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INITIATION OF LIPID PEROXIDATION AS A RESULT OF THE COMBINED ACTION OF FERRIC IRON AND LIGHT ON MEMBRANES

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Abstract – The synergic effect of iron plus blue light on the peroxidation of membrane lipid was investigated, using liposomes made of phospholipid. While strong irradiation did not affect Fe^{+2}-promoted lipid peroxidation that turned out to be O_2-dependent, ferric iron in bright light exerted a pronounced effect on the initiation of lipid peroxidation: this combined action of light and Fe^{+3} on liposomal membranes was apparently independent of O_2. When liposomal samples containing Fe^{+3} were subjected to irradiation, some portions of Fe^{+3} were converted into Fe^{+2}. The extent of the Fe^{+3}-Fe^{+2} conversion increased with increasing time of irradiation, which resembled the dependence of Fe^{+3}-promoted lipid peroxidation on irradiation. Further, it was observed that the effect of irradiation in liposomal samples containing Fe^{+3} was strikingly mimicked by that of Fe^{+2}-addition to the same samples. The obligatory requirement of a suitable Fe^{+3}/Fe^{+2} ratio for the genesis of iron-dependent lipid peroxidation, a controversial proposition, was also confirmed by the observation that lipid peroxidation was substantially enhanced by the addition of a mixture of Fe^{+3} and Fe^{+2}, as compared to the addition of Fe^{+3} or Fe^{+2} alone. The results obtained in this study not only suggest that light acts as an effector for initiating lipid peroxidation, when Fe^{+3} is present in membrane systems, but also imply that any chemical or physical factor that influences the redox states of iron in membranes can play a role in lipid peroxidation reactions.

INTRODUCTION

The involvement of iron in lipid peroxidation of membranes has long been observed. However, the role played by iron in this is still the subject of considerable debate. Although the reactive species immediately responsible for the initiation of iron-catalyzed lipid peroxidation and the underlying mechanism have yet to be elucidated, there now is accumulating evidence that the ratio of ferric to ferrous iron (Fe^{+3}/Fe^{+2}) is critically important in promoting lipid peroxidation.[1,2] A consistent observation is that oxidation of Fe^{+2} or reduction of Fe^{+3} is necessary for supporting iron-promoted peroxidation reactions of membrane lipid; maintenance of the redox states of iron exclusively in Fe^{+2} or Fe^{+3} alone by admixing an excessive amount of an oxidizing or a reducing agent rather results in inhibition of lipid peroxidation.[3-5] The conflicting data in the literature regarding the functions of various oxidizing and reducing agents in connection with lipid peroxidation in the presence of iron might therefore be viewed as arising from the difference of their action in creating the Fe^{+3}/Fe^{+2} ratios in the system studied. In this respect, it seems reasonable to assume that any chemical or physical factor can influence membrane lipid peroxidation as long as it creates or alters the Fe^{+3}/Fe^{+2} ratio.

It is generally accepted that Fe^{+3} is extremely inefficient in bringing about lipid peroxidation, as compared with Fe^{+2} which undergoes autooxidation under aerobic conditions.[1,6,7] However, in a preliminary experiment we were able to detect the occurrence of peroxidation reactions in liposomal suspensions, when incubated with ferric chloride in room light quite a while. Because light can induce the Fe^{+3} to Fe^{+2} conversion when Fe^{+3} is bound or complexed to certain organic ligands,[8] it may be inferred that light creates the Fe^{+3}/Fe^{+2} ratio in systems containing membrane vesicles and Fe^{+3} and thereby act as an effector for Fe^{+3}-dependent peroxidation of lipid. The results obtained in the present study corroborate this inference.

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† *Abbreviations* : ·OH, hydroxyl radical; LOOH, lipid hydroperoxide ; TBARS, thiobarbituric acid-reactive substances

MATERIALS AND METHODS

Liposomes were prepared both from egg yolk L-α phosphatidyl choline (Sigma type VII-E) and from phospholipid separated from soybean hypocotyls by an adaptation of published method.[9] A chloroform stock solution of the lipid in a 100 mL pear-shaped flask was evaporated *in vacuo*. The resulting lipid film was suspended in a N_2-purged phosphate buffer saline (10 m*M* phosphate and 125 m*M* NaCl, adjusted to pH 7.0) to a concentration of 1.0 - 1.5 m*M* by vortexing for 10 min followed by a brief bath sonication and finally by a probe sonication for 3 × 20 sec at 20 kHz using a MSE soniprep 150 ultrasonic disintegrator. All procedures for the liposome preparation were performed under safety light and nitrogen atmosphere.

The amounts of phospholipid in liposomal suspensions were determinedby measuring phosphorous, as in Bartlett.[10] Thiobarbituric acid-reacting substances (TBARS)† formed in liposomal suspensions were assayed according to the method of Buege and Aust.[11] In order to avoid the formation of TBARS catalyzed by iron itself during heating samples, butylated hydroxyl toluene (0.03 volume of 2% ethanolic solution) was added to the thiobarbituric acid (0.375% w/v)-trichloroacetic acid (15% w/v) - HCl (0.25 N) mixture prior to admixing with the liposomal suspensions.

A colorimetric method was employed for the analysis of ferrous iron converted from ferric iron in liposomal suspensions, as described by Mazur *et al.*[12] One hundred μL of 50 m*M* α-α'-dipyridyl solution in ethanol was added to 1 mL liposomal suspensions containing $FeCl_3$; after diluting samples by adding 1 mL ethanol, the resulting OD changes at 520 nm were read.

Photolysis was performed, otherwise stated, with blue light that was obtained from a 1kW Xe-arc lamp (Osram, Muncher, Germany) by using an absorption filter (λ_{max} = 425 nm; effective bandwidth = 98 nm). During irradiation samples were bubbled gently with either air or nitrogen.

All chemicals were purchased from either Sigma Chemical Co (St. Louis. MO), Wako Pure Chemical Ind (Osaka, Japan), or Fluka Chemie AG (Buchs, Switzerland). A Cary 118 C spectrophotometer (Varian, Palo Alto, CA) was used for all spectrophotometric measurements throughout the work.

RESULTS AND DISCUSSION

Liposomal preparations from egg yolk lecithin were subjected to various treatments that were provided by combinations of light (λ_{max} at 425 nm, the effective bandwidth of 98 nm, and the fluence rate at 100 W/m^2), air (1 atm) and iron ($FeCl_2$ or $FeCl_3$ at 100 μ*M*), and then analyzed for the formation of TBARS, that is widely used as an index for the degree of peroxidation of unsaturated fatty acids. The results (Table 1) indicated that, even in the absence of exogenous compounds acting as chelating or redox reagents for iron that have been frequently implicated to be associated with the promotion of iron-catalyzed lipid peroxidation,[13-16] the peroxidation reactions take place in liposomal membranes only if certain conditions are met; that is, the presence of molecular oxygen is required for the initiation to a significant extent of Fe^{+2}-catalyzed peroxidation

Table 1. Production of TBARS in liposomes prepared from egg yolk L-α phosphatidyl choline under varied treatment conditions

Conditions of treatment*			TBARS
Light	Oxygen	Iron	(mmol/mol PC)
-	-	-	20
-	-	Fe^{+2}	55
-	-	Fe^{+3}	54
-	+	-	25
-	+	Fe^{+2}	170
-	+	Fe^{+3}	42
+	-	-	23
+	-	Fe^{+2}	58
+	-	Fe^{+3}	184
+	+	-	38
+	+	Fe^{+2}	180
+	+	Fe^{+3}	165

* The presence and the absence of factors for treatment are denoted by the symbols + and -, respectively. Iron in the form of either $FeCl_3$ or $FeCl_2$ was used. For other treatment conditions, see Materials and Methods. TBARS was measured after 30 min incubations. Data are averages of duplicate measurements.

and the presence of light for that of Fe^{+3}-promoted peroxidation, respectively. As substantiated by the kinetic data, light did not play any meaningful role in lipid peroxidation by ferrous iron (Fig. 1), whereas molecular oxygen did not influence the peroxidation reactions induced by the combination of ferric iron and light (Fig. 2).

Because O_2 can act as an oxidant for Fe^{+2}, not very efficient albeit, as indicated by the autooxidation of Fe^{+2} in aerated solutions, the requirement of O_2 for Fe^{+2}-dependent lipid peroxidation may naturally be interpreted in terms of the oxidative creation of the Fe^{+3}/Fe^{+2} ratio, that is presumed to be a prerequisite for the initiation of lipid peroxidation by iron. On the other hand, the occurrence of Fe^{+3}-dependent peroxidation as a result of the combined action of light and Fe^{+3} is certainly intriguing, for the observation of such phenomenon has never been reported. Assuming that the Fe^{+3}/Fe^{+2} ratio was created through photoreduction of Fe^{+3}, we examined whether Fe^{+2} was produced in liposomal suspensions containing Fe^{+3} upon exposure to light in the near UV to blue region, where Fe^{+3} in aqueous

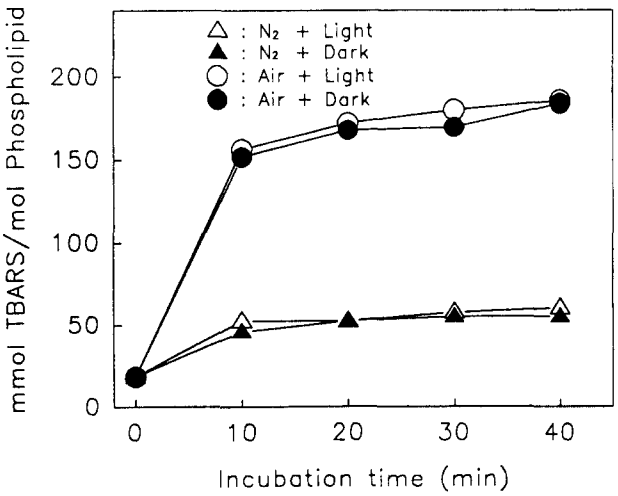

Figure 1. Membrane lipid peroxidation by ferrous iron in liposomes made of L-α phosphatidyl choline from egg yolk. Liposomal samples(0.1μmol phospholipid/mL) containing $FeCl_2$ (0.1μmol/mL) were exposed to blue light (λ_{max} = 425 nm, effective band width = 98 nm, and the fluence rate = 100 W/m²) either in air or in nitrogen atmosphere for the specified periods of incubation at 25°C and immediately subjected to hot acid digestion in the presence of thiobarbituric acid. The amounte of TBARS formed were measured spectrophotometrically. Dark controls were conducted by using samples kept in the dark for the same periods at 25°C For details of the procedures, see Materials and Methods. Data are averages of duplicate measurements.

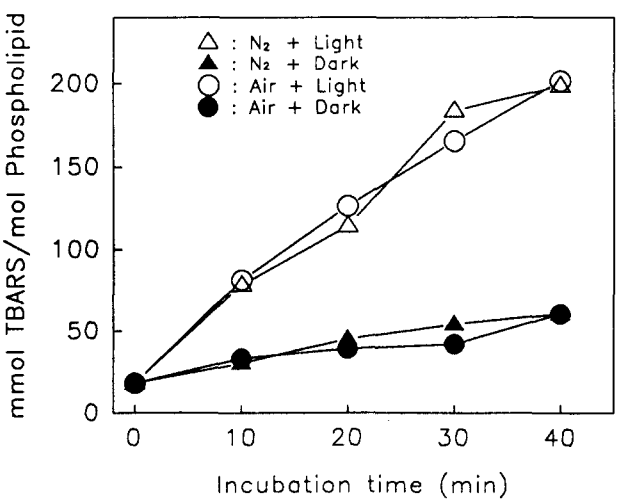

Figure 2. Membrane lipid peroxidation by ferric iron in liposomes. Experimental conditions are the same as in Figure 1 except that $FeCl_3$ (0.1μmol/mL) was added to liposomal suspensions instead of $FeCl_2$. Data are averages of duplicate measurements.

solutions shows a considerable absorption. As expected, photoreduction of Fe^{+3} indeed occurred (Fig. 3). Some portions of Fe^{+3} were rapidly reduced to Fe^{+2} upon the addition of ferric chloride solution into liposomal suspensions (note the production of Fe^{+2} of *ca* 25 mmol/mol lipid, independent of irradiation, that remained nearly constant during the dark incubation). This light-independent reduction of Fe^{+3} might occur through the redox reaction between Fe^{+3} and lipid hydroperoxide (LOOH) pre-existing in lipid molecules. The existence of LOOH can be deduced from a consistent observation that TBARS

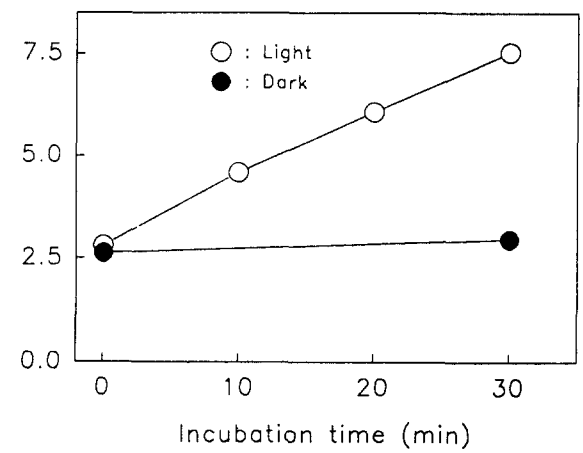

Figure 3. Changes in the concentration of ferrous iron in liposomal suspensions converted from ferric iron by blue light irradiation in the presence of air. Liposomes and incubation conditions are the same as in Figure 1. Data are averages of duplicate measurements.

was formed in liposomes that had not been subjected to the peroxidation-inducing treatments, as shown in Figures 2, 3 and 4. The Fe^{+3}-LOOH reaction has also been proposed to be the primary process for the initiation of lipid peroxidation, if any, catalyzed by Fe^{+3} alone.[1]

For them present, no information is available for addressing a question as to how Fe^{+3} can be reduced by phospholipid. Since some ligands are readily oxidized by the excited Fe^{+3}, as best known, for instance, in the photooxidation of oxalate to CO_2 by Fe^{+3} with high quantum yields in UV and visible light, it would not be totally unreasonable to suppose that Fe^{+3} is photoreduced through the following reaction:

$$LH + Fe^{+3} \xrightarrow{h\nu} L\cdot + H^+ + Fe^{+2}$$

where LH is assumed to be the unsaturated fatty acid constituents of phospholipid and H to be the allylic hydrogens in fatty acid chains containing methylene-interrupted double bonds (note that the allylic hydrogens are intrinsically susceptible to hydrogen abstraction proceeding in the primary process of peroxidation of polyunsaturated fatty acids catalyzed by various reactive species).

Taken as a working hypothesis, the creation of a Fe^{+3}/Fe^{+2} ratio is the immediate cause of promot-ing iron-dependent lipid peroxidation. Therefore, the effect of irradiation of a Fe^{+3}-containing liposomal sample should mimic that ofm addition of Fe^{+2} into the samen sample in the dark. The time-sequential measurements of TBARS production in liposomes, subjected to the respective treatments, clearly revealed that such conjecture was valid, as indicated by very similar kinetic patterns showing rather rapid formation of TBARS upon either irradiation or Fe^{+2} addition (Fig. 4).

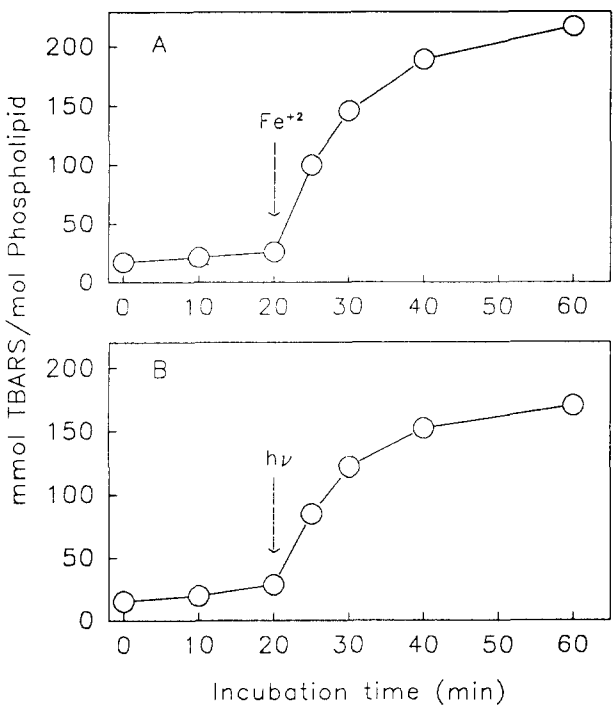

Figure 4. Effects of Fe^{+2}-addition (A) and irradiation (B) on lipid peroxidation in liposome made of L-α phosphatidyl choline in the presence of $FeCl_3$ under aerobic conditions. Liposomal suspensions (0.1 μmol lipid/mL) were admixed with $FeCl_3$ solution (0.1 μmol/mL) and stored in the dark for 20 min at 25°C, and then either irradiated with near UV to visible light ($\lambda > 320$ nm) at 30 W/m^2 for the specified periods or added by $FeCl_2$ solution (0.1 μmol/mL) and kept in the dark. Data are averages of duplicate measurements.

Because all the above results were obtained from liposomal membranes made of only a single type of phospholipid, L-α phosphatidyl choline from egg yolk, they may suffer from lack of physiological relevance. It should therefore be desirable to check whether liposomes prepared with whole phospholipid from certain tissues of a living organism, that are rather suited as a model for biological membranes, produce the compatible results. For this, we used phospholipid isolated form soybean hypocotyls for the preparation of liposomes that consists of phosphatidyl choline (52.5%), phosphatidyl ethanolamine (27.5%), phosphatidyl inositol (6.5%), phosphatidyl glycerol (5.9%) and miscellaneous phospholipids (7.4%). Conforming to our expectation, the synergic effect of Fe^{+3} plus light in liposomes such prepared was virtually identical with that in liposomes prepared only with egg yolk lecithin (Fig. 5; compare this with Fig. 2).

Hydroxyl radical (·OH) has frequently been proposed as the reactive species initiating lipid peroxidation by iron.[16] If such is the case, iron is supposed to be directly involved in certain ·OH-

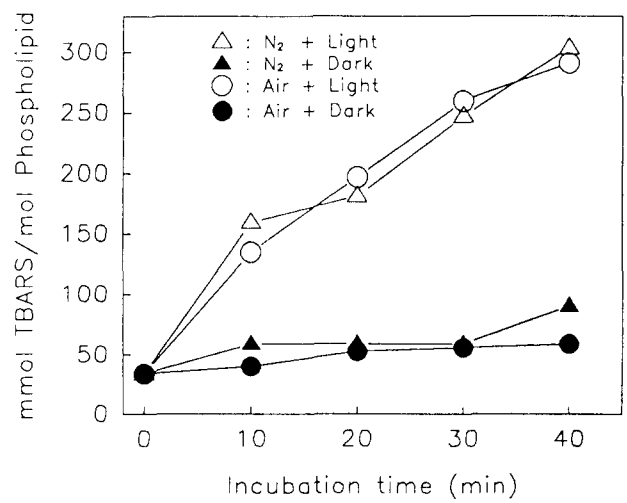

Figure 5. Membrane lipid peroxidation in liposomes made of whole phospholipid from soybean hypocotyls. Experimental conditions are the same as in Figure 1. Data are averages of duplicate measurements.

producing reactions either as a catalyst or as a reactant : the Haber-Weiss reaction and the Fenton reaction are the typical examples of these. However, the candidacy of ·OH for the initiator of the peroxidation reactions in membranes has also been questioned,[17,18] largely based on the observation that catalase, which removes H_2O_2 from reaction systems and thereby inhibits the production of ·OH, and the well-known ·OH scavengers, such as Tris, mannitol and benzoate, fail to protect membrane lipid from peroxidation and, in some cases, even stimulate it.[1,17] Instead, several iron-oxygen complexes, such as perferryl iron (Fe^{+2}-O_2 or Fe^{+3}-O_2^-), ferryl ion (FeO^{+2} or $FeOH^{+3}$) and a ferrous-dioxygen-ferric complex (Fe^{+2}-O_2-Fe^{+3}) have been hypothesized as the alternative to ·OH.[19,20] These hypothetical complexes require oxygen for their formation. This fact is seemingly consistent with the requirement of molecular oxygen for promoting iron-dependent lipid peroxidation in the case that

iron is supplied to liposomal suspensions in the form of Fe^{+2} (Fig. 1). Nonetheless, the concept of the iron-oxygen complexes as the initiating species is apparently not pertinent to the result obtained from the liposome-Fe^{+3} system, subjected to irradiation under anaerobic conditions (Fig. 2).

Leaving such embroiled controversy over the iron-related initiating species to further investigations in the near future, we assayed the production of TBARS in a number of liposomal samples that contained iron at a fixed concentration with varied ferric to ferrous ratios and were incubated in the dark, in order to check whether and to what extent the presence of molecular oxygen affects membrane lipid peroxidation presumably controlled by the Fe^{+3}/Fe^{+2} ratio. As can be noted from the data presented (Fig. 6), it appears evident that,

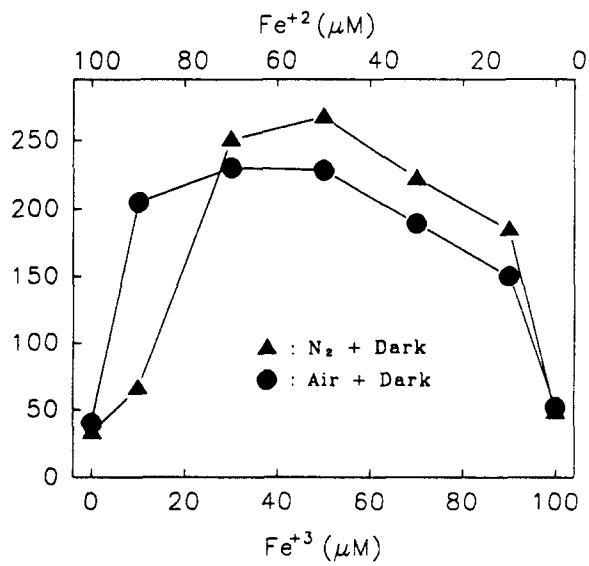

Figure 6. Lipid peroxidation in liposomal membranes catalyzed by various combinations of ferric and ferrous iron. Liposomal samples (0.1 μmol/mL) containing iron (Fe^{+3} + Fe^{+2}) at a concentration of 0.1 μmol/mL with varied ratios of Fe^{+3} to Fe^{+2} from 1/9 to 9/1 were incubated either aerobically or anaerobically at 25 °C in the dark. After 30 min of incubation, the samples were immediately subjected to the assays for TBARS production. Liposomes were made of whole phospholipid from soybean hypocotyls. Data are averages of duplicate measurements.

regardless of the presence or absence of molecular oxygen, the Fe^{+3}/Fe^{+2} ratio is indeed the pivotal, if not the only, parameter determining the rate of the initiation step of lipid peroxidation catalyzed by iron, being the mosteffective when it is 1:1 ratio.

Taken the results presented in this paper all together, the followings are to be concluded: (1) the primary process of lipid peroxidation is induced by a simultaneous supply of Fe^{+3} and blue light to liposome, neither of which *per se* is quite effective in giving rise to the membrane-deteriorating processes; (2) some of Fe^{+3} present in liposomal samples are converted into Fe^{+2} by blue light irradiation, creating the Fe^{+3}/Fe^{+2} ratio which is presumably a prerequisite for the initiation of lipid peroxidation; and (3) the provision of the Fe^{+3}/Fe^{+2} ratio for the system by adding a ferrous iron solution to liposomal suspensions containing Fe^{+3} results in promoting lipid peroxidation, which strikingly mimics the deleterious effect exerted by the combination of light and Fe^{+3} in liposomes.

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REFERENCES

1. Minotti, G. and S. D. Aust (1987) The requirement for iron(III) in the initiation of lipid peroxidation by iron (II) and hydrogen peroxide. *J. Biol. Chem.* **262**,1098-1104.
2. Braughler, J. M., L. A. Duncan, and R. L. Chase (1986) The involvement of iron in the lipid peroxidation. *J. Biol. Chem.* **261**, 10282-10289.
3. Aruoma, O. I., B. Halliwell, M. J. Laughton, G. J. Quinlan and J. M. C. Gutteridge (1989) The mechanism of initiation of lipid peroxidation. Evidence against a requirement for iron(II)-iron(III) complex. *Biochem. J.* **258**, 617-620.
4. Braughler, J. M., R. L. Chase and J. F. Pregenzer (1988) Stimulation and inhibition of iron-dependent lipid peroxidation by desferrioxamine. *Biochem. Biophys. Res. Comm.* **153**, 933-938.
5. Miller, D. M. and S. D. Aust (1989) Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation. *Arch. Biochem. Biophys.* **271**, 113-119.
6. Halliwell, B. and J. M. C. Gutteridge (1990) Role of free radicals and catalytic metal ions in human disease; An overview. *Methods in Enzymol.* **186**, 1-88.
7. Fuji, T., Y. Hiramoto, J. Terao and K. Fukuzawa (1991) Site-specific mechanisms of initiation by chelated iron and inhibition by α-tocopherol of lipid peroxidase-dependent lipid peroxidation in charged micelles. *Arch. Biochem. Biophys.* **284**, 120-126.
8. Chao, C. -C. and A. E. Aust (1993) Photochemical reduction of ferric iron by chelators results in DNA strand breaks. *Arch. Biochem. Biophys.* **300**, 544-550.
9. Muller-Runkel, R., J. Blais and L. I. Grossweiner (1989) Photodynamic damage to egg lecithin liposome. *Photochem. Photobiol.* **33**, 683-687.
10. Bartlett, G. R. (1959) Phosphorous assay in column chromatography. *J. Biol. Chem.* **234**, 466-468.
11. Buege, J.A. and Aust, S. D. (1978) Microsomal lipid peroxidation. *Methods in Enzymol.* **52**, 302-310.
12. Mazur, A., S. Gree, A. Saha and A. Carleton (1958)

Mechanism of release of ferritin iron in vivo by xanthine oxidase. *J. Clin. Invest.* **37**, 1809-1817.
13. Marton, A., S. -R. Noerni, A. Vertes and I. Horvath (1987) The effect of EDTA-Fe (III) complexes with different chemical structure on the lipid peroxidation in brain microsomes. *Biochem. Biophys. Res. Comm.* **145**, 211-217.
14. Fukuzawa, K., T. Fujii and K. Mukai (1991) Peroxide-dependent and independent lipid peroxidations catalyzed by chelated iron. *Arch. Biochem. Biophys.* **290**, 489-496.
15. Miller, D. M., T. A. Grover, N. Nayini and S. D. Aust (1993) Xantine oxidase and iron-dependent lipid peroxidation. *Arch. Biochem. Biophys.* **301**, 1-7.
16. Aust, S. D. and B. A. Svingen (1982) The role of iron in enzymatic lipid peroxidation. In *Free Radicals in Biology* (Edited by W. A. Pyror), Vol. 5, pp. 1-28. Academic Press, New York.
17. Halliwell, B. and J. M. C. Gutteridge (1989) Lipid peroxidation: a radical chain reaction. In *Free Radicals in Biology and Medicin* (Edited by B. Halliwell and J. M. C. Gutteridge), pp. 188-276. Clarendon Press, Oxford.
18. Gutteridge, J. M. C. (1984) Ferrous iron-EDTA stimulated phospholipid peroxidation. *Biochem. J.* **224,** 697.
19. Goddard, J. G. and G. Sweeney (1987) Delayed, ferrous iron-dependent peroxidation of rat liver microsomes. *Arch. Biochem. Biophys.* **259**, 372-381.
20. Minotti,G. (1990) NADPH and adriamycin-dependent microsomal release of iron and lipid peroxidation. *Arch. Biochem. Biophys.* **277**, 268-276.