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A SPIN LABEL STUDY OF LIPID OXIDATION CATALYZED BY HEME PROTEINS

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Summary

Rapid loss of the electron spin resonance signal from a variety of spin labels is observed when ferricytochrome *c* or metmyogloblin are combined with lipids. Evidence is presented that this loss of signal can be used as a sensitive method to study lipid oxidation catalyzed by heme proteins. Under aerobic conditions and with lipids which bind the heme protein, the kinetics of the oxidation process as observed by the spin label method are identical to the kinetics previously observed by measurements of oxygen uptake. Use of pre-oxidized lipids under anaerobic conditions indicates that cytochrome *c* reacts with a product of lipid oxidation. Kinetic studies of the anaerobic reaction indicate that cytochrome *c* reacts rapidly with lipid oxidation products in membrane areas far larger than the area occupied by cytochrome *c*, implying rapid transport of reactive species within the membrane interior in directions parallel to the membrane surface. Under anaerobic conditions, reaction of cytochrome *c* with lipid oxidation products appears to produce a relatively long lived (hours) species located in the hydrophobic portion of the membrane, which is capable of subsequent reaction with lipid-soluble spin labels.

Introduction

In the course of studies by nuclear magnetic resonance (NMR) and electron spin resonance (ESR) of the interaction of cytochrome *c* with model membranes (to be published), we have experienced difficulties with rapid loss of the ESR signal from a nitroxide spin label covalently attached to methionine 65 of cytochrome *c*. Similar losses of ESR signal appear to be a common and sometimes prohibitive problem with the use of spin labels as structural probes (e.g. refs.

Abbreviations: TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl; TEMPO, 2,2,6,6-tetramethylpiperidinooxyl; TEMPO phosphate, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl dihydrogen phosphate; OxL represents a lipid oxidation product(s) which interacts with ferricytochrome *c* to produce new species OxL*. OxL* reacts subsequently with the spin label.

1 and 2). The present paper describes an investigation of the conditions under which the spin label ESR signal rapidly disappeared in our systems. Evidence will be presented that the rapid loss of ESR signal is related to lipid oxidation catalyzed by heme proteins.

The ability of heme proteins to catalyze oxidation of unsaturated lipids has been known for many years [3–5], but the exact nature of this catalysis and its possible biological significance are not well understood. On the other hand, there has emerged in recent years considerable evidence that lipid oxidation is a natural process in the functioning of many biological membranes and that at least some of this oxidation may be due to heme proteins [6–9]. In the past, such oxidation reactions have generally been studied by measurements of oxygen uptake during lipid oxidation or by chemical analysis of the oxidation products. The present experiments show that the loss of ESR signal from spin labels provides an alternative, highly sensitive method to study lipid oxidation catalyzed by heme proteins. An attractive feature of the spin label method is that certain reaction steps can be studied under anaerobic conditions, which may in certain cases lead to a more detailed analysis of the overall oxidation reaction. In the systems described here, anaerobic studies permitted definition of some of the characteristics of intermediates in lipid oxidation reactions, e.g. the life times and the mobility in lipid layers.

Materials and Methods

The nitroxide spin labels 3-(2-iodoacetamide)-2,2,5,5-tetramethyl-1-pyrrolidinooxyl, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl (TEMPOL) and 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl dihydrogen phosphate ester (TEMPO phosphate) were purchased from Syva Associates. 2,2,6,6-Tetramethylpiperidinooxyl (TEMPO), synthesized according to Brière et al. [10], was a gift from Dr. W. Schlegel. Sperm whale skeletal muscle myoglobin was obtained from Calbiochem (Grade A) and used as supplied.

Horse heart cytochrome *c* (Calbiochem, grade A) was further purified by chromatography on SP-Sephadex C25 (Pharmacia) according to the method of Fisher et al. [11]. The column fractions containing the major cytochrome *c* component were freed of salts by dialysis and then lyophilized. Extraneous metal ions were removed by layering an aqueous solution of cytochrome *c* over a chloroform layer containing 8-hydroxyquinoline and stirring slowly, taking care not to mix the chloroform and aqueous layers, at 4°C for 24 h. The chloroform layer was then replaced with fresh chloroform and stirring continued for a further 12 h to remove any 8-hydroxyquinoline in the aqueous phase. After replacement of the chloroform 3–4 times, the aqueous layer was removed, dialyzed and lyophilized. NMR and ultraviolet-visible spectra showed the cytochrome *c* to be native. Cytochrome *c* was labelled at methionine 65 with the spin label 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrolidinooxyl according to previously published methods [12,13].

Lecithin was extracted from fresh egg yolks according to the method of Singleton et al. [14]. To avoid oxidation, the entire procedure was carried out under nitrogen. To remove any extraneous metals, the lecithin was suspended in chloroform/methanol/water (1 : 2.2 : 1, v/v) saturated with EDTA and then

extracted with chloroform followed by chloroform/methanol (3 : 1, v/v) until the aqueous phase clarified. The lecithin was then dried by rotary evaporation under nitrogen, suspended in ethanol and stored in ampules under nitrogen at −20° C. A fresh ampule was used for each experiment.

Bovine heart cardiolipin was obtained from General Biochemical Co. as a solution in hexane. This was stored in ampules under nitrogen at −20° C and a fresh ampule used for each experiment.

Thin-layer chromatography on silica gel with chloroform/methanol/acetic acid/water (25 : 12 : 4 : 2 and 80 : 13 : 8 : 0.3, v/v) of the lecithin and cardiolipin stock solutions gave only a single spot with iodine vapour or $K_2Cr_2O_7$/H_2SO_4 followed by charring. Phosphate analysis of lipids was performed by the method of Bartlett [15]. Lipid concentrations are expressed in molarity of lipid phosphorous. Lipids were analyzed for oxidation by measurement of the 233/215 nm absorbance ratio [16].

Mixed, 1 : 4 by weight, cardiolipin-lecithin vesicles were prepared as follows: The required weights of each lipid in organic solvent were combined and taken to dryness by rotary evaporation under nitrogen. The resulting lipid film was re-suspenden in 5 mM Tris · HCl/1 mM EDTA at pH 8.0 and sonicated with a Branson type B-12 probe sonicator under a stream of nitrogen and with ice bath cooling. Total sonication time was typically 30 min, in periods of 5 min with cooling between sonication periods. The vesicles were then centrifuged at 100 000 × *g* at 10° C for 60 min to remove any larger particles. For experiments with adsorbed cytochrome *c* the protein was dissolved in the pH 8.0 Tris/EDTA buffer and readjusted to pH 8.0. The protein solution was then added dropwise to the sonicated vesicle solution with stirring and readjustment to pH 8.0. When protein was added to the vesicles, the protein-vesicle mixture was centrifuged after addition of the cytochrome *c*.

The vesicles prepared as above were characterized by electron microscopy, Sepharose 4B column chromatography, NMR and ultraviolet-visible absorption. Details will be presented elsewhere as part of structural studies of the vesicles. To summarize, the preparations appeared to consist of single bilayer vesicles of ≈300 Å diameter both with and without adsorbed cytochrome *c*. Ultrafiltration against a membrane which freely passes cytochrome *c* but not vesicles, as well as Sepharose 4B column chromatography, showed that the cytochrome *c* was firmly bound to the vesicles. The optical properties of the bound cytochrome *c* corresponded to those of free cytochrome *c*. Thin-layer chromatography of lipids extracted from fractions of the cytochrome *c*-vesicle preparations after Sepharose 4B column chromatography showed that lecithin and cardiolipin were essentially uniformly distributed in the ratio added. If care was taken to exclude oxygen from the sample and the ratio of protein was not greater than 1 : 80 (mol cytochrome *c*/mol lipid phosphate), the cytochrome *c*-vesicle preparations were stable for at least 5 days at room temperature, as evidenced by electron microscopy and NMR.

For the kinetic studies presented here, cytochrome *c* was added to the 1 : 4 cardiolipin/lecithin vesicles immediately prior to addition of the spin label. Zero time has been taken as the time of addition of the spin label. The zero time intensity of the spin label ESR signal was measured with a separate aliquot of the spin label identically diluted. ESR measurements were made on a Varian

E4 instrument at 9.5 GHz and at ambient temperature. Kinetic data have generally been plotted as $\ln([\text{spin label}]_{\text{time } t}/[\text{spin label}]_{\text{zero time}})$, i.e. $\ln(I/I_0)$, where the peak-to-peak height of the central derivative line in the ESR spectrum (I) has been used as a measure of the spin label concentration. At the lipid concentrations used for kinetic experiments only 1—2% of TEMPO was in the lipid phase, hence partitioning of the spin label between the membrane and aqueous phase [17] did not affect the peak heights. Some variation in the rate of loss of the spin label ESR signal was observed from one preparation to another and it was therefore necessary to run internal controls in each experiment.

Results

(A) Essential components of the reaction leading to the loss of spin label ESR signal intensity

Either with a spin label covalently attached to methionine 65 of cytochrome *c* or with the free spin label TEMPO added to the reaction mixture, the ESR signal rapidly diminished in the presence of cytochrome *c*, oxygen and single bilayer vesicles composed of 1 : 4 by weight cardiolipin-lecithin (Fig. 1). The spin label ESR signal was, however, essentially stable when any one of these three components was omitted from the reaction mixture. Thus, the covalently attached spin label on methionine 65 of cytochrome *c* could be kept from losing ESR intensity by carefully protecting the protein-lipid mixture from atmospheric oxygen during preparation and measurement, but began to show reduced ESR signal when the sample was subsequently exposed to air. Addition of cyanine greatly reduced the rate of loss of TEMPO ESR signal in the presence of lipid, cytochrome *c* and oxygen, suggesting that the heme group of cytochrome *c* is directly involved in the observed interactions (Fig. 2). A similar

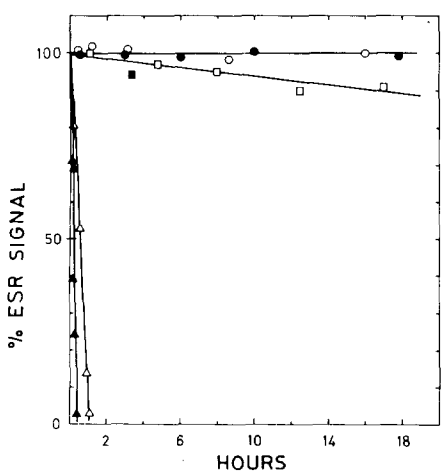

Fig. 1. Loss of spin label ESR signal as a function of time for mixtures of 1 : 4 cardiolipin/lecithin vesicles, cytochrome *c*, cytochrome *c* spin labelled at methionine 65 and TEMPO under air or nitrogen atmosphere: ○, cytochrome *c*, TEMPO, N_2; ●, vesicles, TEMPO, O_2; □, vesicles, spin-labelled cytochrome *c*, N_2; △, vesicles, cytochrome *c*, TEMPO, O_2; ▲, vesicles, spin-labelled cytochrome *c*, O_2.

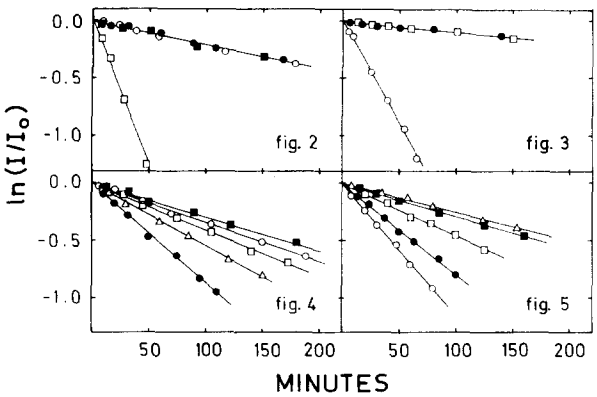

Fig. 2. Effect of cyanide on loss of spin label ESR signal. Plots of $\ln(I/I_0)$ (see Material and Methods) versus time for 1 : 4 cardiolipin/lecithin vesicles ($5.2 \cdot 10^{-3}$ M), cytochrome *c* ($5 \cdot 10^{-5}$ M) and TEMPO ($1 \cdot 10^{-5}$ M) under air: □, vesicles, cytochrome *c*, TEMPO; ○, vesicles, TEMPO; ●, vesicles, TEMPO in 10^{-2} M KCN; ■, vesicles, cytochrome *c*, TEMPO in 10^{-2} M KCN. Cytochrome *c* was equilibrated with 10^{-2} M KCN for 1 h prior to addition to lipid and TEMPO.

Fig. 3. Effect of ionic strength on loss of spin label ESR signal. Plots of $\ln(I/I_0)$ for 1 : 4 cardiolipin/lecithin vesicles ($2 \cdot 10^{-3}$ M), cytochrome *c* ($2 \cdot 10^{-5}$ M) and TEMPO ($1 \cdot 10^{-5}$ M) mixtures under air: ○, vesicles, cytochrome *c*, TEMPO; □, vesicles, TEMPO; ● vesicles, cytochrome *c*, TEMPO in 0.4 M NaCl.

Fig. 4. Effect of lipid concentration on loss of spin label ESR signal. Plots of $\ln(I/I_0)$ versus time for mixtures of cytochrome *c* ($2 \cdot 10^{-6}$ M) and TEMPO ($6 \cdot 10^{-6}$ M) with varying concentrations of 1 : 4 cardiolipin/lecithin vesicles: ○, $2 \cdot 10^{-4}$ M; □, $4 \cdot 10^{-4}$ M; △, $1 \cdot 10^{-3}$ M; ●, $2 \cdot 10^{-3}$ M; ■, $1 \cdot 10^{-3}$ M, no cytochrome *c*. In the absence of cytochrome *c*, the loss of spin label ESR signal was found to be essentially independent of lipid concentration.

Fig. 5. Effect of cytochrome *c* concentration on loss of spin label ESR signal. Plots of $\ln(I/I_0)$ versus time for mixtures of 1 : 4 cardiolipin/lecithin vesicles ($2 \cdot 10^{-3}$ M) and TEMPO ($6 \cdot 10^{-6}$ M) with varying concentrations of cytochrome *c*: ○, $1 \cdot 10^{-5}$ M; ●, $2 \cdot 10^{-6}$ M; □, $4 \cdot 10^{-7}$ M; ■, $4 \cdot 10^{-8}$ M and △, no cytochrome *c*.

rapid loss of ESR signal and inhibition of this loss by cyanide has been found when metmyoglobin is added to the cardiolipin-lecithin vesicles in the presence of air, indicating that although we have primarily investigated cytochrome *c*, other heme proteins are probably capable of the interactions described in this paper.

The ability of cytochrome *c* to cause rapid loss of the ESR signal is dependent on binding of the protein to the lipid membrane. When pure lecithin vesicles were mixed with cytochrome *c* under air, more than 100 times the concentration of cytochrome *c* was necessary to observe rates of loss of the TEMPO ESR signal comparable to the rates observed with an equivalent concentration of the mixed cardiolipin-lecithin vesicles. We attribute this to the fact that positively charged cytochrome *c* binds much more effectively to a negatively charged cardiolipin-lecithin membrane than to a neutral pure lecithin membrane [18]. The necessity for binding of cytochrome *c* to the membrane can also be shown for cardiolipin-lecithin vesicles by performing the experiment with higher salt concentration, which dissociates cytochrome *c* from such membranes [18]. As is shown in Fig. 3, inclusion of 0.4 M NaCl in the reaction mixture effectively inhibits the rapid loss of TEMPO ESR signal. These results

indicate that cytochrome *c* must be bound to the lipid membrane in order to cause rapid loss of the TEMPO ESR signal. As with cytochrome *c*, ultrafiltration (see Material and Methods) showed that myoglobin binds to 1 : 4 cardiolipin-lecithin vesicles, which concurs with the observation that myoglobin also causes rapid loss of TEMPO ESR signal. The necessity of having the protein bound to the lipid membrane in order to catalyze lipid oxidation may also explain some of the confusion in the literature (e.g. refs. 19 and 20) regarding the relative rates of lipid oxidation catalyzed by various heme proteins.

(B) Relation between the loss of spin label ESR signal and lipid oxidation

The observation in the previous section that the presence of lipid was essential for a rapid loss of spin label ESR signal indicated that the observed reaction might involve chemical modifications of the lipids. To further investigate this point, the kinetics of the loss of ESR-observable TEMPO spin label were studied and compared with earlier reports on the kinetics of heme protein-catalyzed oxidation of lipids. Figs. 4 and 5 show that the rate of loss of ESR signal increases at higher concentrations of cytochrome *c* and lipid. That the plots of $\ln(I/I_0)$ in these figures (see Material and Methods) are linear as a function of time indicates that the loss of TEMPO signal is first order in TEMPO. The slopes of the $\ln(I/I_0)$ versus time plots in Fig. 4 have been used to determine the apparent rate constant for TEMPO loss as a function of lipid concentration. By plotting the log of this apparent rate constant versus the log of the lipid concentration, the rate of loss of TEMPO ESR signal is found to depend linearly on the lipid concentration. The data in Fig. 5 can be similarly handled to determine the effect of cytochrome *c* concentrations on the rate of loss of TEMPO ESR signal, yielding the result that this rate of loss increases linearly with approximately the square root of the cytochrome *c* concentration. Thus, the loss of TEMPO ESR signal can be represented by a rate equation of the form: $d[\text{TEMPO}]/dt = -k[\text{cytochrome } c]^{1/2}[\text{lipid}][\text{TEMPO}]$. The dependency on the square root of the cytochrome *c* concentration is the most striking feature of this rate equation. Such a dependency has previously been observed for oxygen uptake during lipid oxidation catalyzed by heme proteins and has been interpreted in terms of free radical mechanisms for such oxidations [20]. Comparison of the amount of ESR signal lost with the amount of cytochrome *c* added indicates a catalytic role for cytochrome *c* in reactions leading to rapid loss of the TEMPO ESR signal. For example, in Fig. 5, after 130 min the increase in total amount of TEMPO ESR signal lost for vesicles with cytochrome *c* at $4 \cdot 10^{-7}$ M compared to vesicles without cytochrome *c* represents 180% of the cytochrome *c* present. In other experiments the total TEMPO loss has exceeded the amount of cytochrome *c* present by more than 4-fold.

In summary, the results in this and the previous section show that the rapid loss of the ESR signal requires the presence of lipid and oxygen and that the cytochrome *c* binds to the lipid membrane. Furthermore, in the reactions leading to rapid loss of ESR signal, cytochrome *c* appears to have a catalytic role and the kinetics for loss of the ESR signal are analogous to those previously observed for lipid oxidation catalyzed by heme proteins. It therefore appears that the loss of spin label ESR signal is intimately linked with heme protein-catalyzed lipid oxidation and hence that this technique can be used to study

such processes. This conclusion is further corroborated by independent evidence on the relation between the extent of lipid oxidation and the loss of ESR signal described in the following section. It is an attractive feature of the potential use of spin label ESR for studies of lipid oxidation that the technique is very sensitive. Experiments can thus be performed with relatively small amounts of lipid and at low levels of total lipid oxidation. An additional advantage over the earlier measurements of oxygen uptake is that certain reaction steps may be investigated under anaerobic condtions. This is illustrated by the experiments described in the following section.

(C) Cytochrome c-lipid interactions leading to loss of spin label ESR signal intensity under anaerobic conditions

Fig. 6 shows the time course of the loss of ESR signal intensity obtained when cardiolipin-lecithin vesicles, which had been slightly "pre-oxidized" by equal, short periods of sonication under nitrogen, air or pure oxygen and subsequently equilibrated for 24 h with a N_2 atmosphere, are mixed with cytochrome *c* and TEMPO under nitrogen and the ESR signal measured in an ESR cell sealed under nitrogen. Under these conditions the vesicles may contain some oxidized lipids, but little or no oxygen is present when the cytochrome *c* and spin label are added. In all cases, the presence of cytochrome *c* results in an initial rapid phase of TEMPO ESR signal loss, followed by a slow loss which has a rate identical to that of the lipid vesicles with no protein present. This latter slow loss appears in large part to be due to processes independent of the vesicles, since similar losses were observed for TEMPO in distilled water. The amount of TEMPO lost in the initial fast phase can be estimated by extrapolating the slow phase loss to zero time and subtracting the extrapolated value from the actual zero time value. When the total loss of TEMPO ESR signal for

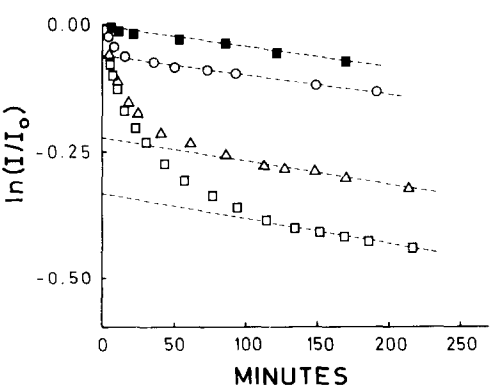

Fig. 6. Loss of spin label ESR signal with "pre-oxidized" lipids under anaerobic conditions. Plots of $\ln(I/I_0)$ versus time for mixtures of 1 : 4 cardiolipin/lecithin vesicles ($4 \cdot 10^{-3}$ M), cytochrome *c* ($4 \cdot 10^{-5}$ M) and TEMPO ($1 \cdot 10^{-5}$ M). To obtain vesicles "pre-oxidized" to varying extents, the lipids were sonicated for 15 min under an atmosphere of (○) nitrogen, (△) air or (□) oxygen. Prior to addition of cytochrome *c* and TEMPO, the vesicles were equilibrated with a nitrogen atmosphere for 24 h. (■) corresponds to vesicles sonicated under oxygen, but without addtion of cytochrome *c*. Air- or nitrogen-sonicated vesicles showed identical behaviour without cytochrome *c*. To ensure anaerobic conditions, TEMPO and cytochrome *c* in nitrogen-flushed buffer were added to the vesicles in a nitrogen box. The resulting mixture was sealed in an ESR cell under nitrogen and kept sealed during the ESR measurements.

vesicles oxidized to various levels is compared to the amount of oxidized lipid, as determined by the 233/215 nm absorbance ratio [16], the amount of loss of TEMPO ESR signal in the fast phase increases linearly with increased lipid oxidation. We have repeatedly observed this relationship in anaerobic experiments, demonstrating that differences in total ESR signal loss cannot be attributed to differences in residual oxygen content. If the 233/215 nm absorbance ratio is used to estimate the total amount of oxidized lipid [16] and this is compared to the total TEMPO loss, in the presence of cytochrome *c* and at low levels of total oxidation (<0.25%), about 20% of the oxidized lipids appear to participate in reactions leading to loss of TEMPO ESR signals. This proportion is not markedly changed either by variation of the TEMPO concentration or by allowing the vesicles to sit for a further 24 h under nitrogen prior to adding cytochrome *c* and TEMPO. From these results we conclude that: (1) cytochrome *c* interacts with some product of lipid oxidation (hereafter denoted OxL), (2) the total loss of TEMPO ESR signal reflects the amount of oxidized lipid potentially reactive in the presence of cytochrome *c*, and (3) the potentially reactive oxidized lipid (OxL) must be present as a species stable at least on the order of days. The high sensitivity of the ESR method is demonstrated by these experiments, which have been performed with ≈ 2 μmol of phospholipid oxidized to about 0.1%, i.e. with less than 1 nmol of OxL.

We have also made a kinetic analysis of the rate of loss of TEMPO ESR signal in the fast phase for the experiments with pre-oxidized vesicles shown in Fig. 6. We have assumed that the rate of loss of TEMPO ESR signal can be represented by a second-order equation of the form $d[\text{TEMPO}]/dt = -k[\text{OxL}^*][\text{TEMPO}]$, where OxL* represents the reactive species which reacts with TEMPO to destroy the ESR signal. We further assumed that the initial concentration of OxL* at zero time determines the total loss of TEMPO signal in the fast phase and hence measurement of loss of TEMPO signal can be used to measure [OxL*] in the course of the reaction. For this model, the second-order rate equation

$$Z \equiv (1/I_f) \ln[I(I_0 - I_f)/I_0(I - I_f)] = kt$$

can be written in terms of the TEMPO ESR intensity at time zero (I_0), at subsequent times (I) and for the final intensity at long times (I_f). Because of the subsequent slow phase loss of TEMPO ESR signal, the value of I_f during the fast phase loss has been estimated as a function of time by extrapolation of the slow phase loss. When the second-order rate function Z is plotted versus time, the data conforms well to a straight line, indicating that the model is appropriate and allowing determination of a second-order rate constant k for the reaction of OxL* with TEMPO. That the data fits this model so well has several implications for the reactions which lead to loss of TEMPO ESR signal. Firstly, since the data fit the second-order model at short times, the reactive species OxL* must be present in its entirety at times short compared to the time at which the first ESR measurement can be made (3—4 min). Secondly, data at long times (80—100 min) also conform to the second-order model, indicating that OxL* must be stable relative to this time period. We interpret this to mean that the interaction of the potentially reactive lipid oxidation products OxL with cytochrome *c* proceeds rapidly (i.e. complete in times $\ll$ 3 min) and

that the reactive species OxL* resulting from the OxL-cytochrome *c* interactions is stable relative to a time period of 100 min.

(D) Species in pre-oxidized vesicles reactive with spin label under anaerobic conditions

The experiments described in this section investigate the nature of the reactive species OxL* which interacts with TEMPO to destroy the ESR signal under anaerobic conditions. One possibility could be that the initially oxidized cytochrome *c* is reduced by interaction with lipid oxidation products and subsequently reduces the TEMPO spin label [21,22]. However, we found by visible absorption at 550 nm that ferricytochrome *c* remained oxidized when it was mixed with "pre-oxidized" vesicles under an argon atmosphere. This excludes direct interaction of TEMPO with reduced cytochrome *c* since the kinetic results of section C indicate that a significant proportion of the cytochrome *c* would have to remain in the reduced state for time periods on the order of 100 min for this mechanism to function.

We have further investigated whether TEMPO interacts directly with oxidized cytochrome *c* or alternatively only with lipids immediately in the neighborhood or bound to cytochrome *c*. This has been tested by experiments measuring the loss of TEMPO ESR signal with "pre-oxidized" vesicles under nitrogen (as in the experiments in Fig. 6), but with varying concentrations of cytochrome *c*. Over the range of cytochrome *c* concentrations possible, the loss of TEMPO ESR signal fit well to the second-order rate equation, Z, and hence the second-order rate constant for reaction of the reactive species OxL* with TEMPO could be measured as a function of cytochrome *c* concentration. The results are shown in Table I. For a 10-fold change in cytochrome *c* concentration, the second-order rate constant remains constant within experimental error, indicating that the rate loss of TEMPO ESR signal is independent of cytochrome *c* concentration under these conditions. Only slight variation in the total amount of TEMPO ESR signal lost in the fast phase was observed (Table I), corroborating the conclusion of section C that interaction of cytochrome *c* with OxL to produce OxL* is rapid. For the results shown in Table I, the total loss of TEMPO ESR signal in the fast phase does not exceed the amount of cytochrome *c* present. However, the variation in cytochrome *c* concentration cannot be easily extended further in the present vesicular system since at lower

TABLE I

KINETIC PARAMETERS FOR LOSS OF TEMPO ESR SIGNAL UNDER ANAEROBIC CONDITIONS IN A SOLUTION CONTAINING $4 \cdot 10^{-3}$ M LIPID, $2 \cdot 10^{-5}$ M TEMPO AND VARIABLE CONCENTRATIONS OF FERRICYTOCHROME *c*

Lipids "pre-oxidized" by sonication under oxygen and mixed with cytochrome *c* and TEMPO as described in legend to Fig. 6.

Cytochrome *c* concentration	k ($M^{-1} \cdot min^{-1}$) *	Total TEMPO loss in fast phase
$4 \cdot 10^{-5}$ M	$4.3 \pm 0.2 \times 10^3$	$4.8 \cdot 10^{-6}$ M
$8 \cdot 10^{-6}$ M	$4.1 \pm 0.4 \times 10^3$	$4.6 \cdot 10^{-6}$ M
$4 \cdot 10^{-6}$ M	$5.0 \pm 0.4 \times 10^3$	$3.9 \cdot 10^{-6}$ M

* Second-order rate constant for loss of TEMPO ESR signal under anaerobic conditions as described in the text.

protein-to-lipid ratios many vesicles may have no bound cytochrome *c* and at higher protein-to-lipid ratios the vesicles are no longer stable. However, in other experiments we have observed total losses of TEMPO in the fast phase which exceeded the amount of cytochrome *c* by up to three times. Furthermore, for the lowest cytochrome *c* concentration shown in Table I, simple statistical considerations (assuming random distribution of OxL and cytochrome *c*) indicate approx. 40% of the vesicles contain more OxL than cytochrome *c*.

Since a good fit to second-order kinetics was found over the entire range of cytochrome *c* concentrations studied, we conclude that cytochrome *c* interacts rapidly with all OxL in vesicles to which it is bound, even if the amount of OxL exceeds the amount of cytochrome *c* for the individual vesicle. The results in this section would support that loss of TEMPO ESR signal occurs subsequently to the cytochrome *c*-OxL interaction and does not directly involve cytochrome *c*. Also, the reaction cannot be restricted to only those oxidized lipids which were initially in the immediate environment of cytochrome *c*.

(E) Use of spin labels with differing lipophilicity to localize the reactive species

To further investigate the nature of the species OxL* in the systems containing lipid, cytochrome *c* and spin label, we have varied the lipophilic character of the spin label used. Corresponding systems containing respectively TEMPO, TEMPOL or TEMPO phosphate (see Materials and Methods section for the structures of these compounds) were compared in these experiments.

Under anaerobic conditions, i.e. with mixtures of cytochrome *c* plus 1 : 4 cardiolipin-lecithin vesicles pre-oxidized by sonication under air (see Fig. 6 legend), TEMPO and TEMPOL show the previously observed fast phase of loss of ESR signal, followed by a loss rate equivalent to that for vesicles alone (Fig. 7). With TEMPO phosphate, the loss of ESR signal is very slow (Fig. 7). Assuming that the slow loss for TEMPO phosphate arises from the same reactions leading to the fast phase loss of TEMPO and TEMPOL, the relative second-order rate constants under anaerobic conditions would be TEMPO =

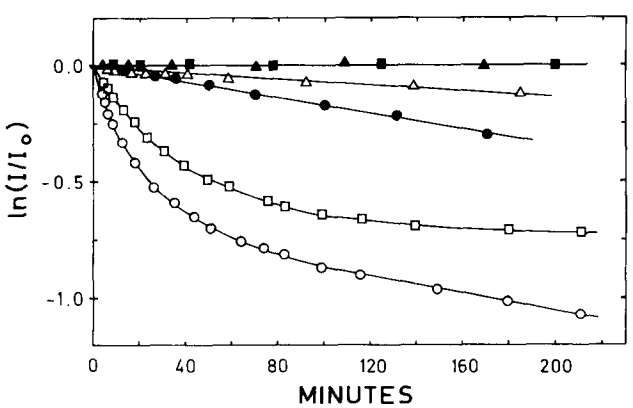

Fig. 7 Effect of type of spin label on loss of ESR signal under anaerobic conditions. Plots of $\ln(I/I_0)$ versus time for various spin labels ($1 \cdot 10^{-5}$ M) with 1 : 4 cardiolipin/lecithin vesicles ($4 \cdot 10^{-3}$ M) and with and without cytochrome *c* ($4 \cdot 10^{-5}$ M). Vesicles "pre-oxidized" by sonication under air were prepared and combined as described in the legend to Fig. 6. ○, ●, TEMPO; □, ■, TEMPOL: △, ▲, TEMPO phosphate; ○, □, △, with cytochrome *c*; ●, ■, ▲, without cytochrome *c*.

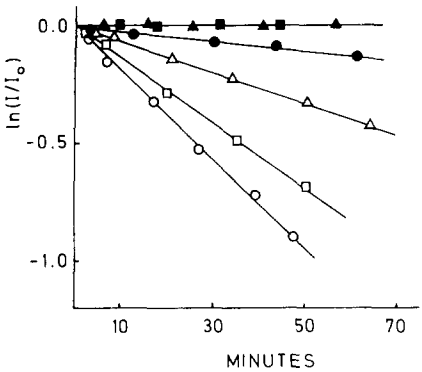

Fig. 8. Effect of type of spin label on rate of loss of ESR signal under aerobic conditions. Plots of $\ln(I/I_0)$ for various spin labels ($1 \cdot 10^{-5}$ M) with 1 : 4 cardiolipin/lecithin vesicles ($2 \cdot 10^{-3}$ M) and with or without cytochrome *c* ($2 \cdot 10^{-5}$ M). ○, ●, TEMPO; □, ■, TEMPOL; △, ▲, TEMPO phosphate: ○, □, △, with cytochrome *c*; ●, ■, ▲, without cytochrome *c*.

1.0, TEMPOL = 0.5 and TEMPO phosphate = 0.015. Corresponding experiments performed under aerobic conditions (Fig. 8) show that the rate of loss of ESR signal from the spin labels TEMPO, TEMPOL and TEMPO phosphate follows the same order as observed under anaerobic conditions, with relative rates of 1.0 for TEMPO, 0.8 for TEMPOL and 0.4 for TEMPO phosphate.

We have directly investigated the lipophilicity of the three spin labels by measuring by ESR the partitioning between the pH 8.0, Tris/EDTA buffer normally used in our experiments and hexane. The relative values found for the partition coefficient (hexane/buffer) were TEMPO = 100, TEMPOL ≈ 0.10, TEMPO phosphate ≈ 0.01. Comparing these relative distribution coefficients with the rate of loss of ESR signals under anaerobic conditions shows that as the lipophilicity of the spin label is increased, the rate of loss of ESR signal also increases. Since there are only minor variations in the redox potentials of such spin labels [23], the present results thus suggest that the rate of reactions leading to the loss of ESR signal is greater in the membrane interior than in the aqueous phase.

The sizeable difference in the relative rate of loss of TEMPO phosphate ESR signal under aerobic (rate = 0.4 of TEMPO rate) and anaerobic (≈0.01 of TEMPO rate) conditions suggests that an alternative reaction mechanism is operative in the presence of oxygen. This mechanism appears to lead to water-soluble reactive species capable of reacting with TEMPO phosphate. As noted above, in the absence of oxygen, the reactive species capable of interacting with spin labels appear to be predominantly in the membrane interior and therefore less accessible to reactions with TEMPO phosphate.

Discussion

From the observed functional characteristics, we formulate the reactions involving the various oxidized lipid species, OxL, OxL* and OxL** in the present experiments in terms of the following scheme:

$$\text{OxL} \xrightarrow{\text{cytochrome } c} \text{OxL*, cytochrome } c \xrightarrow{\text{TEMPO}} \text{OxL**, TEMPO*, cytochrome } c$$

Initially in the vesicle preparation there exists a small proportion of a potentially reactive lipid oxidation product (OxL). When cytochrome *c* is added and binds to the lipids, reaction occurs between cytochrome *c* and OxL, yielding a product(s) OxL* which is capable of reacting with a spin label such as TEMPO. This latter reaction destroys the spin label ESR signal and yields a product(s) TEMPO*. In the presence of oxygen still other reactions appear to lead to spin label loss suggesting that OxL* is also reactive with oxygen.

We have not directly investigated the lipid species (OxL*) or spin label products (TEMPO*) involved in these reactions, but certain species thought to be included in lipid oxidation reactions can be excluded. For example, the stability of OxL (days) and of OxL* (hours) under anaerobic conditions preclude the possibility that these are small free radicals such as superoxide or hydroxide radicals. Under aerobic conditions, where the reactions leading to loss of spin label ESR intensity seem to include water-soluble species, such small radicals may be involved. Lipid peroxides, which will be a significant product of lipid oxidation at low total oxidation levels [24] and which are known to react with heme proteins [25,26], appear to be a possible identity for OxL. If so, OxL* under anaerobic conditions could be a lipid-free radical [24], thus explaining the enhanced reactivity with lipid-soluble spin labels. In the hydrophobic membrane interior, under anaerobic conditions and at low total concentration, so that free radical chain termination mechanisms are less probable, a lipid-free radical might have a relatively long life time [27], as is indicated for OxL* by the experiments in section C. Under aerobic conditions such a free radical could be expected to interact with oxygen to yield other products [24], thus offering a possible explanation for the differences in rate of loss of ESR signal under aerobic and anaerobic conditions for spin labels of varying lipophilicity (section E).

Although the importance of the type of lipid interacting with cytochrome *c* has not been directly investigated in the present experiments (except to show that lipids that bind cytochrome *c* to the membrane must be included), we note that cardiolipin may be particularly effective in promoting reactions of the type observed in the present experiments. Our NMR and ESR studies of the structural organization of the cytochrome *c*-vesicle preparation indicate that the cardiolipin in the vesicles is concentrated near the bound cytochrome *c* (to be published). This together with the high content of unsaturated fatty acids in beef heart cardiolipin [28] (double bonds are necessary for lipid oxidation), suggest that in comparison to other lipids cardiolipin may preferentially interact with cytochrome *c* in the reactions observed here.

A further very interesting point in these studies is the rapidity of the interaction between cytochrome *c* and OxL. The present data indicate that for vesicles having one bound cytochrome *c* molecule, which can be estimated to occupy on the order of 0.5% of the external surface area of the vesicle [29], and having an average of 2—3 equivalents of OxL amongst the 5000—6000 fatty acid chains in a vesicle, the cytochrome *c* interacts with all OxL in times $\leqslant 3$ min (see section C). From the kinetic results in sections C and D it is clear that the reactive OxL is not limited to lipid bound or near to cytochrome *c* and therefore that the cytochrome *c* is capable of rapid interaction with lipid regions far larger than the region directly occupied by the protein molecule.

One possibility is that rapid lateral diffusion of the lipid in the membrane brings all lipid into contact with the cytochrome *c* within short time periods. An alternative could be that cytochrome *c* effectively interacts with lipid molecules well removed from the protein, perhaps through some form of free radical chain mechanism. However, it is apparent that in either case reactive equivalents must be rapidly transported within the hydrophobic membrane interior parallel to the membrane surface. This point, plus the observed production under anaerobic conditions of a long lived reactive species (OxL*) apparently located in the membrane interior, seem to us to have interesting implications for possible modes of functioning in native biological membranes.

In conclusion, the present studies demonstrate that the ESR method can be used to study lipid oxidation processes and further indicate some possible experimental techniques to avoid such processes when the spin label is desired for other purposes. The high sensitivity of ESR allows the lipid oxidation studies to be made with micro-molar amounts of lipids containing very low levels of total oxidation. The ability to eliminate oxygen from the system may allow a more direct study of particular steps in such processes, as demonstrated here for the interaction of cytochrome *c* with lipids. In principle it may also be possible to study the spatial distribution of such processes within a membrane system by use of spin labels that can be restricted to certain membrane regions. As illustrated here and elsewhere [30—32], many processes may lead to loss of spin label ESR signals and hence be accessible to study by ESR methods.

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