The Cooperative Antioxidant Role of Glutathione with a Lipid-soluble and a Water-soluble Antioxidant during Peroxidation of Liposomes Initiated in the Aqueous Phase and in the Lipid Phase*

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A study is made of the effect of GSH as a co-antioxidant with vitamin E during free radical chain autoxidation inhibition studies of dilinoleoylphosphatidylcholine (DLPC) liposomes. Oxidations are initiated in the aqueous phase with azobis(2-amidinopropane hydrochloride) and in the bilayer phase of DLPC with azobis(2,4-dimethylvaleronitrile) under known conditions of the ratio of free radical chain initiation (R). In reactions initiated in the aqueous phase, GSH is not an efficient antioxidant when acting alone; however, in cooperation with vitamin E in the bilayers, it does effect significant extensions of the efficient induction period of vitamin E. Quantitative studies show that GSH “spares” 0.4 molecules of vitamin E in the bilayer/molecule of GSH and therefore terminates approximately 0.8 peroxyl radical chains as a co-antioxidant with vitamin E. In contrast, GSH is not an effective co-antioxidant with an efficient water-soluble antioxidant, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate (Trolox). GSH spares only 0.08 molecules of Trolox/molecule of GSH during autoxidation initiated in the aqueous phase with azobis(2-amidinopropane hydrochloride). The inhibition rate constant for GSH in trapping aqueous phase peroxyls is at least an order of magnitude less than that of Trolox. When peroxidation is initiated in the bilayer phase of DLPC with azobis(2,4-dimethylvaleronitrile), GSH is not an effective co-antioxidant with either vitamin E in the bilayer or Trolox in the water. Comparatively higher ratios of GSH to E (GSH/E = 50) or Trolox (GSH/Trolox = 30) are required to give significant extensions of the E or Trolox induction periods. GSH is estimated to preserve only approximately one vitamin E molecule in the DLPC bilayers. From the kinetic studies and GSH decay studies during inhibition periods, it is concluded that GSH does not act synergistically by regenerating ArOH from the phenoxyl, ArO, radical of vitamin E or Trolox. The mode of antioxidant action of GSH is concluded to be that of trapping peroxyl radicals in the aqueous phase and thereby indirectly sparing vitamin E in the bilayer.

The cooperative interaction of radical chain-breaking antioxidants is important in the protection of biological membranes against free radical damage. The discovery that a water-soluble antioxidant, vitamin C, can interact synergistically to prolong the antioxidant capacity of vitamin E during peroxidations in the hydrophobic phases of bilayers (1–3) and micelles (4) has enhanced current interest in this field. It is important to determine the role of other antioxidants such as sulphydryl compounds in the aqueous phase of membranes in relationship with membrane-bound vitamin E, nature’s major lipid-soluble chain breaking antioxidant (5).

The tripeptide GSH occurs intracellularly in relatively high concentration (6). Although GSH is widely recognized as a protective antioxidant in biological systems (7–14), its antioxidant action in relationship with other antioxidants, notably vitamin E, is not well understood. There is evidence that the antioxidant action of GSH is “vitamin E-dependent,” for example in isolated rat microsomes, and interpretations were given either in terms of regeneration of vitamin E by GSH (15) or that the two function separately to prevent peroxidation of membranes (16). GSH suppressed the azo-initiated autoxidation of liposomes (17) but acted as a prooxidant when the reaction was initiated with Fe²⁺/H₂O₂ (18). Model experiments indicate that GSH can regenerate vitamin E from the α-tocopheroxyl radical in solution (19) and interact with vitamin E during autoxidation (20), but it is not established if such interaction is applicable to biphasic membrane systems.

In order to clarify the antioxidant role of GSH, two basic key questions need be answered. 1) Does GSH operate by direct cooperative (synergistic) interaction with other antioxidants, such as vitamin E, and 2) does GSH trap peroxyl radicals formed in both the lipid and the aqueous phases of membrane systems? To address these questions, quantitative kinetic methods of autoxidation (2,4) are used to determine the effect of GSH alone and in combination with α-tocopherol in the lipid phase of dimyloleoylphosphatidylcholine (DLPC) liposomes and with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate (Trolox) in the water, both known to be efficient antioxidant reactions initiated in either the aqueous or hydrophobic phases of liposomes (21). Peroxidations are initiated under controlled conditions in the aqueous phase with azobis(2-amidinopropane hydrochloride) (ABAP) and in the lipid phase with azobis(2,4-dimethylvaleronitrile) (DMVN). The activity of GSH as a radical-trapping antioxidant is determined by measuring its inhibition rate constant.

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1 The abbreviations used are: DLPC, dihmyloleoylphosphatidylcholine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate; ABAP, azobis(2-amidinopropane hydrochloride); DMVN, azobis(2,4-dimethylvaleronitrile); MLV, multimellar vesicles; ULV, unilamellar vesicles; PT, pressure transducer; O₂, oxygen electrode; E, vitamin E.
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**Experimental Procedures**

Materials—DLPC was obtained from Avanti Polar Lipids. d-a-Tocopherol from Eastman was stored in known concentrations in hexane at -30 °C. Solutions of GSH (Sigma) were prepared immediately before use in argon-degassed phosphate buffer. Trolox was a gift from Hoffmann-La Roche. It was stored at -30 °C, and solutions of known concentration in argon-degassed phosphate buffer were prepared shortly before use. ABAP and DMVN were obtained from Polysciences, Inc. Phosphate buffer was prepared from 0.05 M each of NaH₂PO₄ and Na₂HPO₄ (pH 7.0) in glass-distilled water containing 1 x 10⁻⁴ M Na₂EDTA and passed through Chelex (50-100 mesh, Bio-Rad) to remove traces of heavy metal ions.

Preparations—DLPC solutions were prepared in chloroform and, where required, known amounts of α-tocopherol and DMVN were added. This solution was taken to a film with a stream of argon, and residual solvent was removed under reduced pressure. Degassed phosphate buffer was added (normally the amount of DLPC was 10 mg/ml), followed by vortex stirring, and at least five freeze-thaw cycles in liquid nitrogen to yield multilamellar vesicles (MLV). Unilamellar vesicles (ULV) were prepared by passing these MLV three times through a French pressure cell under argon at 20,000 p.s.i. (22). The MLV and ULV were characterized by their typical ³¹P NMR spectra (23).

Autoxidation and Inhibition Studies—The kinetic experiments were carried out using the sensitive pressure transducer (PT) apparatus described previously (2, 4, 23) and by a Clark-type oxygen electrode (OE) (24). The OE procedure did not give sharp breaks in Trolox induction periods with multilamellar bilayers when reaction was initiated in the bilayer with DMVN. In these cases, the vitamin E induction periods were used to determine the rate of chain initiation (R'). In some experiments the same USF preparation was used on both the PT and OE apparatus to check the reproducibility between the two procedures (vide infra). When oxidations were initiated in the aqueous phase with MLV of DLPC, the initiator (ABAP) was added with the buffer to prepare the bilayers. This avoids the "inhomogeneity" problem which arises when the initiator is not uniformly distributed throughout the bilayers (2). In the PT procedure, the induction period (t) was first measured with vitamin E or with Trolox on a portion of the bilayer preparation, generally 20 mg of DLPC/2.00 ml of aqueous buffer. A second portion of the same bilayer preparation containing initiator and inhibitor was stored frozen in liquid nitrogen during this first inhibition period. This sample was then melted, the GSH added, and the inhibition experiment repeated. A dual channel system was used in the OE procedure so that the two inhibition periods were followed simultaneously. In experiments initiated in the aqueous phase (ABAP), the R' was measured after the vitamin E (or the vitamin E + GSH) induction periods by injecting a known amount of Trolox and measuring again.

Known methods (25, 26) were used to determine the k₈O values for GSH and Trolox. During the inhibition period, the oxygen uptake is given by the integrated rate expression, Δ[O₂] = -k₈O[k₈O[R-H][ln(1 - t/T)]

where k₈ is the propagation rate constant² for autoxidation of DLPC, R-H the substrate, t the time for a measured O₂ uptake during the inhibition period, and T the length of the inhibition period. To measure the GSH concentration during an oxidation, aliquots were removed from the apparatus and the bilayer dispersed by addition to 0.50 M sodium dodecyl sulfate. The resulting clear solution was then analyzed for GSH by Ellman's method (29).

**Results and Discussion**

Peroxidations Initiated in the Aqueous Phase—Some typical oxygen uptake traces obtained using the sensitive PT apparatus for reactions initiated by ABAP in the aqueous phase at 37 °C are shown in Fig. 1. Details giving some typical data and results where α-tocopherol and GSH are co-antioxidants using the PT and the OE method are outlined in Table I.

The presence of GSH causes a poorly defined induction period on the ABAP-initiated autoxidation of DLPC liposomes (Fig. 1, 2), and the rate of oxygen uptake is slow to return to the uninhibited rate. The antioxidant action of GSH differs significantly from vitamin C which is known to be an effective inhibitor of such autoxidations initiated by ABAP (2).

On the other hand, when α-tocopherol was sequestered in the DLPC liposomes and GSH was present in the water, the combination gives a significant extension of the efficient inhibition period typical of vitamin E alone (Fig. 1, compare traces 3 and 4); again the rate is slow to return to the uninhibited rate in trace 4. This effect of GSH on the vitamin E-inhibited peroxidation was investigated quantitatively for GSH/E ratios in the range 10⁻² to 20, and typical results are summarized in Table I.

The product of the extension of the inhibition period due to GSH, (tₑ + GSH) - tₑ, and the rate of peroxyl radical formation, the k₈ per mol of GSH is a measure of the radical trapping capacity of GSH in the presence of E. Comparable results were obtained for this quantity using both the PT and OE procedure, and they indicate that GSH can preserve or spare approximately 0.4 molecules of E/molecule of GSH. Since the former is known to trap two peroxyls/molecule GSH, it is estimated to terminate approximately 0.8 peroxyl chains as a co-antioxidant with E.

It was found previously (2) that vitamin E does not give efficient inhibition of ABAP-initiated peroxidation in multilamellar DLPC liposomes unless the ABAP is added before the liposomes are formed and thereby becomes uniformly distributed as well as the E. When unilamellar liposomes are used, vitamin E gives efficient inhibition of peroxidation even when the initiator, ABAP, is added after the preparation (Fig. 1, 3). In this case, the problem of inhomogeneity of the system, that can arise in multilamellar liposomes, is obviated.

Experiments were also performed to determine if GSH will interact with the water-soluble antioxidant, Trolox, during peroxidation initiated in the water. A result for the GSH/Trolox ratio of 15 is illustrated in Fig. 2A. It is seen that GSH at this molar ratio will extend the effective inhibition period found for Trolox (compare traces 2 and 3). However, GSH is not a very efficient co-antioxidant with Trolox. In this interaction it "spares" only 0.08 molecules of Trolox/molecule of GSH. In other words, relative to Trolox where n = 2 (26), GSH can stop only 0.16 peroxyl radical chains/molecule of GSH.

Two other observations provide insight into the antioxidant action of GSH in the aqueous phase: 1) the inhibition rate constant of GSH (k₈GSH) in trapping peroxyls; and 2) the decay of GSH under typical autoxidation conditions. Plots of oxygen uptake versus -ln(1 - t/T) during inhibition periods observed by GSH and by Trolox give linear results (Fig. 3). Using the slopes of these plots and the k₈ for DLPC under these condi-

² The k₈GSH for DLPC bilayers was recently determined by our rotating sector method (27, 28) to be 36.1 M⁻¹ s⁻¹.
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TABLE I
Effect of GSH on the \(\alpha\)-tocopherol-inhibited (E) peroxidation of DLPC liposomes initiated in the aqueous phase

Runs were done in phosphate buffer, pH 7.0, at 37 °C. The PT method used 28.4 \(\mu\)mol of DLPC in 2.00 ml; the OE method used 7.11 \(\mu\)mol of DLPC in 3.00 ml.

| Run no. | ABAP \(\mu\)mol | Inhibitors | Induction period (\(t\)) | \(R^0\) | \(\tau_{\text{E-GSH}} - \tau_E\) | \(R_X \times \tau_{\text{E-GSH}}\) |
|---------|-----------------|------------|--------------------------|------|-----------------------------|-----------------------------|
| 6 PT    | 9.02            | E 22.6 nmol | 2.66 ms \(\times 10^{-11}\) | 1.20 | 7.06 \(s \times 10^{-3}\)   | 0.394                       |
|         | 3.01            | GSH 7.58 nmol | 9.72 ms \(\times 10^{-11}\) | 0.581|                             |                             |
| 6 OE    | 2.26            | E 6.28 nmol | 5.62 ms \(\times 10^{-13}\) | 0.254| 19.9 \(s \times 10^{-3}\)   | 0.389                       |
|         | 2.26            | GSH 130 nmol | 25.6 ms \(\times 10^{-13}\) | 0.254|                             |                             |
| 8 PT    | 8.12            | E 45.2 nmol | 10.3 ms \(\times 10^{-13}\) | 1.00 | 14.4 \(s \times 10^{-3}\)   | 0.301                       |
|         | 45.2            | GSH 459 nmol | 24.7 ms \(\times 10^{-13}\) | 0.960|                             |                             |

* Runs with the same number refer to the same DLPC bilayer preparation. No. 6 used MLV; no. 8 used ULV.

† The rate of chain initiation measured using Trolox induction periods.

‡ \(\tau_{\text{ext}}\) is the \(\tau_{\text{E-GSH}} - \tau_E\) value.

**Fig. 2.** Autoxidation of DLPC liposomes (28.4 \(\mu\)mol), conditions as in Fig. 1. A, initiation with ABAP (8.16 \(\mu\)mol). 1, uninhibited reaction; 2, inhibited with Trolox (0.045 \(\mu\)mol); 3, inhibited with DMVN (9.50 \(\mu\)mol). B, initiation with DMVN (9.50 \(\mu\)mol). 4, uninhibited reaction; 5, inhibited with Trolox (0.0225 \(\mu\)mol); 6, inhibited with Trolox (0.0225 \(\mu\)mol) and GSH (0.680 \(\mu\)mol).

**Fig. 3.** Plots of oxygen uptake versus \(-\ln(1 - t/T)\) for inhibited autoxidation of DLPC liposomes (28.8 \(\mu\)mol); conditions as in Fig. 1. Initiation was with ABAP (4.06 \(\mu\)mol). 0, for Trolox (0.012 \(\mu\)mol); \(\Delta\), for GSH (0.139 \(\mu\)mol).

**Fig. 4.** Plots of GSH analyses; GSH = amount at time \(t\), GSH0 = initial amount, during inhibited autoxidation of DLPC liposomes (35.2 \(\mu\)mol); conditions as in Fig. 1. \(\Delta\), curve 10, ABAP (49.5 \(\mu\)mol), GSH (4.10 \(\mu\)mol), Trolox (0.225 \(\mu\)mol), and \(\Delta\) indicates end of Trolox inhibition; \(\times\), curve 12, ABAP (4.46 \(\mu\)mol), GSH (15.1 \(\mu\)mol), Trolox (0.016 \(\mu\)mol), and \(\times\) indicates end of Trolox inhibition; 0, curve 11, DMVN (4.75 \(\mu\)mol), GSH (15.0 \(\mu\)mol), \(\alpha\)-tocopherol (0.026 \(\mu\)mol), and \(\times\) indicates end of \(\alpha\)-tocopherol inhibition.

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Peroxidations Initiated in the Bilayer Phase—Experiments designed to determine if GSH can interact with vitamin E when peroxidation is initiated in the bilayer phase reveal that significant extensions of the vitamin E induction period are observed only when ratios of GSH/E greater than 50 are used (Table II). Under these conditions, GSH is very inefficient in its cooperative interaction with E. For example, a typical run such as 10PT used a GSH/E ratio of 56, and a calculation of the Extension \(\times \frac{R_X}{GSH}\) gives approximately \(1 \times 10^{-2}\). Thus, in this system, GSH in the aqueous phase can spare only one
in every hundred vitamin E molecules in the bilayer.

At high GSH concentrations most of it remains unreacted well beyond the termination of the vitamin E inhibition period observed in the same reaction (Fig. 4, curve 11). This result is similar to that observed for initiation in the aqueous phase (curve 12). These results are significant because they show that, at cellular concentrations, depletion of GSH by chain autoxidation of thiyl radicals that, at cellular concentrations, depletion of GSH by chain autoxidation of thiyl radicals  

\[
\text{GS}^\cdot + \text{O}_2 \rightarrow \text{GSOO}^\cdot
\]

where GS\(^\cdot\) is a thiol radical, is not as rapid as oxidative attack at polyunsaturated membrane lipids.

It is of interest to determine if GSH interacts with Trolox in the aqueous phase when peroxidation is initiated in the bilayer phase with DMVN, to compare with the situation illustrated in Fig. 2A for initiation in the water. As shown in Fig. 2B, GSH in a molar ratio of GSH/Trolox = 30 does extend the induction period observed for Trolox alone. Under these conditions, the calculation as above shows that GSH can spare only approximately nine of every thousand Trolox molecules in the aqueous phase. In summary, the results show that, for peroxidation initiated in the bilayer region, GSH is comparatively inefficient in its interaction to preserve either vitamin E in the bilayer or Trolox in the water.

**CONCLUSIONS**

These results illustrate the importance of quantitative kinetic studies of membrane autoxidation and antioxidant action. Specific points are emphasized in the following paragraphs.

1. The lower \(k_{\text{inhib}}\) for GSH compared to Trolox in trapping aqueous peroxyls indicates that GSH is not as effective as known active phenolic antioxidants like vitamin E and Trolox (31) by at least an order of magnitude.

2. However, GSH is effective as a co-antioxidant in cooperative interaction with vitamin E in the bilayer phase, when peroxidation is initiated in the aqueous phase. Here GSH stops 0.8 peroxyl chains. The actual stoichiometric factor for GSH under these conditions is probably 1 if loss due to oxidation is considered (30, 32). It is concluded that the mode of antioxidant action of GSH in this heterogeneous bilayer/aqueous system is due to reaction with the initiating aqueous peroxyl radicals.

\[
\text{GSH} + \text{ROO}^\cdot (\text{aq}) \rightarrow \text{GS}^\cdot + \text{ROOH}^\cdot
\]

GSH spares vitamin E by decreasing peroxyl radical attack on the bilayer, in effect by lowering the rate of chain initiation. Rates at the end of vitamin E inhibition periods do not return smoothly to uninhibited rates because residual GSH retards the \(R_c\).

3. When peroxidation is initiated in the bilayer phase, GSH is not an effective co-antioxidant with either vitamin E in the bilayers nor with Trolox in the water. These results, together with GSH decay studies, show that GSH does not regenerate vitamin E in the bilayers nor Trolox in the water from their respective aryloxy (ArO) radicals. Unlike vitamin C, which exhibits synergistic interaction with both E and Trolox (2), GSH does not compete with peroxyl radicals for the phenolic radicals initially formed from E or Trolox. Some antioxidant action of GSH against bilayer peroxy radicals is attributed to the polarity of peroxy radicals (33) which diffuse to the polar surface where they can be intercepted by GSH.

4. These results have important implications for the role of GSH in biological systems. For example, they support the hypothesis that GSH and vitamin E function separately in the prevention of peroxidation of liver microsomes (16) and account for the qualitative observations that the action of Trolox is “not improved” by GSH in microsomal peroxidation (14). Relatively high concentrations of GSH play a key role in protecting sensitive biological membranes from free radical damage by trapping oxygen radicals in the aqueous phase.

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