle size 5 μm; 4.6 mm × 25 cm; Supelco). Methanol:*tert*-butyl alcohol:40 mM phosphate buffer (60:30:10 by vol) was used as an eluent with a flow rate of 1.0 mL/min, and the hydroperoxides were detected by UV absorption at 234 nm [14]. The multilamellar PC liposomal membranes were prepared as reported previously [15]. PC, AMVN and antioxidants, when used, were incorporated into the membranes simultaneously by dissolving them into the solvent before preparation of the membranes. The unilamellar vesicles were prepared by sonicating multilamellar vesicles with a Branson Sonifier 250. AAPH and SIN-1 were added as an aqueous solution after preparation of unilamellar vesicles. The PC hydroperoxides were analyzed by absorption at 234 nm with an HPLC using LC-Si column (particle size 5 μm; 4.6 mm × 25 cm; Supelco), and methanol:40 mM phosphate buffer (90:10 by vol) was used as an eluent at a flow rate of 1.0 mL/min. The consumption of ASU and α-tocopherol was followed with an HPLC equipped with an electrochemical detector (Kotaki, Co.) which was set at +800 mV. A reversed-phase LC-8 column (particle size 5 μm; 4.6 mm × 25 cm; Supelco) was used, and the eluent was methanol:*tert*-butyl alcohol (90:10 by vol) containing 50 mM $NaClO_4$ at a flow rate of 1.0 mL/min.

### *ESR Analysis*

The ESR spectra were recorded on an X-band JEOL FE1X spectrometer under the following conditions: magnetic field, 329 ± 5 mT; sweep time, 8 mT per min; microwave power, 1 mW; modulation frequency, 100 kHz; and modulation amplitude, 0.02 mT. The experiments were repeated several times and the results of typical experiments are shown. The reproducibilities in the present *in vitro* model experiments were ±5%.

## RESULTS

### *Interaction of ASU with Galvinoxyl Radical*

The rate of reduction of galvinoxyl radical by an antioxidant describes qualitatively its reactivity toward oxygen radicals [12]. The strong absorption spectrum of galvinoxyl in the visible region was diminished by the addition of ASU (data not shown). The absorption spectrum was the same as that observed in the reaction with α-tocopherol (data not shown), suggesting that ASU reduces galvinoxyl by donating a hydrogen atom to galvinoxyl. The rate of reduction can be monitored by following the decay of galvinoxyl with either ESR or visible absorption spectrometer. Figure 2 shows that ASU reduced galvinoxyl in a concentration-dependent manner, but that its rate was much smaller than that of α-tocopherol. Little decay of galvinoxyl was observed in the absence of antioxidant.

Since excess ASU is present, the decay of galvinoxyl is expressed by the following equation:

$$-\mathrm{d[Galvinoxyl]/dt} = k\mathrm{[Galvinoxyl][ASU]} \quad (1)$$

$$= k'[Galvinoxyl] \quad (2)$$

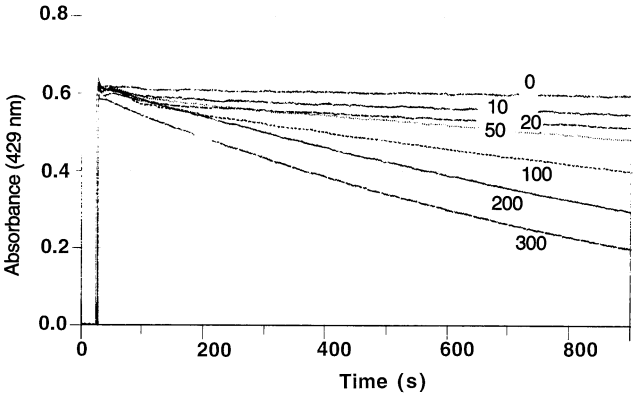


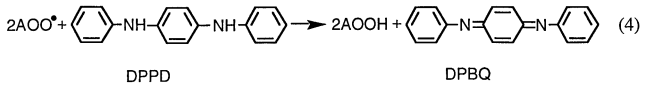

**FIG. 2. Decay of maximum absorption at 429 nm of galvinoxyl by interaction with ASU. The methanol solutions of galvinoxyl (5 μM) and ASU were mixed and the decay of absorption at 429 nm was followed at 37° as described in the "Materials and Methods" section. The numbers in the Figure show the concentration of ASU in μM.**

where $k' = k$[ASU]. The pseudo first-order rate constant $k'$ was estimated from the half-life of galvinoxyl, $\tau_{1/2}$, which is expressed by Eqn 3:

$$\tau_{1/2} = 0.693/k' \quad (3)$$

The results shown in Fig. 2 give $k'$ as $8.66 \times 10^{-4}$ $sec^{-1}$ and $1.38 \times 10^{-3}$ $sec^{-1}$ for 200 μM and 300 μM ASU, respectively. Thus, the second-order rate constant for interaction of ASU with galvinoxyl $k$ is obtained as 4.5 $M^{-1}$ $sec^{-1}$.

DPPD reacts rapidly with the peroxyl radical to give DPBQ (Eqn 4) [13]:




By following the increase in the absorption at 440 nm due to DPBQ, it was found that DPBQ was formed at a constant rate when DPPD was incubated with AMVN under air (data not shown). Adding α-tocopherol to this mixture suppressed the formation of DPBQ, apparently because α-tocopherol competed with DPPD in scavenging peroxyl radicals derived from AMVN. On the other hand, ASU exerted no effect on the formation of DPBQ (data not shown), suggesting that ASU is not capable of competing with DPPD in scavenging peroxyl radicals.

However, it was also found that ASU was consumed when it was incubated with AMVN in acetonitrile in air, suggesting that ASU does react with peroxyl radical but that its reactivity is low. The consumption of ASU observed during the incubation in acetonitrile with AMVN at 37° in air is shown in Fig. 3. In the absence of α-tocopherol, ASU was consumed at a constant rate. With 1.0 mM AMVN, the rates of ASU consumption were $2.69 \times 10^{-10}$ M/sec and $3.63 \times 10^{-10}$ M/sec when the initial ASU concentrations were 5.0 and 10 μM, respectively. When the concentrations of AMVN and ASU were 2.0 mM and 5 μM respectively, the rate of ASU consumption was $4.81 \times 10^{-10}$ M/sec. ASU was spared almost completely by α-tocopherol, while α-tocopherol was consumed at a much faster rate, $1.95 \times 10^{-9}$ M/sec and $1.99 \times 10^{-9}$ M/sec respectively, independent of the absence and presence of ASU. These results suggest that, as observed above, ASU is much less reactive toward peroxyl radical than α-tocopherol and is capable of scavenging only a minor fraction of peroxyl radicals generated from AMVN.

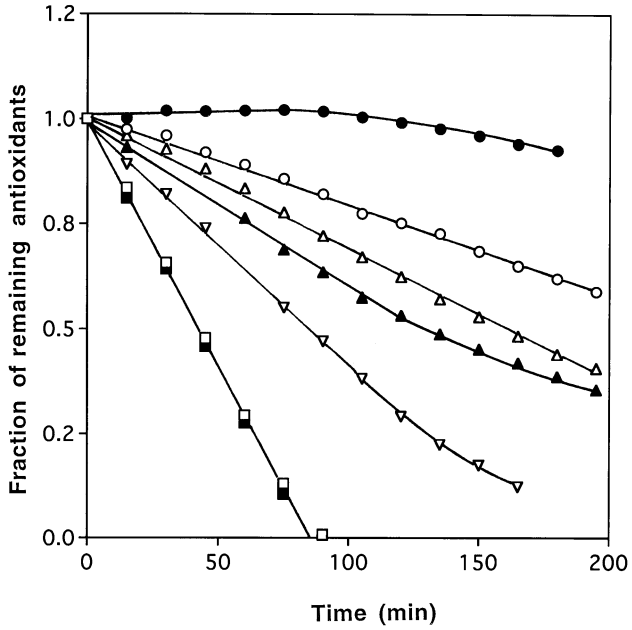



**FIG. 3. Consumption of ASU induced by AMVN. ASU was incubated with AMVN in the absence or presence of α-tocopherol at 37°C in air in acetonitrile, and the decay of ASU (circle and triangle) and α-tocopherol (square) was followed with an HPLC as described in the "Materials and Methods" section. The concentrations of AMVN (mM), ASU (μM), and α-tocopherol (μM) were: △: 1.0, 5.0, 0; ○: 1.0, 10, 0; □: 1.0, 0, 10; ●, ■: 1.0, 10, 10; ▽: 2.0, 5.0, 0; and ▲: 2.0, 1.0, 0.**

### *Inhibition of Oxidation of Methyl Linoleate in Solution*

The oxidation of methyl linoleate induced by free radicals proceeds by a typical chain mechanism to give four kinds of regio- and stereo-isomers of methyl linoleate hydroperoxides having conjugated diene [16]. This system is a convenient one to assay the chemical activity of an antioxidant against lipid peroxidation quantitatively [17]. In the present study, methyl linoleate was oxidized in acetonitrile solution in the presence of lipophilic azo compound, AMVN. The results summarized in Fig. 4 show that ASU acted as a weak antioxidant and retarded the oxidation of methyl linoleate in a concentration-dependent manner, but that it did not give a clear induction period. This shows that ASU acts as a radical-scavenging antioxidant, but that its chemical reactivity toward peroxyl radical is small.

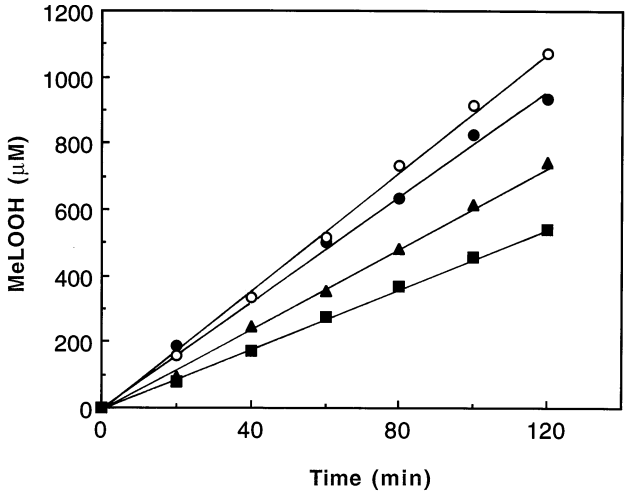



**FIG. 4. Effect of ASU on the oxidation of methyl linoleate in acetonitrile solution. Methyl linoleate (151 mM) was oxidized in acetonitrile with AMVN (0.20 mM) at 37° in air in the absence and presence of ASU, and the formation of methyl linoleate hydroperoxides (MeLOOH) was followed as described in the "Materials and Methods" section. [ASU] = 0 (○); 10 (●); 100 (▲); 200 μM (■).**

On the other hand, as shown in Fig. 5, a small amount of α-tocopherol inhibited oxidation almost completely and gave a clear induction period. The rate of consumption of α-tocopherol was obtained from the results in Fig. 5 as $4.36 \times 10^{-10}$ M/sec. Since one molecule of α-tocopherol scavenges two molecules of peroxyl radicals [17, 18], the rate of radical generation from 0.20 mM AMVN is calculated as $8.72 \times 10^{-10}$ M/sec. On the other hand, the induction period was obtained from the plot of formation of methyl linoleate hydroperoxide against time as 180 min, which gives the rate of chain initiation as $2 \times 5.0 \times 10^{-6}/180 \times 60 = 9.26 \times 10^{-10}$ M/sec, in good agreement with the above value. The average rate of chain initiation by 0.20 mM AMVN is obtained as $9.00 \times 10^{-10}$ M/sec:

$$\text{Rate of chain initiation } R_i = 2\, ek_d[\text{AMVN}] = 9.00 \times 10^{-10}\ \text{M/sec} \quad (5)$$

Thus, $2ek_d$ is obtained as $9.00 \times 10^{-10}/0.20 \times 10^{-3} = 4.5 \times 10^{-6}\ \text{sec}^{-1}$, where $e$ and $k_d$ are the efficiency of chain initiation and rate constant for unimolecular decomposition of AMVN, respectively. This is in reasonable agreement with $2ek_d = 3.9 \times 10^{-6}\ \text{sec}^{-1}$ obtained from the result in Fig. 5.

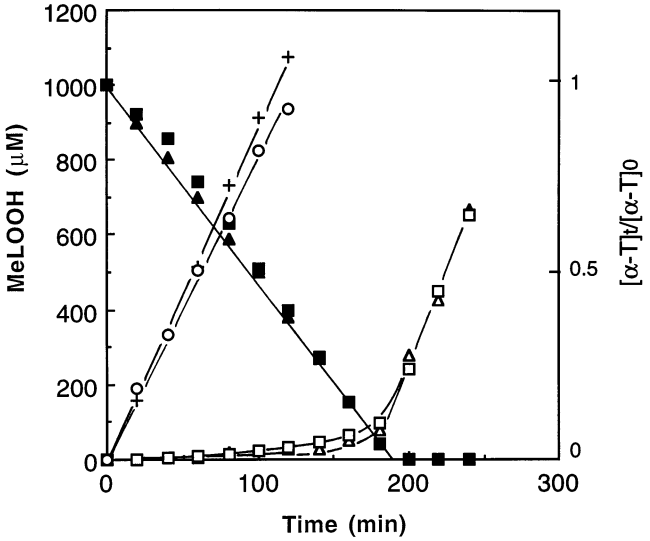



**FIG. 5. Inhibition of oxidation of methyl linoleate by α-tocopherol and ASU. Methyl linoleate (151 mM) was incubated with AMVN (0.20 mM) in acetonitrile at 37° in air (1) without antioxidant (+) or with (2) 5 μM α-tocopherol (△, ▲), (3) 10 μM ASU (○) or (4) 5 μM α-tocopherol and 10 μM ASU (□, ■), and the formation of methyl linoleate hydroperoxide (open marks) and consumption of α-tocopherol (solid marks) were followed. [α-T]*t* and [α-T]o show the concentrations of α-tocopherol at time *t* and zero, respectively.**

### *Inhibition of Oxidation of Soybean PC Liposomal Membranes by ASU*

The oxidation of soybean PC liposomal membranes induced by AMVN proceeds by a free radical-mediated chain mechanism to give PC hydroperoxides as the major primary product [15]. It was found as observed previously [14, 15] that, in the absence of antioxidant, PC hydroperoxides were formed at a constant rate without any induction period (data not shown). The rate of PC hydroperoxide formation was obtained as $4.17 \times 10^{-8}$ M/sec and that of α-tocopherol consumption was calculated as $3.94 \times 10^{-10}$ M/sec, which gives the rate of chain initiation as $7.88 \times 10^{-10}$ M/sec. Then, the kinetic chain length is obtained as $4.17 \times 10^{-8}/7.88 \times 10^{-10} = 53$, indicating that the oxidation of PC in liposomal membranes also proceeds by a chain mechanism. ASU incorporated into PC liposomal membranes suppressed the oxidation in a concentration-dependent manner (data not shown). When 20 μM ASU was added to PC liposomal membranes (the PC/ASU molar ratio being 255/1), the rate of oxidation decreased to 15% of control rate without ASU, whereas the same concentration of free cholesterol exerted little antioxidant effect (data not shown).

The effect of initiating species on the antioxidant activity of ASU against the oxidation of soybean PC liposomal membranes was studied. Hydrophilic radical initiator AAPH and SIN-1 were used. Upon its thermal decomposition in air, AAPH gives peroxyl radicals in the aqueous phase, which attack lipids from outside membranes, while SIN-1 gives nitric oxide and superoxide concomitantly, which rapidly combine to give peroxynitrite and initiate lipid peroxidation [19]. Figure 6 shows the results of oxidations of soybean PC unilamellar vesicles containing 20 μM ASU initiated either by AMVN, AAPH or SIN-1. In every case, PC hydroperoxides were accumulated with time and ASU suppressed the oxidation. The ratio of the rate of oxidation in the presence of ASU ($R_{ASU}$) to that in its absence ($R_O$) gives the relative antioxidant efficacy. As

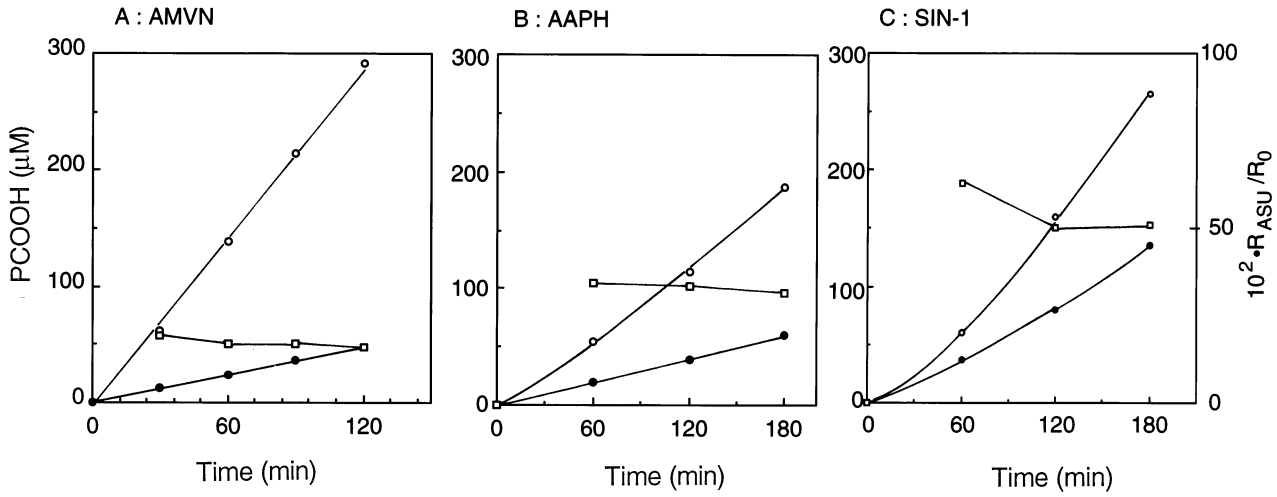



**FIG. 6. Inhibition by ASU of oxidation of soybean PC liposomal membranes induced by either AMVN, AAPH or SIN-1. Soybean PC unilamellar vesicles without or with 20 μM ASU were incubated at 37° in air in the presence of initiator, and the formation of PC hydroperoxides (PCOOH) was followed with HPLC as described in Materials and Methods. AMVN was incorporated into the liposomal membranes, while AAPH and SIN-1 were added as aqueous solution after preparation of vesicles. A:PC = 5.1 mM; AMVN = 1.0 mM; B:PC = 2.8 mM; AAPH = 1.0 mM; C:PC = 2.8 mM; SIN-1 = 20 mM. ●: PCOOH with 20 μM ASU; ○: PCOOH without ASU; □: percentage of inhibition, that is, $10^2 \cdot R_{ASU/R_0}$, where $R_{ASU}$ and $R_0$ are the rate of PCOOH formation with and without ASU, respectively.**

shown in Fig. 6, this ratio was smallest for AMVN-induced oxidation and largest for SIN-1-induced oxidation.

### *Inhibition of Lipid Peroxidation by a Combination of ASU and α-Tocopherol*

The above results suggest that ASU exerts only a weak antioxidant effect against lipid peroxidation in organic solution but substantial antioxidant effect in liposomal membranes. The action of ASU against lipid peroxidation was studied in the presence of α-tocopherol, one of the most abundant and potent lipophilic antioxidants *in vivo*.

As observed in Fig. 5, ASU had little additive effect on the formation of methyl linoleate hydroperoxide and on the consumption of α-tocopherol in the oxidation of methyl linoleate in acetonitrile solution. On the other hand, ASU was found to spare α-tocopherol remarkably in the oxidation of PC liposomal membranes. Figure 7 shows the results of oxidation of soybean PC liposomal membranes induced by AMVN in the absence of any antioxidant and in the presence of either α-tocopherol or ASU or their combination. It shows that individually α-tocopherol inhibited oxidation efficiently, while ASU only retarded oxidation, but that when both antioxidants were present in the membranes simultaneously, ASU spared α-tocopherol quite remarkably and exerted a synergistic effect. ASU spared α-tocopherol in a concentration-dependent manner (data not shown). It was observed by an ESR study that ASU did not reduce α-tocopheroxyl radical (data not shown).

## DISCUSSION

The results obtained in the present study clearly show that ASU is not a potent radical scavenger and that it does not act as an efficient antioxidant in organic solution. However, it does exert substantial antioxidant activity against lipid peroxidation when incorporated into membranes. Its sparing effect of α-tocopherol and its synergistic effect in the membranes are particularly noteworthy.

Most of the conventional antioxidants have reactive phenolic hydrogens or aromatic amines. On the other hand, ASU (Fig. 1) does not have an easily predictable

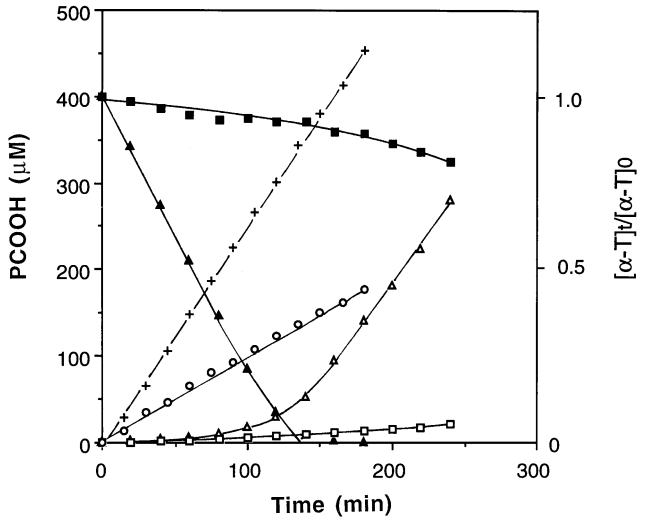



**FIG. 7. Inhibition of oxidation of soybean PC liposomal membranes by ASU, α-tocopherol and their combination. Soybean PC (5.15 mM) multilamellar liposomal membranes were oxidized with AMVN (1.0 mM), and PC hydroperoxide (PCOOH) was measured in the absence (+) and presence of either 10 μM ASU (○) or 3.0 μM α-tocopherol (△) or their combination (□) at 37° in air. The remaining α-tocopherol ([αT]t/[αT]0) was also followed in the absence (▲) and presence (■) of ASU with HPLC as described in the "Materials and Methods" section.**

reactive hydrogen. Consistent with our data, it is therefore conceivable that ASU reacts only slowly with galvinoxyl radical (Fig. 2) or with peroxyl radical (Fig. 3), and that it does not act as a potent antioxidant against lipid peroxidation in solution (Fig. 4). Furthermore, ASU exerted little effect on the action of α-tocopherol in scavenging peroxyl radical (Fig. 3) or in the oxidation of methyl linoleate in acetonitrile solution (Fig. 5). The rates of consumption of ASU and α-tocopherol during the incubation with AMVN in solution also suggest that the inherent chemical reactivity of ASU toward peroxyl radical is poor. As shown above (Fig. 3), the rate of consumption of α-tocopherol was independent of its concentration but was directly proportional to the rate of radical flux. On the other hand, the rate of ASU consumption was dependent on its concentration as well as the concentration of radical initiator. Furthermore, the rate of ASU consumption was much smaller than that of α-tocopherol. These data together show that ASU scavenges only a small fraction of radicals and that its low reactivity does not allow it to compete with other reactions such as the attack of peroxyl radicals upon lipid and bimolecular terminating interactions of peroxyl radicals.

On the contrary, ASU suppressed the oxidation of soybean PC liposomal membranes with moderate activity. Under the same conditions, free cholesterol had no effect. What is more striking is that ASU spared α-tocopherol markedly and, when used with α-tocopherol, prolonged the induction period. The underlying mechanism is not clear at present. McCall and Epps emphasize that the physical effects, such as disorganization of the lipid chains, changes in fluidity, and alteration of membrane thickness are important in determining antioxidant efficacy [3].

Braughler and Pregenzer [6] have studied the effect of ASU on the oxidation of linoleic acid in methanol induced by AMVN and also the interaction of ASU with 2,6-*di-tert*-butyl-5-(4-methoxylphenyl)phenoxyl radical. They have already reported that ASU acted as a weak antioxidant. They measured the rate constants for interaction of ASU with linoleic acid peroxyl radical and the above phenoxyl radical and obtained as $9.4 \times 10^2 \sim 1.8 \times 10^3$ $M^{-1}$ $sec^{-1}$ and $3.3 \times 10^{-6}$ $M^{-1}$ $sec^{-1}$, respectively. The rate constant for scavenging peroxyl radical by the antioxidant can be calculated from the rate of oxidation in the presence of antioxidant and the induction period [6, 17, 18]. However, if the antioxidant does not scavenge peroxyl radicals rapidly enough to inhibit the bimolecular terminating reactions of peroxyls and if it does not give a clear induction period, the above rate constant for inhibition of oxidation cannot be measured accurately. The rate constant ($3.3 \times 10^{-6}$ $M^{-1}$ $sec^{-1}$) for the interaction of ASU with the above phenoxyl radical reported by Braughler and Pregenzer [6] appears to be too small compared with our value for similar phenoxyl radical as well as the rate of chain initiation calculated from Eqn 5 ($4.0 \times 10^3$ $M^{-1}$ $sec^{-1}$) obtained by them [6].

In conclusion, ASU is chemically a weak radical scavenger which acts as a poor antioxidant in solution. However, it exerts a substantial antioxidant effect in membranes and especially as a synergist for α-tocopherol. As stated above, ASU did not regenerate α-tocopherol efficiently by reducing α-tocopheroxyl radical. Thus, the sparing of α-tocopherol by ASU cannot be interpreted by this reduction reaction. The detailed mechanism for such a synergistic effect is not clear at present and is a subject of future study.

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