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Design of Unsymmetrical Azo Initiators to Increase Radical Generation Efficiency In Low-Density Lipoproteins

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Lipid peroxidation studies often employ the use of azo initiators to produce a slow, steady source of free radicals, but the lack of initiators capable of efficiently generating radicals in lipid regions has created persistent problems in these investigations. For example, experiments with symmetrical lipophilic or symmetical hydrophilic azo initiators increasingly suggest that their initiation mechanisms in low-density lipoproteins (LDL) rely upon the presence of α-tocopherol to mediate peroxidation. We report here the synthesis and study of the new unsymmetrical azo compounds **SA-1, SA-2, C-16, C-12,** and **C-8** that decompose over a range of convenient temperatures and improve radical generation efficiency and access to lipid compartments. The half-life for decomposition ($\tau_{1/2}$) of the unsymmetrical initiators at 37°C in methanol covered a range of 121 hours for **SA-1**, 77 hours for **SA-2**, and ≈ 25 hours for the series **C-16, C-12**, and **C-8**. Agarose gel electrophoresis of LDL incubated with these unsymmetrical initiators supports the conclusion that the initiators associate with lipoprotein without disrupting integrity of the particle. The unsymmetical initiator **C-8** when compared to symmetical hydrophilic initiator **C-0** is capable of providing increased peroxidation of LDL, as monitored by formation of cholesteryl linoleate oxidation products and consumption of α-tocopherol. Efficiency of radical generation in lipophilic and hydrophilic compartments was found to be represented with the use of the radical scavenger combination α-tocopherol and uric acid, but not with the use of N,N′-Diphenyl-*p*-phenylenediamine (DPPD) and uric acid. These unsymmetrical initiators, when compared to the widely used symmetrical azo initiators, provide an advantage of free radical production, lipophilic access, and constant radical generation in the investigation of lipid peroxidation in low-density lipoproteins.

Keywords: Initiators/Radical Pair/LDL/Amphiphilic/Peroxidation

DEDICATION: This contribution is dedicated to Professor Etsuo Niki on the occasion of his 60th birthday

INTRODUCTION

Low-density lipoprotein (LDL) is the primary cholesterol transport protein in human blood plasma. Oxidatively modified LDL (oxLDL), including lipid hydroperoxides formed in the oxLDL core, have been found to posses athero-

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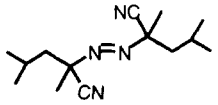
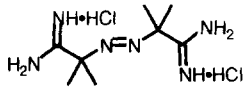
genic properties.[1] Initiation and antioxidant defenses are of key importance to the mechanism(s) of lipid peroxidation, and α-tocopherol (α-TOH) has attracted attention as a peroxidation chain breaker since it is a naturally abundant and potent lipophilic antioxidant.[2] Problems have persisted in investigating the antioxidant mechanism of α-TOH in LDL.[3,4] Initiation of lipid peroxidation in these studies is of key importance. If details of α-TOH antioxidant mechanism are to be determined, it is desirable to efficiently initiate lipid peroxidation under conditions that do not involve tocopherol mediated peroxidation.

Two commonly used methods to initiate LDL oxidation are incubation of LDL with Cu^{2+} solution or incubation with thermally labile azo initiators.[5] The copper-mediated oxidation mechanism of LDL is poorly understood and difficult to control. However, recent evidence supports participation of α-TOH in a Cu^{2+}/Cu^{+} redox cycling that leads to generation of lipid hydroperoxides.[6] Thus, α-TOH is involved in promoting the initiating events of lipid oxidation. Azo initiators, such as **AMVN** (2,2′-Azobis(2,4-dimethylvaleronitrile)) and **AAPH** (2,2′-Azobis(2-amidinopropane)), are often employed to produce a slow, steady source of free radicals by known chemical decomposition mechanisms. Unfortunately, these azo initiators lack the capability of efficiently generating radicals in lipid regions of LDL.[3,7] For example, the increased viscosity of the lipid environment and the macromolecular "cage" nature of a lipoprotein significantly prevents the cage escape of the lipophilic radicals formed in the decomposition of **AMVN**. Studies of the peroxidation of LDL include the use of high concentrations of **AMVN** to produce initiation at a reasonable rate and it has been reported that only 4–5% of radicals generated from **AMVN** actually escape the cage to initiate peroxidation in LDL.[8] Such high initiator concentrations may compromise the integrity of the lipoprotein.



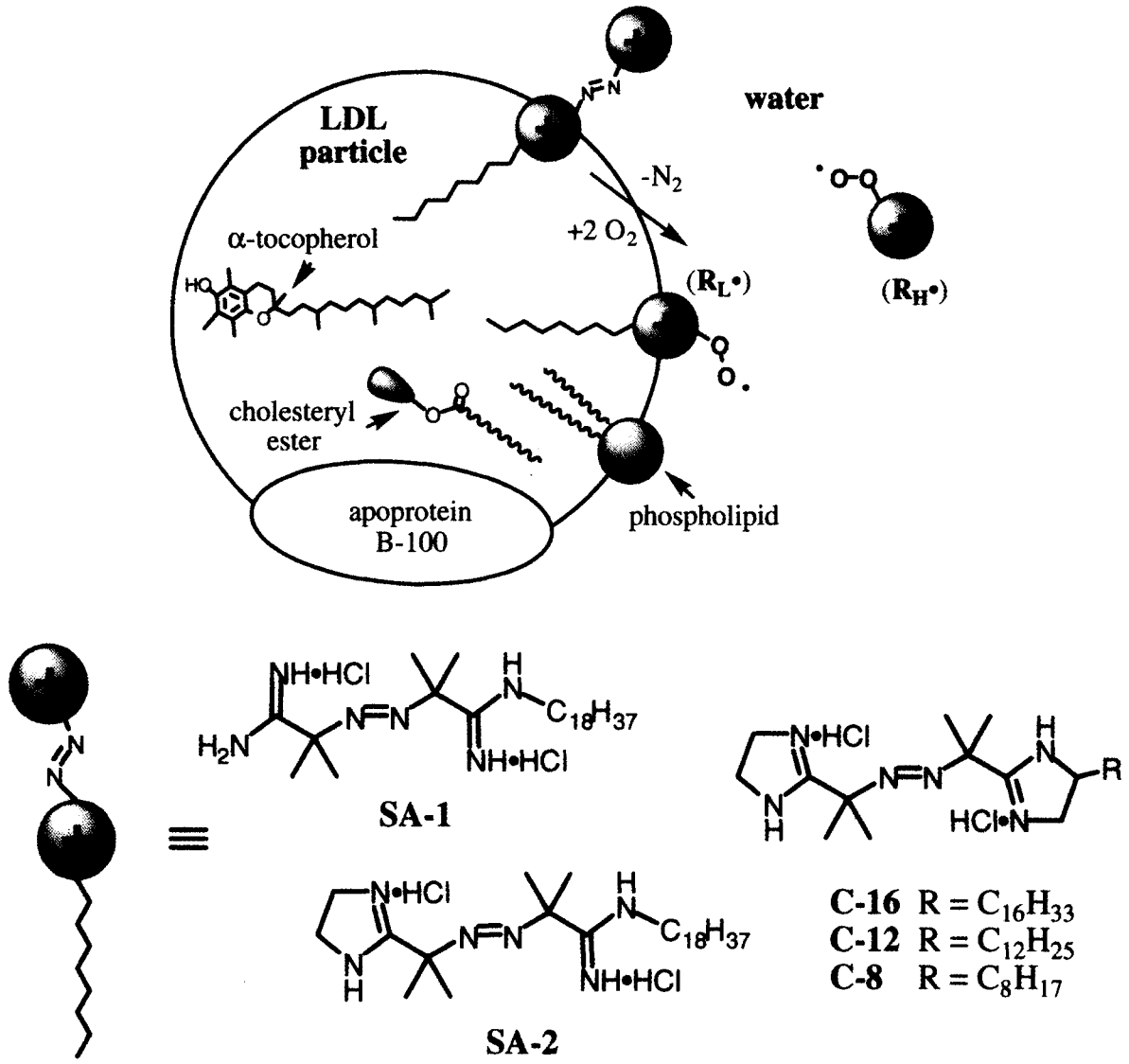
AMVN



AAPH

While the hydrophilic radicals derived from **AAPH** exhibit efficient cage escape in water, the transfer of these radicals into the lipid region of a molecular aggregate is dependent upon some transport mechanism(s). In fact, it has been demonstrated that peroxyl radicals derived from **AAPH** in the aqueous phase do not initiate low-density lipoprotein (LDL) oxidation in the absence of α-TOH.[7] Indeed, there is an increasing body of evidence suggesting that subtle changes in the initiator source, rate of decomposition, or location of radical formation can influence mechanistic pathways of peroxidation.[3]

Given the limitations of existing initiators, there is a need for new compounds that efficiently generate radicals in lipoproteins and other lipid aggregates such as micelles and liposomes. We report here the synthesis and study of the new unsymmetrical azo compounds **SA-1, SA-2, C-16, C-12**, and **C-8** that decompose over a range of convenient temperatures and improve radical access to lipid compartments.[9] These new initiators generate one amphiphilic radical and one hydrophilic radical in the geminate radical pair. The results of this study suggest that these amphiphilic initiators associate with lipoproteins and generate one amphiphilic peroxyl radical, $R_L•$, that partitions in the lipid aggregate while the hydrophilic peroxyl radical, $R_H•$, escapes to the aqueous environment, Scheme 1. The use of the "hydrophobic effect" to separate the geminate radical pair apparently overcomes the difficulties of cage escape and lipid compartment access that plagues the use of symmetrical initiators **AMVN** and **AAPH**.



SCHEME 1

MATERIALS AND METHODS

Materials

Phosphate buffered saline (PBS pH 7.4, 10 mM) was stored over Chelex-100 for at least 24 h to remove trace metal contaminants. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and used without further purification, unless otherwise noted. Plant PC (95%) was obtained from Avanti Polar Lipids and used without further purification. The antioxidants, α-tocopherol and δ-tocopherol, were purchased from Sigma and purified by preparative HPLC 0.5 % 2-propanol in hexanes with UV detection at 292 nm. Solvents were HPLC quality and purchased from commercial sources.

Reactions were carried out in flame dried glassware under an argon atmosphere. Flash chromatography columns were run on silica gel 60 in specified solvent conditions. Thin layer chromatography was performed using 0.2 mm layer thickness silica gel coated plastic (60 F_{254}, EM Industries). Analytical HPLC was carried out on a Waters Model 610 or 600E HPLC with a Waters Model 486 variable wavelength detector, a Hewlett Packard Dual Multi-wavelength 1050 UV detector, or a Bioanalytical Systems LC-4 electrochemical detector connected to a Hewlett Packard 3396 Series III integrator. A single Whatman C-18 column (4.6 mm × 25 cm) was used for reverse phase conditions, and two tandem Beckman 5 μM Ultrasphere columns (4.6 mm × 25 cm) were used for normal phase conditions. Preparative HPLC was conducted on a Waters Model 600E HPLC instrument, with a Waters Model 481 variable wavelength detector. A Rainin Dynamax-60A Si column (21.4 mm × 25 cm) was used with a flow rate of 10.0 mL/min.

^{1}H NMR and ^{13}C NMR were recorded on a General Electric Model QE-300. Chemical shifts are reported in ppm (δ) with respect to TMS (δ=0.0 ppm ^{1}H, and ^{13}C). A Hewlett Packard Model 8452A Diode Array Spectrophotometer with quartz 1 mL teflon stoppered cells was used to determine optical densities.

Initiator Synthesis

2,2′-Azobis(2-amidinopropane)[2-(N-stearyl) amidinopropane] Dihydrochloride, (SA-1).[10,11]

In a 1000 mL three neck round bottom a sample of **AAPH** (22.5 g, 0.083 mol) was dissolved in anhydrous MeOH 800 mL and treated with sodium methoxide (2.6 g, 0.047 mol) and stearyl amine (11.0 g, 0.040 mol) at rt. One neck of the flask was connected to an Argon line that had a hollow glass tube long enough to bubble Ar directly into the solvent. The second neck served as the exhaust over the surface of the solvent. The exhaust line was submersed into an Erlenmeyer flask of water containing a small amount of the indicator methyl red. The remaining neck of the flask was stoppered after the addition of the reactants. The reaction was followed to completion by titration of the trapped NH_3 in water. The solvent was evaporated and the precipitated product filtered and washed with MeOH. The filtrate was then saturated with HCl gas, stirred for 1 h, evaporated, and fractionally recrystallized from MeOH/Et_2O to give a white powder 4.7 g, 22% yield, mp 118°C decomposition. ^{1}H-NMR (300 MHz, CD_3OD): δ 3.47 (t, 2 H); 1.7 (m, 2 H); 1.55 (d, 12 H); 1.3 (m, 30 H); 0.9 (t, 3 H). MS (FAB): calcd. for $[C_{26}H_{55}N_6]^+$ 451.4, found 451.4.

2,2′-Azobis[2-(2-imidazolin-2-yl)propane] [2-(N-stearyl)amidinopropane] Dihydrochloride, (SA-2).[10,11]

In a three-necked flask was dissolved azo initiator **SA-1** (2.5 g, 4.8 mmol) and sodium methoxide (0.23 g, 4.3 mmol) in 25 mL of anhydrous MeOH. After the mixture was stirred for 30 min, ethylene diamine (0.48 mL, 7.1 mmol) was added as argon bubbled through the solution and exhausted into a water trap containing methyl red indicator. The reaction was followed to completion by titration of trapped NH_3. The solvent was evaporated and the resulting solid was washed with MeOH. The filtrate was then saturated with HCl gas, stirred for 1 h, evaporated, and fractionally recrystallized from MeOH/Et_2O to give a white powder 0.5 g, 19% yield, mp 124–130°C decomposition. ^{1}H-NMR (300 MHz, CD_3OD): δ 4.05 (s, 4 H); 3.47 (t, 2 H); 1.7 (m, 2 H); 1.55 (d, 12 H); 1.3 (m, 30 H); 0.9 (t, 3 H). MS (FAB): calcd. for $[C_{28}H_{57}N_6]^+$ 477.5, found 477.44.

2,2′-Azo[2-(2-imidiazolin-2-yl)-propane]-{2-[2-(4-n-octyl)imidazolin-2-yl]-propane} dihydrochloride, C-8
2,2′-Azo[2-(2-imidiazolin-2-yl)-propane]-{2-2-(4-n-dodecyl)imidazolin-2-yl]-propane} dihydrochloride, C-12.
2,2′-Azo[2-(2-imidiazolin-2-yl)-propane]-{2-[2-(4-n-hexadecyl)imidazolin-2-yl]-propane} dihydrochloride, C-16

The synthesis for these unsymmetrical initiators has been previously described in detail.[9]

Initiator Decomposition

The initiator rate of decomposition, k_d, values were measured by following the loss of azo chromophore at 366 nm with a UV/VIS spectrophotometer.[10,12] The k_d for the initiators **AAPH, SA-1, SA-2, C-16, C-12, C-8** and **C-0** were measured in MeOH containing 0.2% concentrated HCl. MeOH experiments were conducted at three temperatures 50°C, 58°C, and 65°C over the course of several half-lives, then extrapolated to calculated k_d at 37°C. Values for k_d in methanol at 37°C were **AAPH** (1.8×10^{-6}), **SA-1** (1.6×10^{-6}), **SA-2** (2.5×10^{-6}) **C-16**(7.4×10^{-6}), **C-12** (8.0×10^{-6}), **C-8** (8.3×10^{-6}), and **C-0** (7.1×10^{-6}). Values for half-life of decomposition were calculated by the following expression: $\tau_{1/2} = \ln 2/k_d$.

LDL Isolation[13]

Whole blood from fasting, normolipidemic healthy subjects was collected in a 450 mL ACD blood collection bag (Baxter) containing the following: 1.61 g dextrose; 1.66 g of sodium citrate dihydrate; 188 mg of anhydrous citric acid; and 140 mg of monobasic sodium phosphate monohydrate. The bag containing blood was centrifuged at 4200 rpm for 10 min at 22°C and the plasma was collected. The low-density lipoprotein (LDL) was isolated from plasma over 15 h by density gradient sequential ultracentrifugation at 14°C using a Beckman Optima LE-80K centrifuge and a Ti 70 rotor. Each spin was preformed at 504,000g for 5.5 h. Lipoproteins were dialyzed extensively against 10 mM PBS (or desalted by passage through two consecutive PD-10 columns),[7] sterilized by passage through a Millex-HA 0.45 μM filter, and stored at 4°C under argon. Protein concentrations of the LDL preparations were determined by the method of Lowry.[14] LDL isolation was confirmed with the use of SDS PAGE separation of associated apoproteins and Beckman LIPO Gel electrophoresis of intact lipoproteins.[15,16]

Gel Electrophoresis

Agarose gel electrophoresis was preformed on either a Ciba-Corning electrophoresis system or a Beckman Instruments Paragon LipoGel system.[16] Lipoprotein concentrations were adjusted to 0.75 mg of protein/mL with PBS and allowed to equilibrate to 37°C for 5 min in a 5 mL flask. To the stirred solution was added initiator in MeOH ≤ 3% v/v (except **C-0** and **AAPH** were in PBS) to give a final **SA-1, SA-2, C-16, C-12**, or **C-8** concentration of 0.125 mM – 0.5 mM. Following addition of initiator (time zero) aliquots for gels were removed after at least 15 minute incubation at 37°C and placed on ice until loaded onto the gel. Up to 3 μL of a LDL sample was loaded into each lane. The gel was then run according to directions, except the electrophoresis was completed at 120 V and run for 45 minutes. The gels were stained with either Fat Red 7B (Corning system) or Sudan Black B (Beckman LipoGel).

LDL Oxidation

Lipoprotein concentrations were adjusted to 0.75 mg of protein/mL with PBS containing 60 μM UA, and allowed to equilibrate to 37°C for 5 min in a 5 mL flask. To the stirred solution was added initiator 0.065 M in MeOH (except **C-0** was in PBS) to give a **C-8**, or **C-0** concentration of 0.5 mM. Following addition of initiator (time zero) aliquots for α-TOH, UA, and cholesteryl linoleate oxidation products were removed at various intervals, and treated as described below.

Aliquots were taken for α-TOH analysis 200 μL, UA analysis 50 μL, and cholesteryl linoleate product analysis 200 μL and placed directly on ice. To the 200 μL α-TOH aliquot was added BHT 30 μL (3 mM in MeOH) and a known amount of the internal standard, δ-TOH (36.5 μM in MeOH). Extraction of all the aliquots was performed with ice-cold MeOH 1 mL and ice-cold hexanes 5 mL in sequence, vortexed vig-

orously after the addition of each solvent (ca. 20 s) and then centrifuged at 1700 rpm for approximately 1 min with the use of an Adams analytical centrifuge.[17] The hexane phase was removed by pipet and concentrated under argon, and then stored at –78°C until analysis. The UA aliquots were simply stored at -78°C until analysis by HPLC. To the cholesteryl linoleate 200 μL aliquot was added BHT 30 μL, the internal standard 13-hydroxyoctadecane,*cis*-9,*trans*-11-dienoate, and the hydroperoxides were reduced to alcohols with the addition of triphenylphosphine 30 μL (25 mM in MeOH). These reduced cholesteryl linoleate oxidation samples were then extracted the same as the α-TOH samples.[17]

HPLC Analysis

Samples of α-TOH were re-suspended in 0.5–1.5 mL of HPLC solvent and analyzed by HPLC with electrochemical detection.[18] The mobile phase was composed of methanol/reagent alcohol 60:40 and lithium perchlorate 20 mM. Electrochemical detector settings were +0.60 – +0.65 V with a range setting of 0.5 μA. Flow rate was 1.0 or 1.5 mL/min which provided good separation of δ-TOH and α-TOH within 7 minutes. External standard curves were created by injecting 5–25 μL of working standard (α-TOH 9.0 μM and δ-TOH 12.0 μM in mobile phase) intermittently between sample injections of 15–75 μL. Columns were flushed with MeOH/H_2O 50:50 after each use.

Uric acid standards were prepared from a stock solution (1.25 mM UA in PBS pH 7.4). The stock solution was diluted with mobile phase to produce a working standard 2.25 μM UA. The mobile phase consisted of filtered and degassed sodium acetate buffer 40 mM, pH 4.75, Na_2EDTA 0.54 mM, dodecyltriethylammonium phosphate 1.5 mM (Regis Chemical Co., Morton Grove, IL), and 7.5 % MeOH. Electrochemical detector settings were +0.60 – +0.65 V with a range setting of 50 nA and the flow rate was 1.5 mL/min. The standard curves were generated by intermittently injecting 5–25 μL working standard between sample injections. Samples of UA were diluted with 1.0–2.5 mL of mobile phase and 15 μL was injected. Columns were flushed with MeOH/H_2O 50:50 after each use.[19]

Cholesteryl linoleate reduced oxidation products were analyzed as described previously.[17] Briefly, the evaporated samples were resuspended in mobile phase consisting of 0.5% 2-propanol in hexanes. Injection onto two tandem Beckman 5 μm Ultrasphere columns with a flow rate of 1 mL/min provided separation of the four regioisomeric cholesteryl linoleate alcohol products (reduced from the hydroperoxides).

Scavenging Efficiencies

The generation of free radicals in micelles was carried out at 37°C in air using, Triton X-100 0.1 M. In a typical experiment methyl linoleate 8.3 μL (neat) and either N,N′-Diphenyl-*p*-phenylenediamine (DPPD) 50 μL (15 mM in MeOH) or α-TOH 150 μL (~4.5 mM in MeOH) were mixed in a 5 mL flask. Next, **C-8** 150 μL (0.065 M in MeOH) was added, swirled, and followed by addition of surfactant (0.125 M in PBS pH 7.4). (Note that in the case of water soluble **C-0**, the initiator was added in PBS pH 7.4 after the addition of surfactant, and pure MeOH 150 μL was added in place of the initiator solution in methanol.) Finally, uric acid 600 μL (1.25 mM in PBS pH 7.4) and PBS 100 μL was added bringing the total volume to 5 mL. The dispersions were thoroughly mixed by vortexing 2 minutes. Experiments containing DPPD as the lipophilic antioxidant did not require extraction of the micellular mixture as required with α-TOH. The loss of DPPD was monitored by directly measuring the increase in absorbance at 440 nm, which corresponds to formation of N,N′-diphenyl-*p*-benzoquinone diimine (DPBQ) after each

molecule of DPPD reacts with two molecules of peroxyl radicals.[4]

The generation of free radicals in MLV liposomal membranes was carried out at 37°C in air using plant phosphatidyl choline (PC) 10.3 mM. PC and lipid soluble components (α-TOH or DPPD and initiator, except for **C-0**) were dissolved in MeOH and placed in a 5 mL flask. The MeOH was evaporated in vacuo to obtain a thin film. PBS buffer 4.4 mL and UA 0.6 mL (1.25 mM in PBS) were added and the PC film was slowly peeled off by shaking and then vortexing to obtain white, milky liposome suspensions.[20] Aliquots for α-TOH and UA were removed at various intervals, and treated as described above. Aliquots for DPPD were also removed and extracted in the same way as α-TOH samples before resuspension of the evaporated sample in MeOH and analysis by UV at 440 nm.

RESULTS AND DISCUSSION

The new initiators reported here were prepared by displacement of ammonia from the amidine functional group of the parent compound, **AAPH**.[10] This commercially available parent compound has been widely used in LDL oxidations and it decomposes at convenient temperatures to generate water soluble peroxyl radicals.[8] Addition of an alkyl chain by reaction of **AAPH** with stearyl amine provided the unsymmetrical azo initiator **SA-1** (stearyl amidine-1). Synthesis of **SA-2** was accomplished by exchange of ethylene diamine on the unsubstituted amidine of **SA-1**. These unsymmetrical initiators were purified by crystallization from methanol-ether solvent mixtures. In short, the synthesis for **C-16, C-12**, and **C-8** involves a similar exchange of ethylene diamine on to **AAPH**-followed by displacement of the remaining acyclic amidine with a 1,2-diamine.[9]

Kinetics for initiator decomposition were determined by measurement of the disappearance of the azo absorption in the UV at 366 nm.[10,12] Experiments with **C-0** in methanol solvent gave good first order kinetics over three half-lives. Rate constants for **C-0** decomposition, k_d, were determined at 65°C, 2.3×10^{-4} s^{-1}, $\tau_{1/2} = 0.8$ hr; 58°C, 1.1×10^{-4} s^{-1}, $\tau_{1/2} = 1.8$ hr; and 50°C, 3.9×10^{-5} s^{-1}, $\tau_{1/2} = 5.0$ hr. Extrapolation of these rate constants to 37°C gives a value of $k_d = 7.1 \times 10^{-6}$ s^{-1}, $\tau_{1/2} = 27.1$ hr at this temperature. Rate constants were also determined for **AAPH, SA-1, SA-2, C-16, C-12**, and **C-8**.

The variation of initiator structure from acyclic amidine, **SA-1**, to the cyclic imidazolines, **SA-2** and **C-16**, provides a range of half-life for decomposition ($\tau_{1/2}$). Cyclization to the imidazoline counterpart was previously shown to have a significant effect on the rate of decomposition of azo initiators.[12] For example, **C-0** (similar to **C-16** structure except symmetrical with R = H) has a half-life for decomposition approximately four times shorter than **AAPH**. Figure 1 presents values for the determined $\tau_{1/2}$ of each initiator in methanol at 37°C. Comparison of the initiators to **SA-1**, shows that inclusion of one imidazoline, **SA-2**, provides a $\tau_{1/2}$ 1.6 times shorter, and introduction of two imidazolines, **C-16**, gives a $\tau_{1/2}$ 4.7 times shorter than **SA-1**. Thus, the series of unsymmetrical azo initiators presented here provide a range of decomposition rates that can be chosen for the specific requirements of peroxyl radical production. For slow radical flux or experiments at temperatures above 37°C the initiator **SA-1** ($\tau_{1/2} = 121$ hours) would be ideal. However, if high radical flux is necessary the unsymmetrical initiator series **C-16, C-12**, and **C-8** ($\tau_{1/2} \approx 24$ hours) generate almost five times as many peroxyl radicals under the same conditions.

The unsymmetrical azo initiators being amphiphilic interact with lipoprotein particles differently than the symmetric water soluble initiators **AAPH**, and **C-0**. Symmetrical water soluble initiators largely decompose in aqueous regions and produce peroxyl radicals that are also hydrophilic. The initiating capability of these hydrophilic peroxyl radicals in LDL oxida-

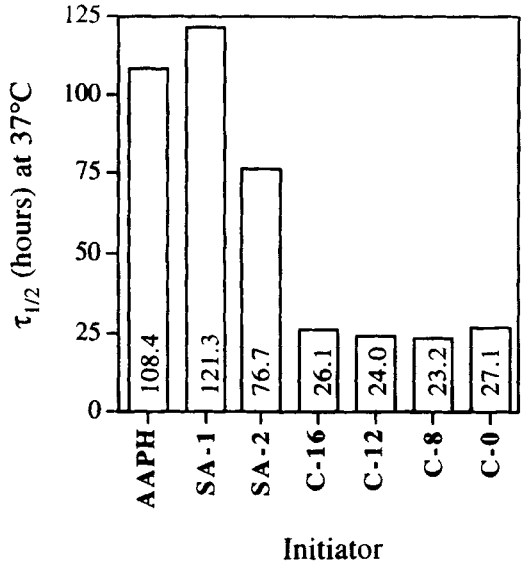


FIGURE 1 Half-life for Decomposition of Azo Initiators in Methanol. Rate constants for initiator decomposition in methanol were determined at 65°C, 58°C, and 50°C by disappearance of the azo absorption in the UV at 366 nm. Extrapolation of these rate constants to 37°C gives values for kd and $\tau_{1/2}$ at this temperature

tions has been reported to be dependent upon α-TOH concentrations within the LDL particle, suggesting that **AAPH** derived peroxyl radicals have very little, if any, direct access to the lipids in the core of a LDL.[3] On the other hand, amphiphilic unsymmetrical initiators likely associate with the LDL phospholipids, which are also amphiphilic. However, the surfactant character of the unsymmetrical initiators could not be overlooked. Addition of too much unsymmetrical initiator to a lipoprotein solution would certainly compromise integrity of the LDL particles. Therefore, additions of any unsymmetrical initiators to LDL solutions were kept to a minimum (≤ 0.5 mM initiator with LDL concentrations at 0.75 mg protein/mL). Decomposition of the initiators **SA-1, SA-2, C-16, C-12**, and **C-8** generate one hydrophilic peroxyl radical ($\mathbf{R_H}$•) and one amphiphilic peroxyl radical ($\mathbf{R_L}$•). Ideally, the $\mathbf{R_L}$• peroxyl would have enough lipophilic character to allow access to lipids in the core and monolayer regions of a LDL.

Agarose gel electrophoresis supports the conclusion that the unsymmetrical azo initiators associate with LDL without destroying particle integrity. LDL solutions were incubated with initiator for 15 min at 37°C and loaded onto an agarose on Mylar gel for lipoprotein analysis.[16] In Figure 2, electrophoresis shows that LDL treated with unsymmetrical azo initiators **SA-1, SA-2, C-16**, and **C-12** yields lipoprotein bands that migrate more toward the negative electrode than untreated LDL. Association of the positively charged unsymmetrical azo initiators would logically alter the overall electronic character of a lipoprotein particle, thus more attraction toward the negative electrode is reasonable if LDL contains the charged initiators. The results suggest a concentration dependence, as noted in Figure 2 panel A with the incremental migration of **SA-1** at 0.125 mM, 0.25 mM, 0.5 mM, and 1.0 mM. Between the **SA-1** concentrations of 0.5 mM and 1.0 mM there was a significant change in migration. This result could be interpreted in two ways:[21] (1) The **SA-1** initiator at higher concentrations compromises the integrity of the LDL particle. (2) The **SA-1** initiator, which has a poor solubility in buffer near 1.0 mM, may precipitate from the solution before incorporating into the LDL. The **C-16, C-12**, and **C-8**series of initiators, Figure 2 panel B, also shows an interesting trend. Longer alkyl chains provide a greater shift of the LDL band to the negative electrode. This effect is likely a result of how much initiator associates with the LDL particle. For example, the **C-8** initiator has greater water solubility than **C-16**; accordingly, **C-8** may partition less into the phospholipid monolayer and more into the aqueous phase. Incubation of LDL with the symmetrical water soluble initiators, **AAPH** (Figure 3, lane 1) and **C-0** (data not shown) shows no change in electrophoretic mobility. Although **C-8** does not closely associate with LDL, it still generates an amphiphilic peroxyl

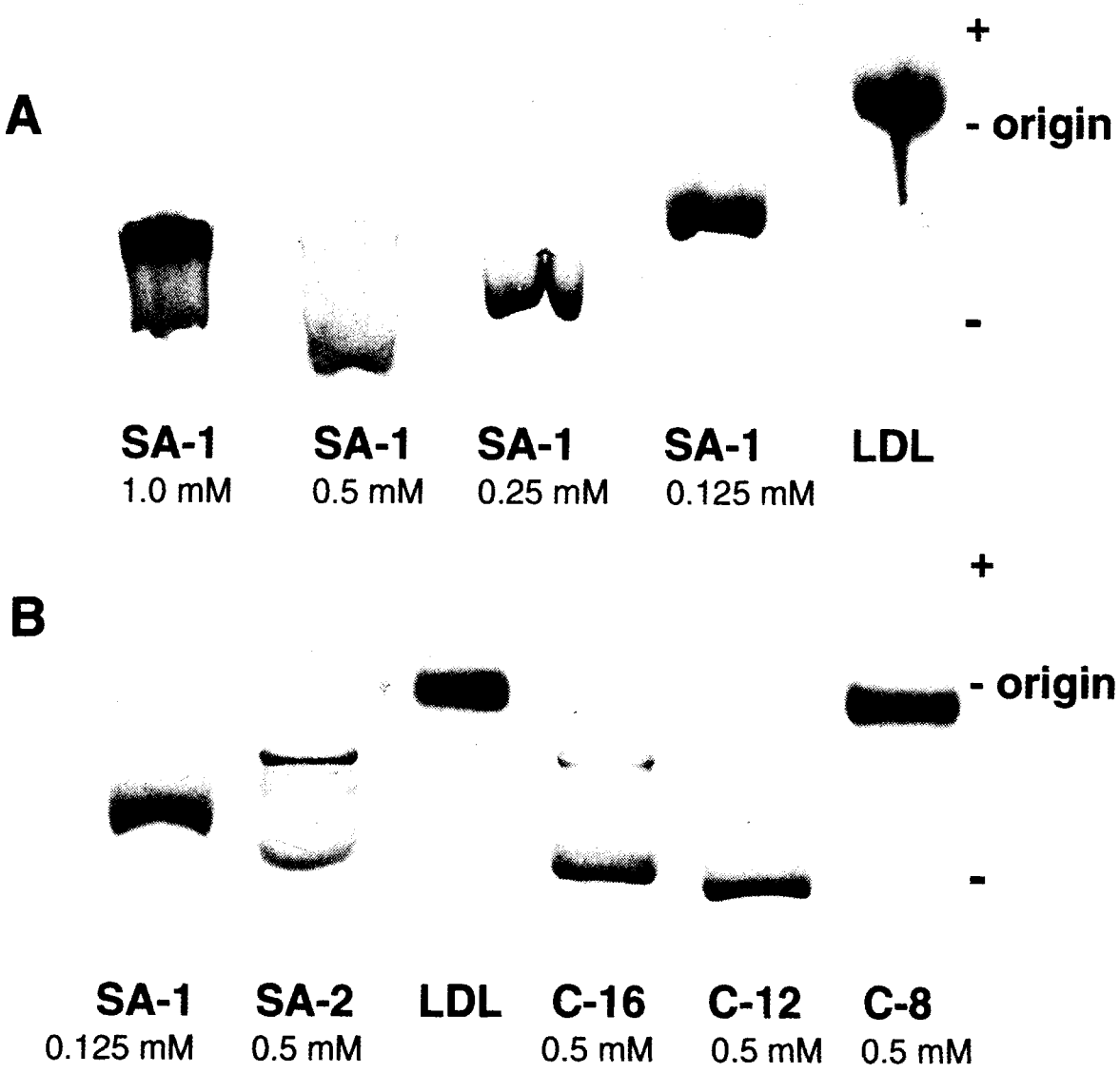


FIGURE 2 Agarose Gel Electrophoresis of Unsymmetrical Azo Initiator Treated LDL. LDL solutions were incubated with initiator for 15 min at 37°C and loaded onto a Corning agarose gel (panel A) or a Beckman Instruments LipoGel (panel B). Untreated LDL, panel A lane 5 and panel B lane 3, migrates toward the positive electrode from the origin. LDL incubated with positively charged unsymmetrical initiators, as indicated, demonstrate varying levels of migration toward the negative electrode compared to untreated LDL

radical $\mathbf{R_L}$• that has greater partitioning into a LDL than hydrophilic $\mathbf{R_H}$• peroxyl.

Electrophoretic mobility of LDL changes over the course of an oxidation with the use of either the well known **AAPH** or new unsymmetrical **SA-1** initiator. Figure 3 displays these changes over the time course of an oxidation. Note that as the **AAPH** initiated oxidation progresses, the LDL band gradually migrates more toward the positive electrode.[5] **SA-1** shows a similar effect, however, the initial location of the LDL band was shifted toward the negative electrode from

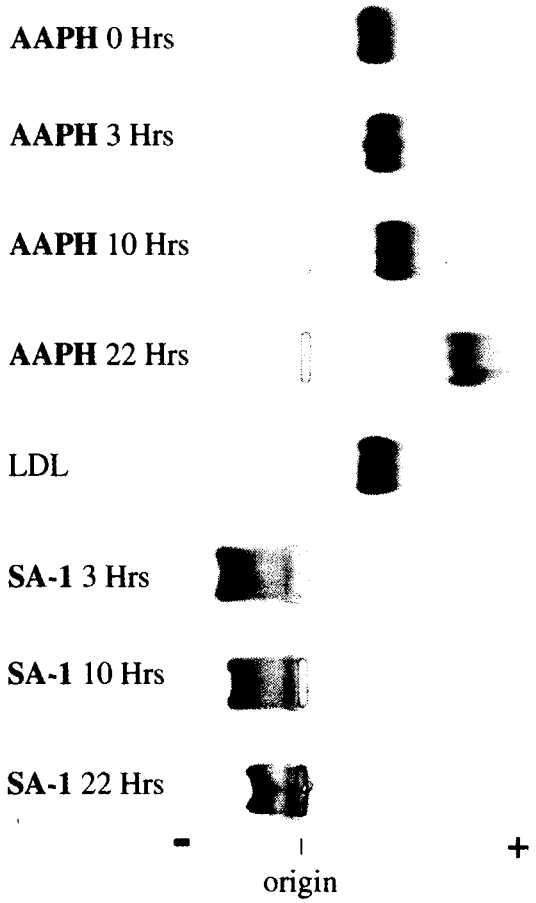


FIGURE 3 Agarose Gel Electrophoresis of LDL Oxidized by **AAPH** or **SA-1**. After initiation of LDL oxidation at 37°C by **AAPH** (0.5 mM) or **SA-1** (0.5 mM), aliquots were taken at intervals and stored on ice until run on this Corning agarose gel. Lane 5 is untreated, unoxidized LDL and migrates from origin towards the positive electrode as expected. As both **AAPH** and **SA-1** oxidations progress the LDL bands shift increasingly toward the positive electrode

addition of the positively charged initiator. This data also supports that addition of unsymmetrical initiators to LDL solutions does not compromise the integrity of the particle.

Product analysis profiles from **C-0** and **C-8** initiated LDL oxidations demonstrate that unsymmetrical initiators provide a potential advantage over symmetrical initiators. The experiments in Figure 4 panel A (**C-0** initiation) and panel B (**C-8** initiation) were run under identical conditions except for the initiator added. Consumption of α-TOH was significantly different between the two experiments. After five hours of oxidation initiated by **C-0**, only ≈ 28% of α-TOH was consumed (crosses). On the other hand, in **C-8** initiated **LDL** oxidation, more than 97% α-TOH was consumed in the same time period. This increase in α-TOH consumption is attributable to the increased access of the **C-8** derived $\mathbf{R_L}\bullet$ peroxyl radical into the LDL. The cholesteryl linoleate oxidation products also increase faster for the **C-8** initiated LDL oxidation. Squares and circles show the concentration of the 13 (*trans, cis*) and 9 (*trans, cis*) cholesteryl linoleate alcohol products formed during oxidation followed by reduction with triphenylphosphine. The 13 (*trans, trans*) and 9 (*trans, trans*) reduced cholesteryl linoleate hydroperoxide products (the alcohols) are represented by diamonds and triangles, respectively. The increasing quantities of *trans, trans* products during the **C-8** initiated oxidation reflect the decrease in hydrogen atom donating ability as α-TOH is consumed over the course of an oxidation.[17] These results suggest that **C-8** more efficiently generates initiating peroxyl radicals that have access to LDL antioxidants and lipids than does **C-0**.

$$\mathbf{R'_L - N = N - R'_H} \xrightarrow{k_d} [\mathbf{R'_L \bullet N_2 \bullet R'_H}] \xrightarrow{e} \xrightarrow{O_2} \mathbf{R_L \bullet + R_H \bullet + N_2} \quad (1)$$

$$\text{Rate of free radical flux} = 2e \bullet k_d[\text{initiator}] = 2d[\text{DPPD(or } \alpha - \text{TOH)}]/dt + 2d[\text{uric acid}]/dt \quad (2)$$

Radical generation efficiency was monitored by the use of two different radical scavenger combinations, either N,N′-Diphenyl-*p*-phenylenediamine (DPPD) and uric acid (UA) or α-TOH and uric acid. Both DPPD and α-TOH are lipid soluble antioxidants that scavenge radicals primarily in the lipid regions of an aggregate such as LDL, while uric acid is a water soluble antioxidant that traps radicals in the aqueous regions of lipid emulsions.[4,22] Partitioning of

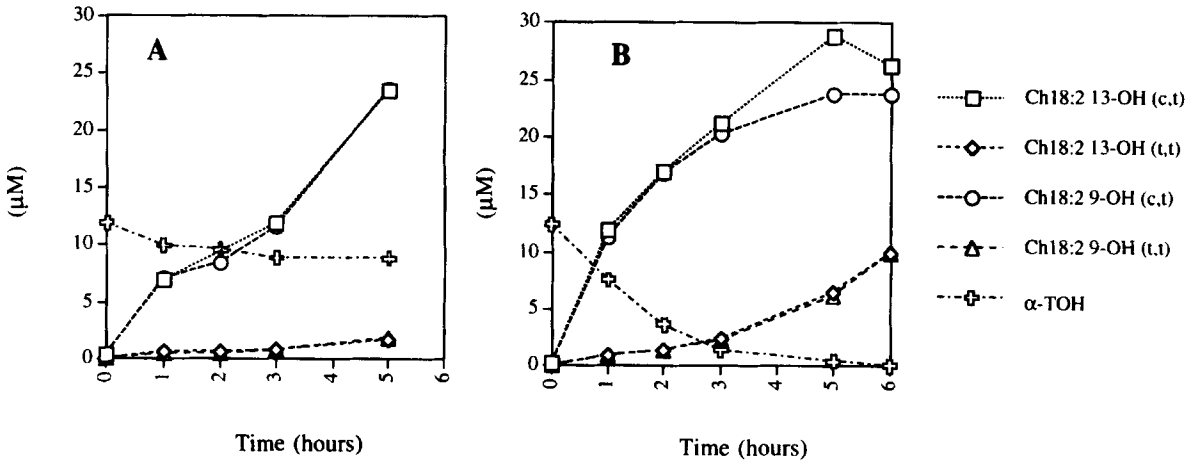


FIGURE 4 Oxidation Product Analysis From **C-0** and **C-8** Initiated LDL. LDL (0.75 mg protein/mL) was initiated at 37°C by the addition of either **C-0** (solution in phosphate buffer) 0.5 mM final concentration, or **C-8** (dissolved in methanol ≤ 3% v/v) 0.5 mM final concentration. Aliquots for cholesteryl linoleate hydroperoxide analysis were reduced to the respective alcohol products by triphenyl phosphine during extraction. The reduced cholesteryl linoleate oxidation products were separated by normal phase HPLC with 0.5% 2-propanol in hexanes. Consumption of α-TOH was determined by reverse phase HPLC with electrochemical detection

the radical formed from the initiator between lipophilic antioxidant (DPPD or α-TOH) and hydrophilic antioxidant (UA) presumably gives information about the locus of the scavenged radical in the emulsion. Each of the antioxidants presented here are known to be efficient radical chain breakers that scavenge two free radicals per antioxidant molecule.[4,22,23]

Values for observed initiator free radical flux, as measured by consumption of scavengers, are given by $2\,e \bullet k_d$ [initiator], where *e*is the radical escape fraction from the initial radical pair formed in the decomposition, and k_d [initiator] is the rate of geminate radical pair formation, eq 1. Two radicals are generated per initiator decomposition, and an efficiency fraction of 1.0 means that all radicals formed from an initiator escape from the solvent cage and react with a radical scavenger. Equation 2 separates the total radical generation into lipophilic and hydrophilic components. The sum of the rates of scavenger consumption from lipophilic antioxidant (DPPD or α-TOH) and hydrophilic antioxidant (UA)would be equal to the rate of initiator decomposition times the escape fraction ($e \bullet k_d$) since the initiator generates two radicals and each antioxidant consumes two radicals.

Initial experiments to monitor radical generation in lipophilic and hydrophilic regions of lipid aggregates used Triton X-100 micelles and phosphatidyl choline (PC) liposomes. These lipid aggregates were chosen because they are in common use, easy to make, and provide a broader scope in evaluation of the advantages of unsymmetrical initiators. Consumption rate of DPPD was determined by the increase in the UV at 440 nm, which corresponds to formation of N,N′-diphenyl-*p*-benzoquinone diimine (DPBQ) after each molecule of DPPD reacts with two molecules of peroxyl radicals.[4] Rates of consumption of α-tocopherol and uric acid were measured by monitoring the disappearance of these compounds by HPLC.[18,19] In a typical micelle experiment, initiator (~2 mM), Triton X-100 (0.1 M), α-tocopherol (or DPPD) (~0.1 mM), uric acid (~0.1 mM), and methyl linoleate (~5 mM) were vortexed in pH 7.4 phosphate buffer. The resulting solution or emulsion was

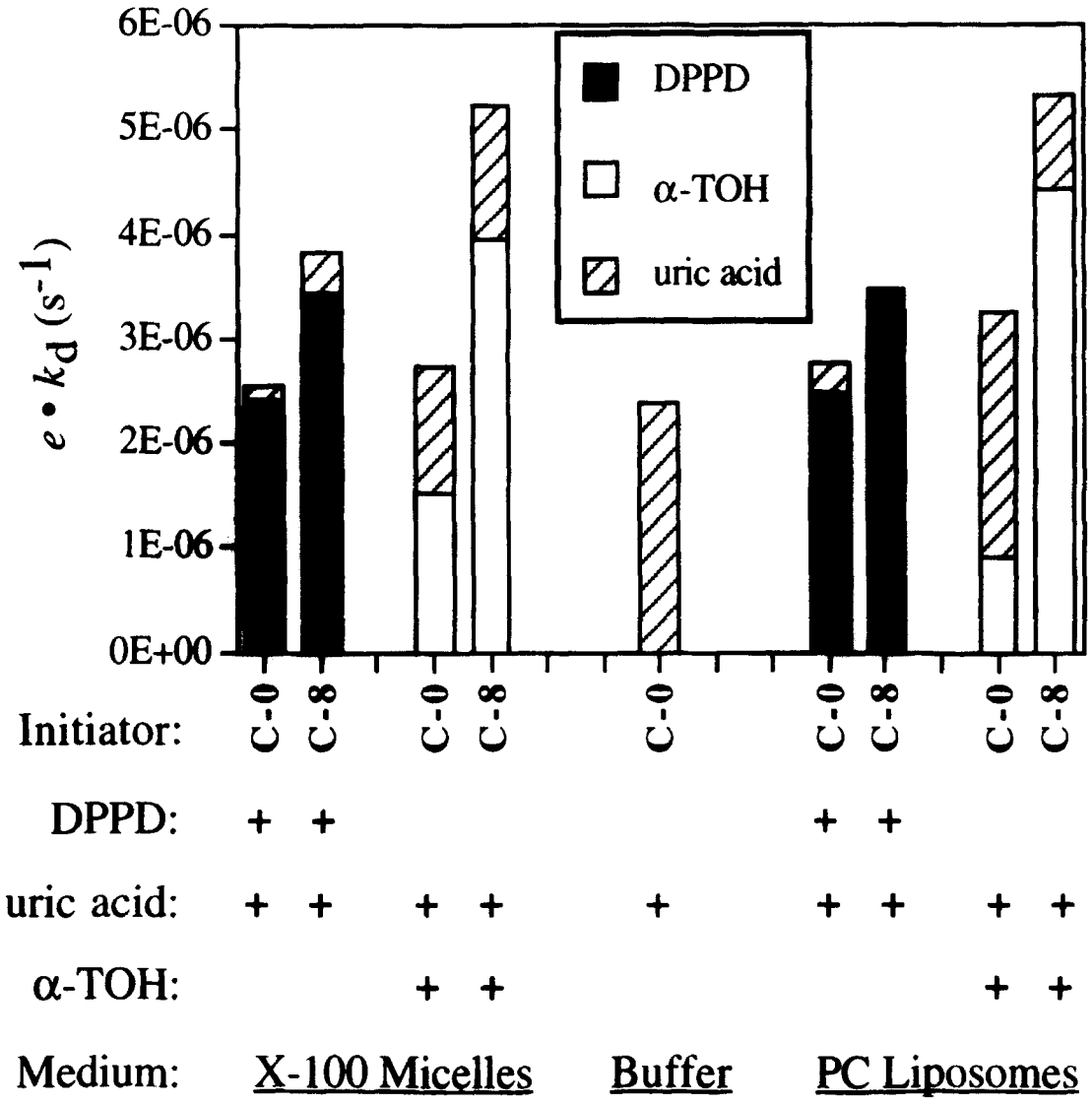


FIGURE 5 Lipophilic/Hydrophilic Radical Scavenging by DPPD, α-tocopherol, and Uric Acid. Proportions of radicals scavenged by lipophilic antioxidants DPPD or α-TOH and by hydrophilic antioxidant UA. Triton X-100 micelles were (0.1 M) containing initiator (~2 mM), DPPD (or α-TOH) (~0.1 mM), uric acid (~0.1 mM), and methyl linoleate (~5 mM) in pH 7.4 phosphate buffer at 37°C. Liposomes were phosphatidyl choline (10.3 mM) containing the amounts of initiator and antioxidants as the micelle experiments

kept at 37°C for several hours (< 0.1 half-lives) during which time aliquots were taken and analyzed for α-tocopherol and uric acid. For liposome experiments, phosphatidyl choline (10.3 mM) was substituted for Triton X-100. Rates of tocopherol (or DPPD) and urate consumption were determined and $e \bullet k_d$ values were calculated by the use of equation 2.

Results from experiments with the antioxidant combination of DPPD and UA suggest that DPPD does not function the same as α-TOH when used in combination with UA. The following data in Figure 5 support this conclusion: (1) In both micelles and liposomes, lipophilic scavenging reported by DPPD is very high with only a small portion of scavenging from the hydrophilic scavenger uric acid. (2) Uric acid is a good scavenger when used alone with **C-0** in phosphate buffer. (3) The antioxidant combination of α-TOH and UA display significantly different lipophilic/hydrophilic partitioning and overall scavenging compared to the DPPD and UA combination. The data suggest that there is an interconnection between UA and DPPD across the aqueous/lipid interface. In other words, uric acid appears to be maintained or regenerated at the expense of DPPD. Thus, the antioxidants α-tocopherol and uric acid were chosen to best represent radical scavenging in lipophilic and hydrophilic compartments respectively. The widely used antioxidant ascorbic acid is known to reduce α-tocopheroxyl radicals (α-TO•) and regenerate α-TOH across the interface of micelles, liposomes, and LDL.[8,24]Uric acid does not exhibit this regenerating capability with α-TOH in any system so far investigated.[22]

Further experiments to investigate the advantages of these new unsymmetrical initiators are underway. Detailed experiments addressing the efficiency of radical generation in a variety of media is currently in press.[9] Continued experiments are directed at assessing the role of α-TOH in an unsymmetrical azo initiator induced oxidation of LDL. These new initiators may well overcome the problems of free radical production and lipophilic access encountered with the use of the symmetrical azo initiators **AAPH** and **AMVN**.

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