Biochemical Pharmacology, Vol. 36, No. 21, pp. 3629–3633, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

OXYGEN RADICAL DAMAGE TO DNA BY RIFAMYCIN SV AND COPPER IONS

GREGORY J. QUINLAN and JOHN M. C. GUTTERIDGE*

Division of Chemistry, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, U.K.

(*Received* 20 *February* 1987; *accepted* 12 *May* 1987)

Abstract—The hydroquinone moiety of the antibiotic rifamycin SV reacts with molecular oxygen to form reduced oxygen intermediates such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). The antibiotic semiquinone is also formed. Rifamycin SV in the presence of iron and copper salts can lead to the formation of the highly reactive hydroxyl radical ($OH^\cdot$) which degrades the sugar deoxyribose. This damage is substantially inhibited by the enzyme catalase and scavengers of the hydroxyl radical such as formate, mannitol and thiourea. When linear duplex DNA is substituted for deoxyribose only rifamycin SV and copper ions substantially degrade DNA with release from the DNA molecule of thiobarbituric acid-reactive products. Damage to DNA by rifamycin and copper ions is significantly inhibited by catalase but poorly inhibited by scavengers of the hydroxyl radical consistent with a site-specific radical reaction of the DNA molecule.

Several biological properties of rifamycin SV are known to resemble those of the metal chelating agent 1,10-phenanthroline. Here, we show that similarities extend to an unusual chemical property whereby thiobarbituric acid-reactive material is released from DNA in the presence of a copper salt.

The rifamycins are lipophilic antibiotics produced by the micro-organism *Nocardia mediterranei* [1]. They have a naphthohydroquinone ring spanned by a long aliphatic bridge; the latter participating in binding to RNA polymerase [2]. Bacterial DNA-dependent RNA synthesis is strongly inhibited by low concentrations of the rifamycins and inhibition closely resembles that produced by the metal chelating agent 1,10-phenanthroline [3].

The hydroquinone moiety (QH_2) of rifamycin SV reacts with molecular oxygen to form reduced oxygen intermediates such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). The drug semiquinone ($QH^\cdot$) is also produced (equations 1–3)

$$QH_2 + O_2 \leftrightharpoons QH^\cdot + O_2^- + H^+ \quad \{1\}$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \{2\}$$

$$QH^\cdot + O_2 \rightarrow Q + O_2^- + H^+ \quad \{3\}$$

Oxidation of the hydroquinone form of the drug, known as "SV" to the quinone (Q) form "S" is greatly accelerated by the presence of divalent metal ions [3, 4] particularly transition metal ions. Rifamycin SV in the presence of iron or copper ions can lead to hydroxyl radical ($OH^\cdot$) formation as measured by the ability of $OH^\cdot$ to degrade the sugar deoxyribose [5].

Rifamycin SV was found to be particularly useful as an agent against organisms such as *Mycobacterium tuberculosis* [6]. However, renewed interest in the drug has centred around its reported antiviral [7], anti-inflammatory [8] and immunosuppressive properties [9]. In the present paper, we report that rifamycin SV in the presence of copper ions can substantially degrade the DNA molecule and release fragments containing aldehydic groups.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Rifamycin SV sodium salt, superoxide dismutase (bovine erythrocyte), catalase (bovine liver), albumin (human fatty acid free), 2-deoxy-D-ribose, 1,10-phenantholine, DNA (calf thymus type 1), DNA (*E. coli* type VIII) were from the Sigma Chemical Company (Poole, Dorset). Agarose (electrophoresis grade) Koch-Light (Colnbrook, Bucks). All other chemicals were of the highest purity available from BDH Ltd (Poole, Dorset). Malondialdehyde (MDA) was prepared as previously described [10].

Degradation of deoxyribose and DNA. The following reagents were added to new clean plastic tubes in the order indicated; 0.2 ml of phosphate–saline buffer (0.1 M sodium phosphate in 0.15 M NaCl) pH 7.4 or Tris buffer (0.1 M) pH 9.2, 0.2 ml DNA (1 mg/ml in distilled water), or 0.2 ml deoxyribose (10 mM), 0.1 ml of rifamycin SV (1 mg/ml). Where indicated in the appropriate figures or tables, 0.1 ml of proteins or scavengers were added. The reaction was started by the addition of 0.1 ml of metal salt (see figures and tables) and resulting mixtures were incubated for 2 hr at 37° in a shaking waterbath. In experiments where DNA was degraded by 1, 10-phenanthroline the procedure previously described [11] was adopted.

Development of TBA-reactivity. After incubation of samples, 0.5 ml of thiobarbituric acid (1% w/v in 0.05 M NaOH), and 0.5 ml of 28% w/v trichloroacetic acid (TCA) were added for DNA degradation studies and the low levels of TBA-reactivity measured by fluorescence. However, for deoxyribose degradation 2.8% w/v TCA was substituted for 28% TCA and the high levels of TBA-reactivity formed were measured by absorbance. The tube contents were transferred from plastic to glass tubes and heated to 100° for 15 min to develop the colour.

The resulting TBA chromogen was extracted into 1.5 ml of butan-1-ol and the clear organic layer used for both spectrofluorimetric and spectrophotometric measurements.

Thiobarbituric acid adduct fluorescence. Fluorescence in the butanol extracts was measured at 553 nm following excitation at 532 nm in a Perkin Elmer MPF 4 spectrofluorimeter. Units of fluorescence are shown as Relative Fluorescence Intensity (RFI) calibrated against a standard rhodamine B (3 μM) as previously described [10]. Absorbance values were kept below 0.05 to avoid inner-filter effects [12].

Electrophoretic separation of damaged DNA. An agarose gel (0.65% w/v) was prepared in sodium phosphate buffer pH 7.8 (0.1 M) containing EDTA (5 mM). Samples were loaded into wells cut into the gel, each containing 50 μl of the incubated DNA test solution pre-mixed with an equal volume of liquid agarose. The gel was run in the above buffer for 24 hr at 11 mA. Resulting bands of DNA were viewed under u.v. light after development in ethidium bromide (5 μg/ml). *E. coli* DNA was used for the studies.

Separation of rifamycin SV and rifamycin S by HPLC. Separation of the rifamycins was performed in acetonitrile (far u.v. grade) containing 35% v/v phosphate (0.1 M potassium salt) at a flow rate of 1 ml/min. The column (250 mm × 4.6 mm) was packed with ODS silica (5 μ) (Shandon Southern). Separation of 20 μl samples (as prepared for the oxygen uptake experiments) was monitored at 216 nm.

Oxygen uptake measurements. A Clarke-type electrode (Hanstech Ltd) was used in these experiments. The electrode was calibrated according to the manufacturers instructions with 100% air saturation at 35° equal to 0.219 μ moles of oxygen/ml. To the reaction vessel was added 1 ml of distilled water, 0.4 ml of phosphate saline buffer (as previously described) pH 7.4, 0.4 ml of rifamycin SV (1 mg/ml). The electrode was allowed to stabilise before 0.2 ml of appropriate metal salt (1 mM) was added. Readings were taken from the point of metal salt addition.

Detection of rifamycin SV oxidation by spectrophotometry. To a cuvette were added 0.8 ml of phosphate–saline buffer (as previously described) pH 7.4, 0.4 ml of formate (200 mM) or thiourea (20 mM), 0.1 ml of rifamycin SV (1 mg/ml) and 0.2 ml of copper salt (1 mM). The resulting changes in absorbance were monitored at 525 nm for 10 mins.

All results shown are the mean of three or more experiments which differed by less than ±5%.

RESULTS

In a previous study it was shown that rifamycin SV (RSV) oxidised rapidly at pH 9.2 in the presence of manganous ions, to form rifamycin S (RS) [4] and this was confirmed in our experiments at pH 9.0 (Fig. 1). However, under our experimental conditions copper ions were more effective at stimulating oxidation of RSV at pH 7.4 (Fig. 1), measured as both uptake of oxygen and the formation of RS.

The sugar deoxyribose was not degraded by RSV

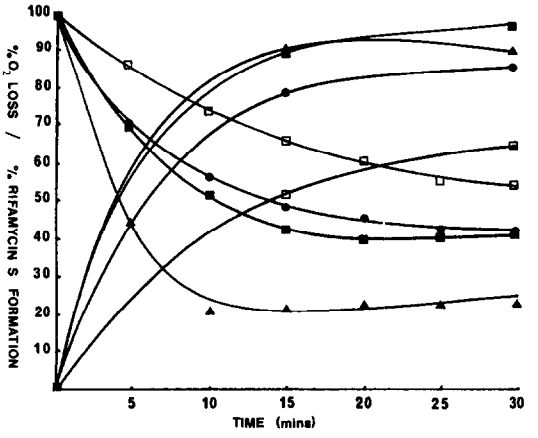

Fig. 1. pH-dependent oxidation of rifamycin SV in the presence of copper and manganese ions. O_2 values decrease from 100%. Rifamycin S values increase from 0%. ■, Cu^{2+} (0.1 mM) at pH 9.0; ▲, Mn^{2+} (0.1 mM) at pH 9.0; ●, Cu^{2+} (0.1 mM) at pH 7.4; □, Mn^{2+} (0.1 mM) at pH 7.4. Rifamycin S formation was measured by HPLC using % area for calculation of values.

and manganous ions whereas RSV and copper ions were extremely effective at bringing about degradation (Table 1). This copper-dependent degradation of deoxyribose was substantially inhibited by the addition of catalase and the enzyme's protective activity was mostly destroyed by heat-denaturation of the protein (Table 1). Albumin and superoxide dismutase were partly inhibitory but this was probably due to non-specific copper-binding by the proteins since heat-denaturation of them did not abolish their activity (Table 1). The hydroxyl radical scavengers mannitol, thiourea and formate significantly decreased deoxyribose degradation in the presence of copper ions and rifamycin SV whereas urea, added as a control for non-specific scavenging effects, had little or no protective action (Table 1). Thiourea and formate but not mannitol, however, greatly accelerated the oxidation of RSV to RS in a buffer, containing copper ions, at pH 7.4 (Fig. 2). This acceleration of oxidation could be reduced by adding mannitol to the reaction at concentrations five times that of formate and 50 times that of thiourea (Fig. 2). The addition of superoxide dismutase to the same reactions was without effect (data not shown).

When linear duplex DNA was incubated with copper ions and RSV they were able to bring about the release of TBA-reactive material from it. The ability of other metal ions to do this was also examined (Table 2). Ferrous ions released some TBA-reactive material from DNA in the absence of RSV and copper ions released TBA-reactive material from RSV in the absence of DNA (Table 2). However, RSV and copper ions produced substantially more TBA-reactive material from DNA. The fluorescent characteristics of the TBA adducts formed from products released from DNA deoxyribose and RSV are shown in Fig. 3. As can be seen their spectra are indistinguishable from the TBA adduct formed by the 3-carbon molecule malondialdehyde (MDA).

Table 1. Degradation of deoxyribose by rifamycin SV and copper ions the effect of inhibitors

	TBA reactivity after 2 hr incubation at 37°	
	A532 nm	% Inhibition
Blank (Deoxyribose + RSV)	0.024 (subtracted)	
Control (Deoxyribose + Cu^{2+} + RSV	0.226	
Control + superoxide dismutase (0.06 mg/ml)	0.171	24%
Control + superoxide dismutase Heat denatured	0.142	37%
Control + catalase (0.06 mg/ml)	0.012	95%
Control + catalase Heat-denatured	0.130	43%
Control + albumin (0.06 mg/ml)	0.155	32%
Control + albumin Heat-denatured	0.160	29.2%
Control + formate (50 mM)	0.032	86%
Control + mannitol (50 mM)	0	100%
Control + urea (5 mM)	0.185	18%
Control + thiourea (5 mM)	0	100%

Concentrations shown are the final reaction concentrations. Details of the reaction mixture are given in the Methods Section.
RSV = Rifamycin SV.

Gel electrophoresis clearly shows the damage resulting from incubation of DNA with a mixture of copper and RSV (Fig. 4). For comparison the effect of a 1,10-phenanthroline–copper ion mixture is also shown. Degradation of DNA by the RSV–copper complex (Monitored as TBA fluorescence) was strongly inhibited by catalase but not by the addition of other proteins (Table 3). The hydroxyl radical scavengers, thiourea, mannitol and formate did not protect the DNA against damage by copper and RSV (Table 3).

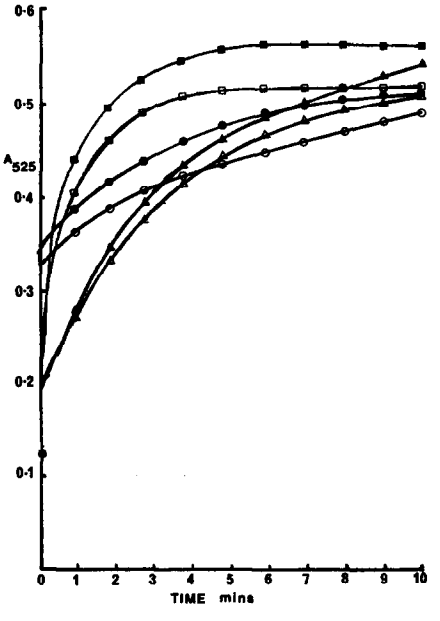

Fig. 2. Oxidation of rifamycin SV in the presence of copper ions, thiourea or formate at pH 7.4. The inhibitory effect of mannitol. ■, Rifamycin SV + Cu^{2+} (0.1 mM) + formate (40 mM); □, rifamycin SV + Cu^{2+} (0.1 mM) + formate (40 mM) + mannitol (200 mM); ▲, rifamycin SV + Cu^{2+} (0.1 mM); △, rifamycin Sv + Cu^{2+} (0.1 mM) + mannitol (40 mM); ●, rifamycin SV + Cu^{2+} (0.1 mM) + thiourea (4 mM); ○, rifamycin SV + Cu^{2+} (0.1 mM) + thiourea (4 mM) + mannitol (200 mM). Final reaction concentrations are shown. Details of the assay procedure are given in the Methods section.

DISCUSSION

The inhibitory characteristics of rifamycin SV towards the initiation of RNA synthesis were described by Scrutton in 1971 as "resembling those of the metal chelating agent 1,10-phenanthroline" [3]. It is now well established that a complex of 1,10-phenanthroline and copper (II) degrades DNA in the presence of reducing agents [13–16]. Here, we show that similarities between rifamycin SV and 1,10-phenanthroline extend to several other chemical properties.

At a pH value of 7.4 copper ions are the most effective of the metal ions tested, in promoting oxidation of RSV with the accompanying formation of damaging oxygen radicals. In agreement with previous studies manganous ions do not appear to participate in Fenton chemistry to form $OH^{\cdot}$ radicals that damage the sugar deoxyribose [17]. However, they do promote the rapid oxidation of RSV [3, 4]. Damage to deoxyribose by copper and RSV was substantially inhibited by catalase and, to some extent, by all proteins added including the heat-denatured form of catalase. This partial inhibition can be ascribed to a general metal-binding antioxidant property of proteins when added to oxygen radical reactions stimulated by copper ions [18]. The importance of hydrogen peroxide, as shown by catalase inhibition of deoxyribose degradation suggests that hydroxyl radicals are formed by a Fenton-type reaction and this is further supported by the inhibitory activity of mannitol, thiourea and formate. Urea, as expected, showed little or no inhibitory action when added as a control for non-specific scavenging effects.

Table 2. Release of TBA-reactivity following damage to DNA by rifamycin SV and transition metal ions

Metal ion	Relative fluorescence intensity units (RFI) Ex 532 nm Em 553 nm		
	DNA + metal ion	RSV + metal ion	DNA + RSV + metal ion
Cu^{2+} (0.1 mM)	3	44	160
Cu^{2+} (0.08 mM)	3	41	146
Cu^{2+} (0.04 mM)	3	40	121
Cu^{2+} (0.02 mM)	3	36	75
Cu^{2+} (0.01 mM)	3	35	49
Fe^{3+} (0.1 mM)	8	6	11
Fe^{2+} (0.1 mM)	37	7	21
Co^{2+} (0.1 mM)	3	7	5
Mn^{2+} (0.1 mM)	3	5	8
Cr^{6+} (0.1 mM)	4	15	15

Reaction conditions are described in the Methods section and final reaction concentrations of metal ions are shown.

RSV = Rifamycin SV.

The TBA absorbance value of the DNA, RSV and copper mixture showing 160 RFI units was 0.059 (A532 nm).

Formate and thiourea but not mannitol, however, greatly accelerated the oxidation of RSV in a simple buffer at pH 7.4 containing copper ions. This rapid oxidation could be reduced by adding higher concentrations of mannitol than either the formate or thiourea present and, since superoxide dismutase had no inhibitory effect on the oxidation of RSV by these scavengers it suggests that OH· radicals are in some way involved. It is possible that radicals formed from formate and thiourea are able to reduce the metal ions present and increase the rate of RSV oxidation, e.g. reaction of OH· with formate gives a highly reducing formate radical.

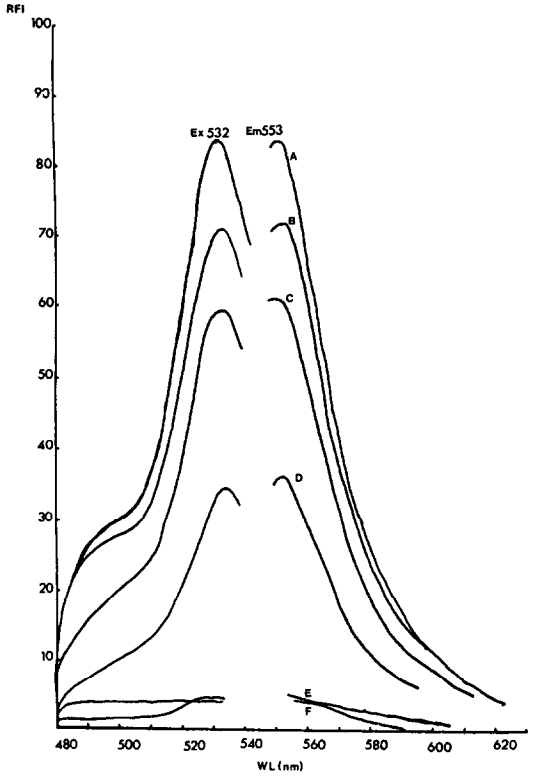

Fig. 3. Fluorescent scans of the TBA adducts formed from copper damage to DNA, and rifamycin SV. Fluorescent scans were made at 532 nm excitation and 553 nm emission. (A) Malondialdehyde standard; (B) copper–phenanthroline damage to DNA; (C) copper–rifamycin SV damage to DNA; (D) copper damage to rifamycin SV; (E) DNA + rifamycin SV; (F) DNA + copper ions. The reaction conditions leading to formation of TBA-reactivity are fully described in the Methods sections.

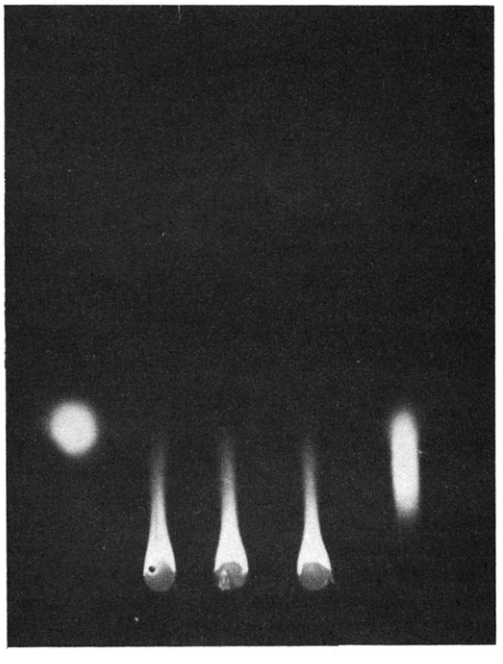

Fig. 4. Separation of copper-damaged DNA by gel electrophoresis. The gel shows from left to right: (1) DNA incubated with copper salt, phenanthroline and mercaptoethanol; (2) DNA incubated with copper ions; (3) DNA incubated with rifamycin SV; (4) DNA control; (5) DNA incubated with copper ions and rifamycin SV.

Table 3. Degradation of DNA by rifamycin SV and copper ions: the effect of inhibitors

	TBA-reactivity after 2 hr incubation at 37°	
	RFI Units	% Inhibition
Blank (DNA + RSV)	4 (subtracted)	
Control (DNA + Cu^{2+} + RSV)	166	
Control + superoxide dismutase (0.06 mg/ml)	154	7%
Control + superoxide dismutase heat-denatured	128	23%
Control + catalase (0.06 mg/ml)	10	94%
Control + heat-denatured catalase	143	14%
Control + albumin (0.06 mg/ml)	147	11%
Control + heat-denatured albumin	138	16.9%
Control + formate (50 mM)	111	33%
Control + mannitol (50 mM)	166	0
Control + urea (5 mM)	166	0
Control + thiourea	148	11%

Details of the reaction conditions are given in the Methods section and values shown are final reaction concentrations. RSV = Rifamycin SV RFI = Relative fluorescence intensity units.

Linear duplex DNA was only degraded by RSV in the presence of copper ions. Like 1,10-phenanthroline [16], RSV and copper ions release TBA-reactive material from the deoxyribose moiety of DNA, a property so far shared only with the antitumour antibiotic bleomycin in the presence of ferrous ions [19]. The release of TBA-reactive material from DNA by copper and RSV suggests that binding takes place between the drug–metal complex and DNA. Ferrous ions were able to release some TBA-reactive material from DNA in the absence of RSV and copper ions could release TBA-reactive material from RSV in the absence of either DNA or deoxyribose. The latter finding suggests that the RSV molecule itself could be source of reactive aldehydes. The fluorescent properties of the various TBA adducts were spectroscopically indistinguishable from that formed by an authentic sample of malondialdehyde.

Damage to DNA by copper and RSV is dependent on the generation of hydrogen peroxide in the reaction since catalase strongly inhibits the damage observed. A similar pattern, with poor protection given by hydroxyl radical scavengers, is also seen when a copper–phenanthroline complex degrades DNA [16], suggesting that the reaction on the DNA molecule leading to $OH^{\cdot}$ radical formation is site-specific and not amenable to interruption by scavengers unless they have some special ability to approach the site of radical formation. It might also suggest that the $OH^{\cdot}$ radical is not responsible for damage to DNA. Alternatives, in copper reactions, to the $OH^{\cdot}$ radical have been proposed and these include a $CuH_2O_2^+$ complex [20] and the Cu(III) form [20, 21], both of which could lead to DNA damage [22]. Like site-specific $OH^{\cdot}$ radicals neither of these species would be scavenged by mannitol, thiourea or formate.

The antibiotic rifamycin SV and its analogue rifampicin (data not included) are able to degrade DNA *in vitro* in the presence of copper ions. It is highly likely that the drug would also cause DNA damage *in vivo* making it a potentially more "toxic" agent than hitherto thought.

REFERENCES

1. P. Sensi, *Res. Prog. Org. Biol. Med. Chem.* **1**, 337 (1964).
2. W. Wehrli and M. Staehelin, *Bact. Rev.* **35**, 290 (1971).
3. M. C. Scrutton, *FEBS Lett.* **78**, 216 (1977).
4. Y. Kono, *J. Biochem.* **91**, 381 (1982).
5. G. J. Quinlan, M. Spitali and J. M. C. Gutteridge, in *Free Radicals Oxidant Stress and Drug Action* (Ed. C. Rice-Evans) Richelieu Press, London (1987).
6. P. Mison and T. Trnka, *Collect Czech. Chem. Commun.* **37**, 1049 (1972).
7. C. G. Engle, E. Lasinski and J. Gelzer, *Nature, Lond.* **228**, 1191 (1970).
8. F. Caruso, F. Montrone, M. Fumagalli, C. Patrono, S. Santandrea and C. Gandini, *Ann. Rheum. Dis.* **41**, 232 (1982).
9. J. E. Kasik and M. Monick, *Antimicrob. Agents Chemother.* **19**, 134 (1981).
10. J. M. C. Gutteridge and T. R. Tickner, *Analyt. Biochem.* **91**, 250 (1978).
11. J. M. C. Gutteridge, *Biochem. J.* **218**, 983 (1984).
12. J. M. C. Gutteridge, *Free Rad. Res. Comms.* **1**, 173 (1986).
13. D. S. Sigman, D. R. Graham, V. D'Aurora and A. M. Stern, *J. biol. Chem.* **254**, 12269 (1979).
14. K. A. Reich, L. E. Marshall, D. R. Graham and D. S. Sigman, *J. Am. chem. Soc.* **103**, 3582 (1981).
15. B. G. Que, K. M. Downey and A. G. So, *Biochemistry* **19**, 5987 (1980).
16. J. M. C. Gutteridge and B. Halliwell, *Biochem. Pharmac.* **31**, 2801 (1982).
17. J. M. C. Gutteridge and J. V. Bannister, *Biochem. J.* **234**, 225 (1986).
18. J. M. C. Gutteridge and S. Wilkins, *Biochim. biophys. Acta* **759**, 38 (1983).
19. M. T. Kuo and C. W. Haidle, *Biochim. biophys. Acta* **335**, 109 (1973).
20. K. V. Ponganis, M. A. DeAranjo and H. L. Hodges, *J. inorg. Chem.* **19**, 2704 (1980).
21. G. R. A. Johnson, N. B. Nazhat and R. A. Saadalla-Nazat, *J. Chem. Soc. Chem. Commun.* 407 (1985).
22. S. Goldstein and G. Czapski, *J. Free. Rad. Biol. Med.* **1**, 373 (1985).