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REDOX CYCLING OF BLEOMYCIN–Fe(III) BY AN NADH-DEPENDENT ENZYME, AND DNA DAMAGE IN ISOLATED RAT LIVER NUCLEI

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Abstract—Isolated rat liver nuclei were incubated aerobically with bleomycin (BLM) and $FeCl_3$ in the presence of NADH. An increase in NADH and oxygen consumption was observed accompanied by DNA cleavage as shown by gel electrophoresis. Malondialdehyde (MDA) was also formed, which partly derived from DNA indicating an oxidative cleavage mechanism. BLM and NADH were obligatory to provide these effects, whereas $FeCl_3$ could be omitted, without a complete loss of the activities mentioned above. This was explained by the presence of some iron in the nuclei.

NADPH was consumed to a lesser extent compared to NADH and was less effective with respect to O_2 consumption and MDA formation.

It could be excluded that mitochondrial or microsomal contaminations in nuclear preparations were responsible for the effects observed.

The results suggest that the BLM–Fe(III)-complex can be repeatedly reduced (redox cycled) by NADH- (and NADPH-) dependent reductases of liver nuclei to BLM–Fe(II) which is known to form reactive oxygen species and to damage DNA. It is concluded that the enzymatic reduction of a BLM–metal complex in the cell nucleus may be an essential step in the cytotoxic activity of bleomycin.

Bleomycins (BLM)† are a class of glycopeptide antibiotics with cytostatic and cytotoxic properties which form complexes with a variety of metal ions [1]. The reduced BLM–iron-complex activates oxygen and induces DNA single and double strand breaks which are considered to be responsible for the action of the drug [2–5]. However, the mechanism of activation of the BLM–iron complex in whole cells is still not clear. Since Fe^{3+} is available for bleomycin *in vivo*, a biological redox system is necessary, which is able to reduce the resulting BLM–Fe(III)-complex and which is further able to establish a redox cycle.

Substances like glutathione, ascorbate or xanthine oxidase have also the capability to reduce BLM–Fe(III) *in vitro* [6, 7]. On the other hand, microsomes or isolated microsomal NADPH-cytochrome P-450 reductase have been shown to catalyze a redox cycle of the BLM–iron complex [8–11]. But it is rather unlikely that oxy radicals formed during enzyme-catalyzed redox cycling of BLM in the endoplasmic reticulum are responsible for DNA damage in the cell nucleus.

We wondered whether constitutive enzymes of the cell nucleus are able to catalyse a redox cycle of the bleomycin–iron complex as shown for adriamycin, a quinonoid anticancer drug [12], since such a redox cycle in close proximity to DNA could be essential for the action of the drug. In a preliminary experiment we found that, in isolated cell nuclei, NADPH and O_2 consumption were stimulated by BLM and $FeCl_3$ [3]. In the present study we incubated cell nuclei isolated from rat livers with BLM, $FeCl_3$ and NADH. Furthermore, we measured MDA formation as a parameter for DNA cleavage [3]. DNA degradation was also shown by gel electrophoresis.

MATERIALS AND METHODS

Bleomycin was purchased from Mack (Illertissen, F.R.G.). It was composed of 55–70% BLM-A_2, 25–32% BLM-B_2 and minor amounts of other bleomycins of the A and B group. NADH, NADPH, glucose-6-phosphate, succinic acid, maleic acid, TES, agarose (Type I) and ethidium bromide were from Sigma (Muenchen), cytochrome *c* was purchased from Boehringer (Mannheim). All other chemicals were obtained from Merck (Darmstadt) and were of analytical grade.

Cell nuclei were prepared according to the method of Fleischer and Kervina [14] as previously described [13]. Male Wistar rats of 160–200 g body weight were starved overnight and killed by decapitation. All subsequent steps were carried out either at 4° or 0°. The livers were perfused with 0.9% NaCl and homogenized in 0.33 M sucrose. The 960 g_{av} pellet of the homogenate was subjected three times to high density sucrose centrifugation. The resulting nuclei were washed with 1 M sucrose and 0.25 M sucrose (each containing 1 mM $MgCl_2$) respectively, were suspended in TES–$MgCl_2$ buffer (25 mM TES and 2.5 mM $MgCl_2$, pH 7.5) and stored in an ice bath. All experiments described in this paper were performed within a period of 48 hr after the isolation procedure was accomplished. Mitochondria and

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† Abbreviations used: BLM, bleomycin; MDA, Malondialdehyde.

microsomes were prepared from the 960 *g* supernatant of the liver homogenate [15, 16].

Protein contents of nuclear, microsomal and mitochondrial suspensions were determined according to Lowry *et al.* [17]. DNA was estimated according to Burton [18].

Glucose-6-phosphatase activity of nuclear and microsomal suspensions (both in 0.25 M sucrose) was determined simultaneously, according to the method of Swanson [19] using Na–maleate buffer (100 mM, pH 6.2).

Succinate–cytochrome *c* reductase activity of nuclear and mitochondrial suspensions (both in 0.25 M sucrose) was determined by the method of Fleischer and Fleischer [20].

For determination of NADH or NADPH consumption and MDA formation, incubations were carried out at 37° in shaken, stoppered glass tubes (15 ml Corex tubes or similar size). The standard incubation contained 25 mM TES (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mM $FeCl_3$, 0.1 mg/ml BLM, 0.5 mg/ml nuclear protein and 0.15 mM NADH (or NADPH) in a final volume of 0.5–1.0 ml. The reaction was started with the addition of NADH (or NADPH) after 5 min preincubation at 37°. At given times, aliquots were removed and the NADH (or NADPH) content was measured fluorimetrically.

Parallel, aliquots of the incubation were mixed with ice-cold trichloroacetic acid solution (10%), centrifuged and the MDA present in the supernatant was determined by the thiobarbituric acid assay [21]. The presence of MDA was confirmed by comparison of the red pigment with that formed by authentic MDA on HPLC.

In some experiments nuclei were replaced by mitochondrial or microsomal suspensions in TES–$MgCl_2$ buffer.

In order to perform the incubation anaerobically, nuclear pellets were gassed with nitrogen for 5 min and the reaction started by resuspending them in an oxygen-free mixture containing all other premixed ingredients. After 30 min the incubation vial was opened and gassed with synthetic air (80% N_2, 20% O_2) for 1 min followed by aerobic incubation. For control experiments a portion of the oxygen-free suspension was aerated at $t = 0$.

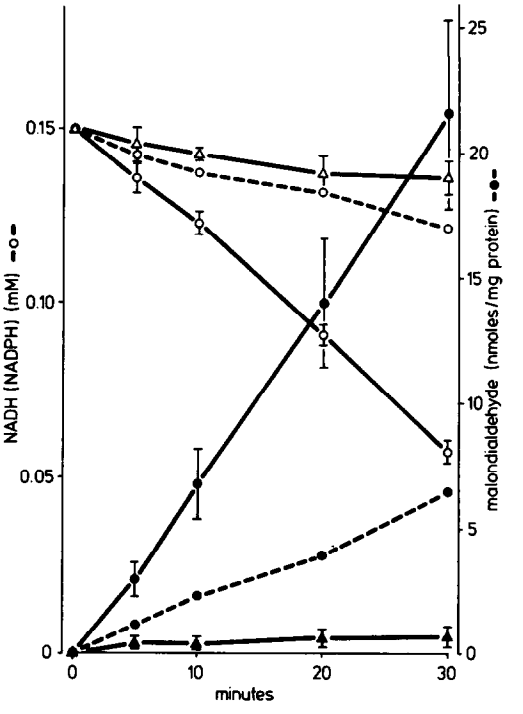

Fig. 1. Time course of BLM–Fe(III)-induced NADH consumption and MDA formation by isolated rat liver nuclei. Nuclei (0.5 mg protein/ml) were incubated in a final volume of 1 ml with BLM (0.1 mg/ml), $FeCl_3$ (0.1 mM) and NADH (NADPH) (0.15 mM) for 30 min at 37°. Mean values ± SD (N = 3). Open symbols: NADH (NADPH) consumption; solid symbols: MDA formation. ○—○, ●—● complete system; △—△, ▲—▲ minus BLM, minus $FeCl_3$. Dashed curves: complete system containing 0.15 mM NADPH instead of NADH (mean values of two experiments).

O_2 consumption was measured by a Clark oxygen electrode.

Incubation mixtures for gel electrophoresis contained 0.5 mM NADH (NADPH) instead of

Table 1. BLM–Fe(III)-induced NADH consumption and MDA formation by nuclei

	NADH consumption (nmoles/ml/30 min)	MDA formation (nmoles/ml/30 min)
Complete system	92.9 ± 2.9	10.8 ± 1.9
Minus bleomycin, minus $FeCl_3$	14.6 ± 5	0.4 ± 0.2
Minus bleomycin	17.9 ± 5	0.7 ± 0.2
Minus $FeCl_3$	32.1 ± 6.4	1.8 ± 1.1
Minus NADH	—	1.0*
Minus nuclei	0*	0*
Minus O_2	22.1*	0.8*
Minus O_2 for 30 min, plus O_2 for 30 min†	120*	14.5*

Nuclei (0.5 mg protein/ml) were incubated with BLM (0.1 mg/ml), $FeCl_3$ (0.1 mM) and NADH (0.15 mM). In control experiments compounds were omitted as indicated. Treatment of nuclear suspension and incubation mixture to obtain anaerobic conditions did not affect the activity of the nuclei and any of the compounds (control data not shown).

Mean values ± SD (N = 3) except where otherwise indicated.

* Mean values of two experiments.

† Value obtained by further 30 min aerobic incubation after the anaerobic treatment.

0.15 mM. Samples taken at given times were mixed with SDS and EDTA and were subjected to gel electrophoresis on 1% agarose. Electrophoresis and ethidium bromide staining were performed using standard methods.

RESULTS

In the presence of BLM–Fe(III) NADH consumption was highly increased compared to controls (Fig. 1). The thiobarbituric assay of the incubation mixture revealed that MDA was formed (Fig. 1). In both cases NADPH showed a less pronounced effect. Table 1 shows that BLM was absolutely necessary for these effects whereas in the absence of $FeCl_3$, NADH consumption and MDA formation still took place to some extent probably due to traces of iron present in nuclei. Furthermore, oxygen was required and the formation of MDA was strictly dependent on NADH.

NADH consumption and MDA formation increased parallel to increasing concentrations of BLM and $FeCl_3$. They were also dependent on the amount of suspended nuclei (data not shown).

Figure 2 demonstrates the degradation of nuclear DNA induced by BLM, $FeCl_3$ and NADH. The electrophoretic mobility of the nuclear DNA increased indicating the occurrence of (double) strand breaks. After 20–30 min nuclear DNA was almost completely degraded. Similar to the results presented above, no DNA damage was observed when BLM or NADH were omitted whereas, in the absence of iron, nuclear DNA was still degraded to some extent. The degradation of nuclear DNA also depended on the presence of oxygen (data not shown). Similar but less pronounced damage of DNA was observed when NADPH was used instead of NADH.

Figure 3 demonstrates oxygen consumption of nuclei which was greatly enhanced when BLM, Fe(III) and NADH were added. As in previous experiments, NADPH was less active and BLM was able to stimulate O_2 consumption to some extent without the addition of $FeCl_3$.

Since microsomes and mitochondria contain enzymes which may redox cycle the BLM–Fe(III)-complex in the presence of NADH or NADPH, the purity of the nuclear preparations with regard to these organelles was examined. Based on the activities of succinate cytochrome *c* reductase and glucose-6-phosphatase respectively of the isolated nuclei, mitochondrial and microsomal contamination was less than 1% (data not shown).

Furthermore, we could not detect a BLM–Fe related NADH consumption in mitochondrial suspensions of 0.005 mg protein/ml. On the basis of

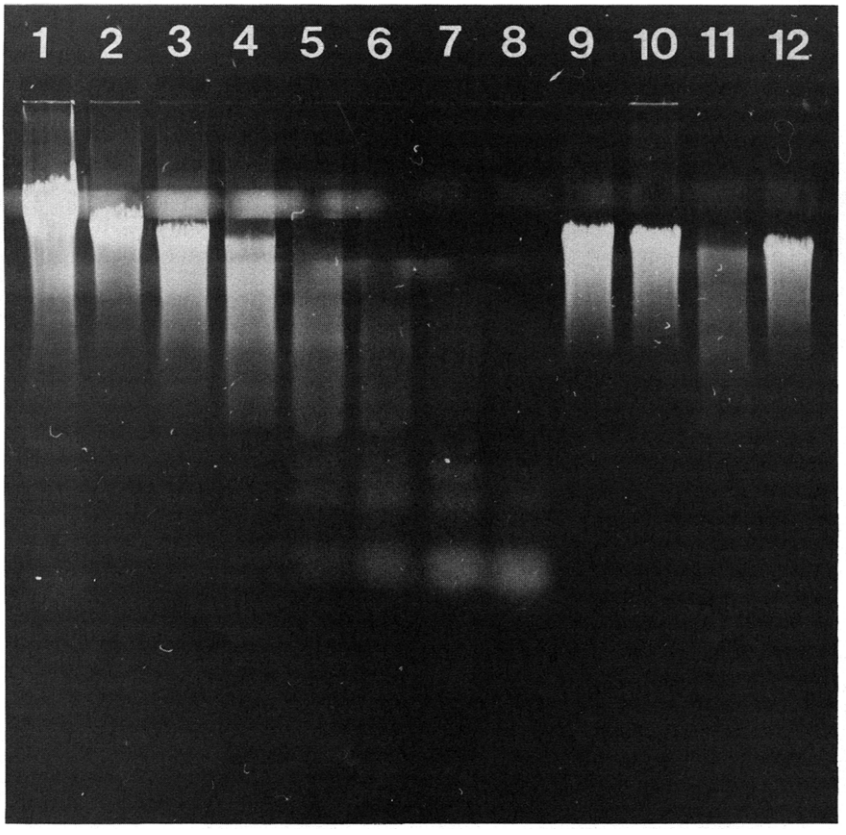

Fig. 2. Agarose (1%) gel electrophoretic patterns of ethidium bromide stained rat liver nuclear DNA (0.5 mg nuclear protein/ml) after treatment of the nuclei with BLM (0.1 mg/ml), $FeCl_3$ (0.1 mM) and NADH (0.5 mM) for 0, 2, 5, 10, 15, 20 and 30 min (lanes 2–8). Lane 1, untreated nuclear DNA. Lanes 9–12, 30 min treatment in the absence of BLM/$FeCl_3$ (9), of BLM (10), of $FeCl_3$ (11) and of NADH (12).

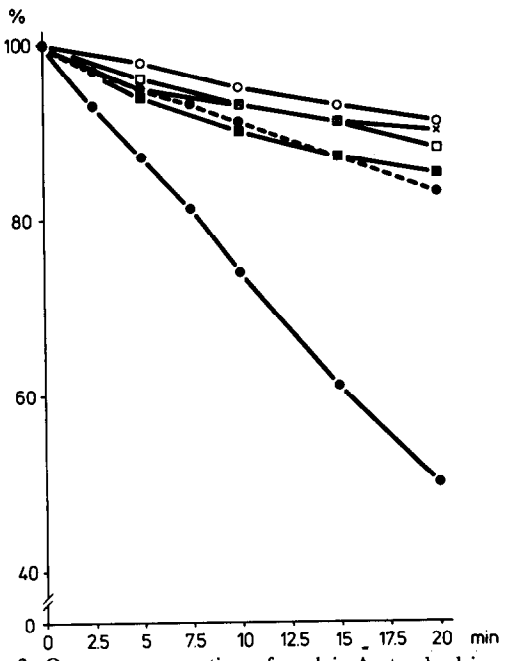

Fig. 3. Oxygen consumption of nuclei. A standard incubation mixture (5 ml) was incubated at 37° in a tube (15 ml Corex) in a water bath and stirred by a miniature magnetic rod. An oxygen electrode was dipped in the suspension and the tube was sealed with parafilm. The amount of oxygen was calculated by setting the initial value = 100% which corresponded to air saturation. ●—● complete system; ■—■ minus $FeCl_3$; □—□ minus BLM; ×—× minus NADH, minus NADPH; ○—○ minus BLM, minus $FeCl_3$. Dashed curve: complete system with NADPH (0.15 mM) instead of NADH.

these data an involvement of mitochondria in the experiments described in this paper could be excluded.

In microsomal suspensions incubated with BLM, Fe(III) and NADH, MDA is formed which is probably due to lipid peroxidation as already shown for NADPH [22]. Since it can be expected that MDA formed in nuclei during incubation with BLM–Fe(III) is at least partially originated from the nuclear envelope, we compared BLM–Fe(III) mediated MDA formation in a nuclear suspension of 0.5 mg protein/ml and a microsomal suspension of 0.1 mg protein/ml, both of them having the same lipid content [14]. When incubated under standard conditions for 120 min the nuclei formed 20.7 nmol/ml MDA whereas the microsomes produced 13.7 nmol/ml. The significantly higher amount of MDA formed in the nuclei must be accounted to DNA oxidation.

Better evidence that MDA is a DNA degradation product stem from experiments performed with (U-^{14}C)-thymidine prelabelled nuclei of a melanoma cell line: ^{14}C-MDA was detected after incubation with BLM, $FeCl_3$, and NADH, whereas no radioactivity was associated with nuclear lipids (data not shown).

DISCUSSION

It has long been known that microsomes incubated aerobically with NADPH or NADH, bleomycin, ferric ions and DNA catalyze chain breakage [8, 23, 24]. Reduction of the BLM–Fe(III)-complex [9] and DNA cleavage [9, 10, 25] was also observed when isolated microsomal NADPH–cytochrome P-450 reductase was used instead of microsomes.

Recently, we observed that the NADPH and O_2 consumption of liver cell nuclei was stimulated when incubated in the presence of an oxidized BLM–iron-complex [13]. We concluded that NADPH-cytochrome P-450 reductase present in the outer nuclear envelope [26] is responsible for a redox cycle of BLM–Fe(III/II).

In this paper we present data which reveals that NADH is an even better cofactor than NADPH with respect to the catalysis of the redox cycle of BLM–Fe(III/II) and concomitant DNA cleavage. The activity and/or the amount of the NADH-dependent enzyme responsible for this redox cycle must be significantly higher compared to NADPH–cytochrome P-450 reductase present in nuclear suspensions. Since the involvement of a mitochondrial enzyme is excluded, NADH–cytochrome b_5 reductase which has also been found in the nuclear envelope [27] may be the enzyme responsible for the NADH-dependent redox cycling of the BLM–iron complex. However, it is still not excluded that other classes of reductases can also catalyse redox cycling of the BLM–iron-complex.

MDA formation suggests that strand breaks are formed by oxidation of the deoxyribose moiety of nuclear DNA as shown in model experiments with DNA or polydeoxynucleotides and the BLM–Fe(II)-complex [3–5, 28–31].

The dependence of these effects on oxygen once again indicates the involvement of active oxygen species in the action of the drug. However, on the basis of this data it cannot be decided whether the reduction of BLM–Fe(III) to BLM–Fe(II) takes place under anaerobic conditions or not. The low NADH consumption observed in the anaerobic incubation might also be due to traces of oxygen present in the suspension since it is very difficult to remove oxygen from a nuclear suspension entirely. Only very low amounts of MDA were formed under these conditions, but gel electrophoresis revealed some DNA damage (results not shown).

Independent of the mechanism and the nature of the nuclear enzymes involved in redox cycling of BLM–Fe(III/II) the results show that bleomycin-induced DNA cleavage can be highly stimulated by NADH which is normally present in the cytoplasm in high concentrations. Furthermore, some NADH comsumption, MDA production and DNA degradation is observed without addition of external $FeCl_3$. Since in the complete absence of iron, BLM would not be activated [32], we suggest that bleomycin chelates iron (or other metal) ions already present in the nuclei. This is not surprising since iron is very abundant in the cell. On the other hand, these ions can not originate from our reagents which, including TES-buffer, were free of iron.

It is therefore conceivable that under physiological conditions bleomycin forms a complex with iron which is redox cycled by nuclear, mainly NADH-dependent, reductases. The reduced BLM–iron-complex would bind and activate O_2 whereby the nuclear DNA is oxidized and strand breaks occur.

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REFERENCES

1. R. M. Burger, J. Peisach and S. B. Horwitz, *Life Sci.* **28**, 715 (1981).
2. H. Kuramochi, K. Takahashi, T. Takita and H. Umezawa, *J. Antibiot., Tokyo* **34**, 576 (1981).
3. R. M. Burger, A. R. Berkowitz, J. Peisach and S. B. Horwitz, *J. biol. Chem.* **255**, 11832 (1980).
4. A. P. Grollman, M. Takeshita, K. M. R. Pillai and F. Johnson, *Cancer Res.* **45**, 1127 (1985).
5. N. Murugesan, Ch. Xu, G. M. Ehrenfeld, H. Sugiyama, R. E. Kilkuskie, L. O. Rodriguez, L.-H. Chang and S. M. Hecht, *Biochemistry* **24**, 5735 (1985).
6. E. A. Sausville, J. Peisach and S. B. Horwitz, *Biochemistry* **17**, 2740 (1978).
7. W. E. Antholine and D. H. Petering, *Biochem. biophys. Res. Commun.* **90**, 384 (1979).
8. N. Yamanaka, T. Kato, K. Nishida and K. Ota, *Cancer Res.* **38**, 3900 (1978).
9. M. E. Scheulen, H. Kappus, D. Thyssen and C. G. Schmidt, *Biochem. Pharmac.* **30**, 3385 (1981).
10. R. E. Kilkuskie, T. L. Macdonald and S. M. Hecht, *Biochemistry* **23**, 6165 (1984).
11. I. Mahmutoglu and H. Kappus, *Biochem. Pharmac.* **34**, 3091 (1985).
12. N. R. Bachur, M. V. Gee and R. D. Friedman, *Cancer Res.* **42**, 1078 (1982).
13. H. Kappus and I. Mahmutoglu, in *Biological Reactive Intermediates III—Mechanisms of Action in Animal Models and Human Disease* (Eds. J. J. Kocsis, D. J. Jollow, Ch. M. Witmer, J. O. Nelson and R. Snyder) p. 273. Plenum Press, New York (1986).
14. S. Fleischer and M. Kervina, *Meth. Enzymol.* **31**, 6 (1974).
15. F. C. Guerra, *Meth. Enzymol.* **31**, 299 (1974).
16. H. Remmer, H. Greim, J. B. Schenkman and R. W. Estabrook, *Meth. Enzymol.* **10**, 703 (1967).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. K. A. Burton, *Biochem. J.* **62**, 315 (1956).
19. M. A. Swanson, *Meth. Enzymol.* **2**, 541 (1955).
20. S. Fleischer and B. Fleischer, *Meth. Enzymol.* **10**, 406 (1967).
21. J. A. Buege and S. D. Aust, *Meth. Enzymol.* **52**, 302 (1978).
22. H. Kappus, H. Muliawan and M. E. Scheulen, in *Microsomes, Drug Oxidations and Drug Toxicity* (Eds. R. Sato and R. Kato), p. 555, Japan Scientific Societies Press, Tokyo/Wiley–Interscience, New York (1982).
23. M. A. Trush, E. G. Mimnaugh, E. Ginsburg and T. E. Gram, *J. Pharmac. exp. Ther.* **221**, 152 (1982).
24. D. R. Bickers, R. Dixit and H. Mukhtar, *Biochim. biophys. Acta* **781**, 265 (1984).
25. M. E. Scheulen and H. Kappus, in *Oxygen Radicals in Chemistry and Biology* (Eds. W. Bors, M. Saran and D. Tait) p. 425. Walter de Gruyter, Berlin (1984).
26. Y. Sagara, T. Harano and T. Omura, *J. Biochem., Tokyo* **83**, 807 (1978).
27. I. Kano and D. W. Nebert, *Eur. J. Biochem.* **109**, 25 (1980).
28. L. F. Povirk, *Biochemistry* **18**, 3989 (1979).
29. J. M. C. Gutteridge, *FEBS Lett.* **105**, 278 (1979).
30. L. Giloni, M. Takeshita, F. Johnson, Ch. Iden and A. P. Grollman, *J. biol. Chem.* **256**, 8608 (1981).
31. J. C. Wu, J. W. Kozarich and J. Stubbe, *Biochemistry* **24**, 7562 (1985).
32. B. Halliwell and J. M. C. Gutteridge, *Meth. Emzymol.* **105**, 47 (1984).