Introduction

The protection that antioxidants confer against attack by free radicals is crucial to the survival of cells and organisms. Some enzymic systems e.g. superoxide dismutase (SOD) and catalase interact specifically with oxygen free radicals. Other enzymes, e.g. thioredoxin reductase, diaphorase and the glutathione-regenerating enzymic systems, reduce thiols. Non-enzymic antioxidants, which are not as specific, may also scavenge other organic and inorganic radicals. Depending on whether they act mainly in the aqueous phase or in the hydrophobic region of membranes, antioxidants are classified as water-soluble (e.g. ascorbic acid, urate) or lipid-soluble (e.g. ubiquinols, retinoids, carotenes, flavonoids, tocopherols). Some radical scavengers (e.g. carotenoids, retinoids) are not recyclable and may propagate further radical reactions, whereas others are recycled via cascades of enzymic systems or other non-enzymic antioxidants. Antioxidant activity can be transferred from the aqueous to the lipid phase (ascorbic acid→tocopherol), just as radical chain reactions can be propagated from the aqueous environment into the membrane. The free-radical scavenging activity of antioxidants depends largely on the kind of radicals formed (oxygen-derived, carbon-centered, thiyl-, alkoxyl-, phenoxyl- or peroxyl-radicals) and on the environment of the reaction (hydrophilic, lipophilic, at the surface or in the core of membranes). Measurements of antioxidant potency and the stoichiometry of radical scavenging [1] may vary considerably because of these factors. In order to evaluate radical-scavenging activity in different environments and by varying methods, studies were carried out using the phycoerythrin assay in aqueous phase [2], the cytochrome c assay in hexane and in dioleoyl-phosphatidylcholine (DOPC) liposomes, and the chemiluminescence assay with luminol in DOPC liposomes. Interactions and recycling were measured in low-density lipoproteins (LDL) using e.s.r. spectroscopy.
Materials and methods

Chemicals
The chemicals 2,2'-azobis(2-amidopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Polysciences, Inc. (Warrington, PA). Cis-parinaric acid and Trolox<sup>8</sup>, an α-tocopherol water-soluble homologue, were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ascorbate and DOPC were purchased from Sigma Chemical Company (St. Louis, MO), and h.p.l.c. grade organic solvents from Fisher Scientific (Fair Lawn, NJ). The following compounds were kind gifts: B-phycoerythrin (Prof. A. N. Glazer, Department of Microbiology, University of California, Berkeley, CA), carotenoids and retinoids (Hofmann-LaRoche Inc., Nutley, NJ), α-tocopherol (Henkel Corp., LaGrange, IL), dihydrolipoic acid (ASTA Medica, Frankfurt am Main, Germany). All other reagents were commercial products of analytical grade.

Assays
The phycoerythrin assay in aqueous phase was carried out according to DeLange and Glazer [2]. Cis-parinaric acid was assayed in hexane and in DOPC liposomes as described [5]. The α-tocopherol concentration was 1.25–25 μM, probucol was 25–250 μM, and ascorbate was 2.5 μM.

The chemiluminescence assay of luminol in DOPC liposomes was carried out as follows. Generation and interactions of peroxyl radicals in DOPC liposomes were assayed by the chemiluminescence produced in the presence of luminol. The incubation medium contained: DOPC liposomal suspension (2.5 mM) in Tris/HCl buffer, pH 7.4, at 40 °C; luminol, 150 μM; and AMVN, 2.5 mM. Lipophilic antioxidants were incorporated into liposomes by sonication for 60 s at 4 °C. The reaction was started by the addition of AMVN.

E.s.r. spectroscopy was accomplished as described previously [6] using a concentration of α-tocopherol in LDL of 12 nmol/mg protein and probucol of 80 nmol/mg protein, corresponding to 4.0 mg. If present, ascorbate was of 1.4 mM and dihydrolipoic acid (DHLA) of 0.8 mM concentration.

Results

The phycoerythrin fluorescence-based assay in aqueous system
In the phycoerythrin assay, AAPH-derived peroxyl radicals induced an immediate and almost linear decrease in fluorescence. The water-soluble antioxidants ascorbic acid and Trolox<sup>8</sup> completely protected phycoerythrin against peroxyl radicals until the antioxidants were consumed. DHLA was less protective, whereas the lipid-soluble α-tocopherol provided good protection after a delay. However, ‘delay’ means incomplete protection. Similarly, β-carotene and other lipid-soluble antioxidants also led to incomplete, although concentration-dependent, inhibition of phycoerythrin decay.

Cis-parinaric acid fluorescence-based assay in hexane
In hexane, cis-parinaric acid has an excitation maximum at 304 nm and an emission maximum at 421 nm. After addition of AMVN, the fluorescence intensity decreased linearly unless the reaction was inhibited by a lipid-soluble antioxidant, such as α-tocopherol or β-carotene. However, retinyl-palmitate did not prove effective in this system. The stoichiometry of lipid-soluble antioxidants is given in Table 1.

Cis-parinaric acid fluorescence-based assay in DOPC liposomes
Both Trolox<sup>8</sup> and α-tocopherol produced a delay in the loss of cis-parinaric acid fluorescence. Tocopherol was significantly more effective than Trolox<sup>8</sup>. β-Carotene was very efficient in radical scavenging in the liposomal system, whereas the effect of retinyl-palmitate was much less pronounced, being similar to that of DHLA. Ascorbate was not effective (Table 1) and did not enhance the effect of DHLA.

Chemiluminescence assay in DOPC liposomes
Interaction of luminol with peroxyl radicals generated in AMVN-containing DOPC liposomes at 40°C gave a characteristic chemiluminescence response that was not observed when AMVN, luminol or DOPC liposomes were omitted. AMVN-induced chemiluminescence of luminol was not sensitive to oxygen-radical quenchers (SOD, dimethylsulphoxide) or to the iron chelator, desferoxamine. However, the lipid-soluble scavenger of peroxyl radicals, α-tocopherol, if incorporated into the liposomes, produced inhibition of the chemiluminescence response with a half-maximum effect of 7.5–10 μM. β-Carotene (half-maximum effect at 37 μM), retinoic acid (half-maximum effect at >200 μM) and retinyl-palmitate (half-maximum effect at 160 μM) were less effective in this system, but DHLA (half-maximum effect at 12 μM) was almost as potent as tocopherol.

Interaction between antioxidants was demon-
Table 1
Reaction stoichiometry with peroxyl radicals (moles scavenged per mole)

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Parinaric acid</th>
<th>Chemilum.</th>
<th>Chemistry/No. of conj. double bonds</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phycoerythrin</td>
<td>30</td>
<td>0.2</td>
<td>11</td>
<td>Lipid</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>16</td>
<td>0.3</td>
<td>1</td>
<td>Lipid</td>
</tr>
<tr>
<td>Retinoids</td>
<td>0.25</td>
<td>0.05</td>
<td>5</td>
<td>Lipid</td>
</tr>
<tr>
<td>Tocopherol</td>
<td>2</td>
<td>1</td>
<td>Phenol/0</td>
<td></td>
</tr>
<tr>
<td>DHLA</td>
<td>0.4</td>
<td>0.6</td>
<td>Dithiol/0</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>1.1</td>
<td>0</td>
<td>Transient</td>
<td>Vinyl/double bond</td>
</tr>
</tbody>
</table>

Carotenoids tested: ß-carotene, cryptoxanthin, lutein, lycopene, zeaxanthin.
Retinoids tested: retinyl palmitate, retinoic acid, retinol.
*Trolox, a water-soluble analogue, exhibits a stoichiometry of 2 in the aqueous phycoerythrin assay.

Figure 1
Scheme of tocopherol and probucol recycation

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione

strated for ascorbic acid, DHLA and tocopherol. Human LDL was used because the content in endogenous vitamin E is sufficient for e.s.r. signal detection. However, if exogenous tocopherols, tocotrienols, chromanoxols (Trolox<sup>*</sup>), or even probucol were added, the recycling behaviour was similar, but the antioxidant potency varied. α-Tocopherol (ED<sub>50</sub>, 7.5–10 μM) exerted 10–15-fold higher antioxidant efficacy than probucol (ED<sub>50</sub>, 120 μM). Both phenolic compounds were protected by the addition of ascorbate. This protection could be considerably improved if DHLA was present in addition. In this scenario, ubiquinols and glutathione (GSH) should be mentioned because in vivo they are known to participate in the recycling mechanisms and could easily be included in the scheme depicted in Figure 1.

All of these interactions are enhanced and regenerated by enzymic recycling of ascorbic acid and GSH (Figure 2). Even without enzymic support, in our systems DHLA efficiently recycled ascorbic acid and protected tocopherol. In vivo DHLA is recycled itself via diaphorase, which is a partial enzymic activity in some dehydrogenases and can be isolated from these multienzyme complexes. In addition to their specific cofactor activity in enzymes (i.e. lipoamide in dehydrogenases together with diaphorase activity, thioredoxin in ribonuclease reductase together with thioredoxin reductase activity, or the disulphide-dependent reductases in the vitamin K cycle), thyl, and especially dithyl antioxidants, support the general GSH and ascorbic acid redox pools. This is achieved primarily either by the low redox potential (lipoic acid/DHLA = −0.29 V) or -SH/S–S– exchange [7].

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DHLa diminished the signal height of the probucol radical by 20%. Ascorbate delayed the appearance of the probucol radical for approximately 20 min. Between 20 and 30 min, a mixed ascorbyl-probucyl radical signal existed.

If DHLa and ascorbate were present, the ascorbyl radical signal was maintained by DHLa for almost 80 min and the signal of the probucol radical did not appear earlier.

Discussion
In the aqueous system, the radical-scavenging activity of lipid-soluble antioxidants (tocopherol, carotenoids and retinoids) was not dependent on structure, but only on lipophilicity. These antioxidants did not produce complete protection against peroxy radicals formed by thermolysis of AAPH. The water-soluble antioxidants Trolox® and ascorbic acid were most effective in the aqueous environment. DHLa, which has an intermediate solubility (10-15 times higher in octanol than in water), exhibited peroxy-radical scavenging activity in the phycoerythrin assay. Trolox® and α-tocopherol, which have the same radical-scavenging mechanism due to identical chromanol ring systems, differed considerably in their efficacy as a result of their differing solubilities.

Cis-parinaric acid in hexane
It may be questioned why this artificial system was used in our investigations. The difficulty in establishing stoichiometric rates of radical scavenging in a natural hydrophobic environment, such as a biological membrane or at least a liposomal system, made it necessary to evaluate these rates in an isotropic solution system, and then to correlate them to models, where water/membrane and intramembrane compartmentation determine radical-scavenging activities. In an organic solvent under our experimental conditions, the peroxy radical formation from AMVN is $3.5 \times 10^{-5}$ [AMVN]$^{-1}$ s$^{-1}$ (3.2 in hexane; 4.3 in methanol) [8, 9]. These findings may be used to estimate rates of radical formation and the propagation rates of radical chain reactions in the hydrophobic environment of membranes. The stoichiometry of all carotenoids containing 11 conjugated double bonds can be given in hexane solution as 30 mol peroxy radicals being scavenged by 1 mol of carotenoid. Retinoids containing only five conjugated double bonds are totally ineffective in this system. α-Tocopherol reveals a stoichiometry of 2 moles of radicals being scavenged per mol of antioxidant.

DOPC liposomes
Fluorescence assay
Fluorescence or fluorescence quenching of cis-parinaric acid is the most suitable model for lipid peroxidation, as parinaric acid itself is a polyunsaturated fatty acid. The radical attack becomes visible in the region where polyunsaturated acyl residues are attacked. Our findings suggest that the
rate of radical formation in the liposomal system is similar to that in hexane solution. Thus, the stoichiometry can be given as 1 mole of peroxyl radical scavenged per mole of α-tocopherol in liposomal systems. This agrees with our measurements in the cis-parinaric acid system. According to this correlation, β-carotene exhibits a stoichiometry of 16 (Table 1).

**Chemiluminescence assay**

In this system, α-tocopherol provided effective protection against peroxyl radical attack, with a half-maximum effect at 7.5 μM concentration.

Addition of ascorbic acid to the AMVN-containing DOPC liposomes incubated in the presence of luminol completely quenched the chemiluminescent response. This quenching effect was transient, and the chemiluminescence curve followed its typical time course after the ascorbate was consumed.

Provided the rate of radical formation from AMVN at 40°C is in the same range in DOPC liposomes as in organic solvents (3–5 × 10⁻⁶ [AMVN]⁻¹ s⁻¹), a stoichiometry of 1 mole peroxyl radicals consumed by 1 mole α-tocopherol would be obtained in DOPC liposomes, as detected by cis-parinaric acid fluorescence. One mole Trolox® scavenges 0.5 moles peroxyl radicals, one mole retinyl-palmitate and DHLA scavenges up to 1 mole, and one mole β-carotene scavenges up to 16 moles.

The differences in stoichiometry between parinaric-acid fluorescence and luminol chemiluminescence (Table 1) may be because cis-parinaric acid is reacting in the hydrophobic part of the membrane, while luminol reacts closer to the surface of the membrane.

**Conclusions and further considerations of biological membranes**

That aqueous and lipophilic systems demonstrate different potencies for the same antioxidants suggests that radical-scavenging capacity does not depend only on molecular features, but also on interactions, in both artificial and biological systems. We studied various systems to evaluate radical-scavenging capacity in different environments. However, we know that even these improved data do not take into account all of the interactions between the aqueous and lipophilic compartments, especially within the membranes, of biological antioxidant systems. In addition to radical-scavenging capacity, antioxidant activity depends on recycling of the scavenger.

In this context, three types of antioxidants and their interactions should be discussed, namely, phenols, thiols and ascorbic acid. The most effective antioxidant systems appear to be wide-spread cascades of relatively unspecific non-enzymic radical-scavenging compounds such as tocopherols (or other phenols [10], including ubiquinols), ascorbate and thiols, which are recycled primarily by interactions with one another (and therefore propagate radical-scavenging chain reactions that counteract radical chain reactions) but also by specific enzymic systems. It is this interplay that gives advantage to the antioxidant systems over the perpetuation of radical-generating events. The mitochondrial respiratory enzyme complex I to complex IV provides the best example for optimal handling and control of radicals: a single electron is separated from the proton and transferred to oxygen via highly reactive oxygen-radical species to produce water.

The LDL system [11] not only serves as a model for measuring free-radical scavenging but also is closely related to (patho)physiology, since LDL oxidation and uptake by macrophages strongly depends on the balance of pro- and antioxidants in the plasma and in LDL. Plasma is readily depleted of endogenous antioxidants, especially of vitamins C and E, during LDL oxidation, and vitamin substitution or application of antioxidant drugs (e.g. probucol or possibly dihydrolipoic acid) may protect against this oxidation. Vitamin E content in LDL plays a crucial role in protection against radical attack: oxidation cannot be detected before tocopherol is used up. Hence, the maintenance of vitamin E content in LDL may be the major challenge for preventing LDL oxidation and atherogenesis. This might be achieved by exogenously added vitamin E, by protection of the endogenous tocopherol system in LDL, or by support of tocopherol recycling via administration of recycling antioxidants.

Pathological implications of nitric oxide, superoxide and peroxynitrite formation
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Introduction
Approximately 6 years ago, nitric oxide was identified as the endothelium-derived relaxing factor (EDRF), the endogenous vasodilator mimicked by nitroglycerin and nitroprusside [1-4]. An astonishing variety of tissues synthesize nitric oxide, which has important roles in the control of systemic blood pressure, respiration, digestion, penile erection, platelet aggregation, cerebral blood flow and neuronal synaptic plasticity [5]. Furthermore, nitric oxide, or a secondary oxidant derived from it, contributes to the microbicidal and tumoricidal activities of activated macrophages and neutrophils [6, 7]. Endothelium and neurons produce nitric oxide by a calmodulin-activated enzyme, which oxidizes arginine to citrulline and requires biopterin, NADPH and oxygen [8, 9]. The biopterin remains tightly bound to the enzyme and may be recycled within it. Nitric oxide synthase also contains FAD, FMN and ferric haem as essential cofactors. The macrophage enzyme is not regulated by calmodulin, and gene sequences reveal significant differences between the brain, endothelial and macrophage enzymes [10].

Pathological conditions can substantially up-regulate the production of nitric oxide. Stimulated macrophages and neutrophils produce significantly greater fluxes of nitric oxide than endothelium by the inducible calcium-independent nitric oxide synthase. Endotoxin (lipopolysaccharide) and cytokines induce the calcium-independent nitric oxide synthase in many tissues that do not normally produce nitric oxide [11]. For example, the acute hypotension associated with septic shock may be mediated by the expression of the inducible nitric oxide synthase [12].

If nitric oxide is a major cytotoxic agent, then why is nitric oxide generated for signal transduction not toxic? Although the amounts of nitric oxide produced for signal transduction are significantly lower than those produced by macrophages the continuous daily formation of nitric oxide in brain and endothelium will result in vastly greater exposure to nitric oxide (measured as time x concentration) than the transient activation of inflammatory cells for a few hours. In part, the answer is that the toxicity of nitric oxide at low concentrations has been over-estimated due to extrapolation from the rapid formation of other more reactive nitrogen oxides at high concentrations. In millimolar concentrations, nitric oxide rapidly reacts with oxygen to form nitrogen dioxide (NO₂) as well as the strong two-electron oxidants, N₂O₃ and N₂O₄, by dimerization of nitric oxide and nitrogen dioxide. However, the rate of formation of NO₂ is determined by the third order reaction:

\[ k_3 [NO] [O_2] \]  

Consequently, the reactivity of nitric oxide falls rapidly with the square of nitric oxide concentration. At low concentrations, similar to those which can be produced in vivo, nitric oxide can be administered safely in the gas phase for weeks to treat pulmonary hypertension [13]. While the orange-brown colour of concentrated nitrogen dioxide is formed immediately when gaseous nitric oxide contacts air, this reaction is at least 10 million times slower at the maximum physiological nitric oxide concentrations (about 0.1 μM) [14, 15]. In a simple in vitro system, over an hour is required for physiological concen-