Biochemical Pharmacology, Vol. 34, No. 5, pp. 669–674, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

FREE RADICAL PRODUCTION FROM NORMAL AND ADRIAMYCIN-TREATED RAT CARDIAC SARCOSOMES

PAUL J. THORNALLEY* and NICHOLAS J. F. DODD
Department of Biophysical Chemistry, Paterson Laboratories, Christie Hospital & Holt Radium Institute, Manchester M20 9BX, U.K.

(*Received* 20 *June* 1984; *accepted* 6 *September* 1984)

Abstract—The production of hydroxyl radicals in rat myocardial sarcosomes treated with adriamycin was demonstrated by the electron spin resonance technique of spin trapping. Using the spin trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), the formation of a hydroxyl radical spin adduct was observed in adriamycin-treated rat heart sarcosomes with NADPH as co-factor. Oxygen, NADPH and sarcosomal protein were absolute requirements for hydroxyl radical production. Hydroxyl radical spin adduct formation was not inhibited by the metal ion chelators diethylenetriaminepenta-acetic acid (DETAPAC) or desferrioxamine, or by addition of superoxide dismutase but could be inhibited by addition of catalase and high concentration of the hydroxyl radical scavengers mannitol and N-acetylcysteine. Hydroxyl radical production in adriamycin-treated rat myocardial sarcosomes appears to arise from the reductive metabolism of adriamycin by an NADPH-dependent quinone reductase—NADPH: cytochrome P450 reductase; the reduced quinone (semiquinone) reduces oxygen to hydrogen peroxide, probably via superoxide, although this was not detected. The hydrogen peroxide appears to react directly with adriamycin semiquinone, although involvement of traces of iron in a Fenton type of reaction cannot be excluded. From the observations it is suggested that adriamycin-induced cardiotoxicity is an oxidative pathology arising from intracellular generation of relatively high levels of hydroxyl radicals.

Adriamycin is one of the most important examples of 5,12-naphthacenedione derivatives which have found clinical use as antitumor drugs [1]. The pharmacological use of adriamycin is limited, however, by toxic side effects—one of which is cardiotoxicity [2]. Clinical manifestations of these cardiotoxic effects, ECG abnormalities, myocardial fibrosis and necrosis, cardiomegaly and heart failure [3]. The major morphological changes observed in myocardial cells, following administrations of adriamycin, are swelling and calcification of mitochondria, and disruption of the sarcoplasmic reticular membrane [3].

The cardiac sarcoplasmic reticulum appears to be an important site of metabolism of antitumor quinone drugs; cardiac NADPH cytochrome P-450 reductase (EC 1.6.2.4) has been implicated as the active metabolic site in this process [4]. Doroshow has recently shown that adriamycin stimulates both oxygen consumption and superoxide (as judged by superoxide dismutase-inhibitable reduction of acetylated cytochrome *c*) in rat heart sarcosomal fractions [5]. Both oxygen consumption and superoxide production required NADPH, and both were inhibited by $NADP^+$ and sulphydryl reagents—observations consistent with the involvement of sarcosomal NADPH cytochrome P450 reductase in adriamycin-mediated oxygen reduction to superoxide.

Adriamycin semiquinone (Q^-) reacts rapidly with oxygen to give superoxide (O_2^-):

$$Q^- + O_2 \rightarrow Q + O_2^-$$

and at physiological pH the equilibrium is well over to the right [6]. Winterbourn [7, 8] reported that superoxide and adriamycin semiquinone can reduce ferricytochrome *c* and indicated that addition of superoxide dismutase may suppress both superoxide-mediated and adriamycin semiquinone-mediated reduction of ferricytochrome *c*. Rapid removal of superoxide, by the dismutase will decrease the steady state concentration of both superoxide and semiquinone. Consequently in such systems the superoxide dismutase-inhibitable reduction of ferricytochrome *c* represents both superoxide- and semiquinone-mediated reduction of cytochrome.

Doroshow [5] indicated that hydrogen peroxide is produced from the aerobic metabolism of adriamycin in rat heart sarcosomes. Adriamycin semiquinone has been reported to react with hydrogen peroxide to produce hydroxyl radicals [9, 10]. The protection of the heart muscle against adriamycin-induced toxicity by sulphydryl compounds (e.g. N-acetylcysteine [11]) suggests that hydroxyl radical production stimulated by adriamycin in the rat heart myocardium may have an important role in the development of adriamycin-induced cardiotoxicity. So ideally, we wish to follow superoxide and hydroxyl free radical production in physiologically functioning rat heart sarcosomes treated with adriamycin.

A technique which may be used to observe oxygen radical production from adriamycin-treated myocardial sarcosomes is the electron spin resonance

* Address for correspondence: Dr P. J. Thornalley, Department of Pharmaceutical Sciences, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET, U.K.

(ESR) technique of spin trapping [12]. In principle, the spin trapping technique can give both an independent measure of superoxide formation, and can also follow hydroxyl radical formation, by monitoring the ESR spectra of superoxide and hydroxyl radical spin adducts in rat heart sarcosomal incubations. Superoxide can be measured without recourse to superoxide dismutase-inhibitable effects.

In this report, the spin trapping of free radicals produced by rat heart sarcosomal fractions, treated with NADPH and adriamycin, in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) is described. Spin trapping data was corroborated by oxygen consumption measurements.

Free radical production from adriamycin-treated rat heart mitochondria is the subject of a further report [13].

1. MATERIALS AND METHODS

1.1 *Experimental animals.* Male Wistar rats weighing 180–200 g were supplied by the Paterson Laboratories Animal Services Department (Christie Hospital, Manchester, U.K.). From the time of weaning, these animals were maintained on a diet of 'Economy diet', Labshore (Poole, Dorset, U.K.) with water available *ad libitum*. Rat heart was chosen for study as the rat appears to develop acute and chronic cardiac toxicity after anthracycline treatment that is very similar to anthracycline-induced cardiomyopathy in humans [14].

1.2 *Materials.* Adriamycin (doxorubicin) hydrochloride, mannitol, NADPH, $NADP^+$, NADH, diethylenetriaminepenta-acetic acid (DETAPAC), and histidine were supplied by Sigma Chemical Co. Ltd. (Poole, U.K.). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich Chem. Co. Ltd., Gillingham, Dorset. Desferrioxamine mesylate was purchased from Ciba Laboratories, Horsham, Sussex, England.

Bovine catalase was supplied by Sigma. Human copper–zinc superoxide dismutase was a generous gift from Dr. J. V. Bannister (Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford, U.K.) and had an activity of 3300 units/mg [15].

1.3 *Preparation of rat sarcosomes.* Experimental animals were killed by cervical dislocation. The cardiac ventricles were excised, blotted dry, trimmed of connective tissue and then cut into fine pieces while being kept on ice at 0°. To prepare heart sarcosomes, the ventricle tissue was washed vigorously with 100 mM KCl at 0° containing 5 mM histidine pH 7.3 and were then homogenised for 2 min in 4 vol. of KCl/histidine buffer at 4°. The sarcosomal fraction was obtained by differential ultracentrifugation of the tissue homogenate as described by Martonosi [16] and resuspended before use in 150 mM potassium phosphate buffer, pH 7.4, containing 100 μM DETAPAC.

1.4 *Oxygen consumption.* Oxygen consumption was measured at 37° with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.).

1.5 *Electron spin resonance.* ESR spectra were recorded on a Varian E109, dual cavity, ESR spectrometer. The sample temperature was 22°. For spin trapping experiments, rat heart sarcosomal fraction incubations were prepared containing 100 mM DMPO and the ESR spectrum of the incubation was recorded with time. Typical spectrometer settings for recording the ESR spectra are: field set 3290G, field scan 200G, modulation frequency 100 kHz, modulation amplitude 1.0G, receiver gain $1–5 \times 10^4$, scan time 4 min, time constant 0.3–1.0 sec, microwave frequency 9.24 GHz, microwave power 20 mW. The time course of spin adduct production was followed by setting the applied magnetic field for the top of a peak in the first derivative ESR spectrum of the spin adduct (with zero field scan) and following the signal amplitude with time. Spectra shown are typical of 6 separate sarcosomal preparations.

2. RESULTS

2.1 *Free radical production from adriamycin-treated rat heart sarcosomes.* The incubation of sarcosomal protein (1 mg/ml) with the spin trap DMPO (100 mM), NADPH (1 mM) and adriamycin (100 μM) at 22°, gave the ESR spectrum reported in Fig. 1. This spectrum has ESR parameters: $g = 2.0050$, $a_N = a_H = 14.9$G. These parameters are consistent with those for the ESR spectrum of the hydroxyl radical spin adduct of DMPO, 5,5-dimethyl-2-hydroxypyrrolidino-1-oxyl (DMPO-OH) [17]. A typical time course of DMPO-OH production in adriamycin-treated sarcosomal fractions is shown in Fig. 2, curve *a*, with the spread of maximum DMPO-OH signal intensities given in Table 1. To confirm that DMPO-OH is produced from hydroxyl radicals produced by the adriamycin-treated sarcosomal fraction with NADPH careful controls were performed to detect components of DMPO-OH production (if any) formed by hydroxyl radical-independent routes [18] and by the decomposition of the superoxide spin adduct [19].

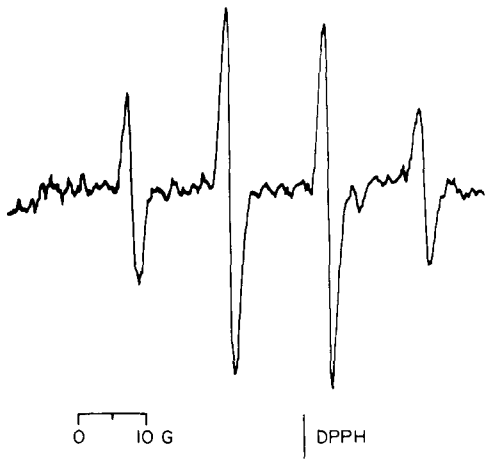

Fig. 1. Free radical production from adriamycin-treated rat heart sarcosomes. Reaction mixture: 1 mg/ml rat heart sarcosomal protein, 1 mM NADPH, 100 mM DMPO, 100 μM adriamycin, 1 mM DETAPAC in 150 mM potassium phosphate, pH 7.4. The ESR spectrum was recorded after 10 min at 22°. Scan time 4 min ESR Spectral parameters: $g = 2.0050$, $a_N = a_H^\beta = 14.9$ G.

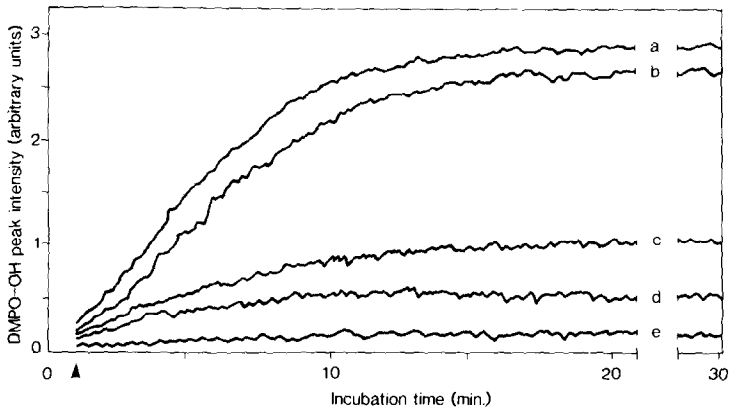

Fig. 2. The time course of DMPO–OH formation from adriamycin-treated rat heart sarcosomes. Reaction mixtures contain: 1 mg/ml rat heart sarcosomal protein, 1 mM NADPH, 100 mM DMPO and 1 mM DETAPAC in 150 mM potassium phosphate, pH 7.4. Further additions were: *a*—100 μM adriamycin, *b*—100 μM adriamycin + 0.1 mg/ml superoxide dismutase, *c*—No further additions, *d*—100 μM adriamycin + 100 mM mannitol and *e*—100 μM adriamycin + 500 units/ml catalase. Time course curves were recorded by setting a zero field scan and an applied magnetic field for the top of the downfield peak in the ESR shown in Fig. 1. Incubations were at 22°.

Firstly, addition of 100 mM mannitol to the adriamycin-treated sarcosomal fraction incubation used in Fig. 1 gave a large decrease (*ca.* 80%) in observed DMPO–OH production—compare Fig. 2, curves *a* and *d*. This is quantitatively consistent with the expected competition between DMPO (100 mM) and mannitol (100 mM) for hydroxyl radicals—given the high reactivities of both DMPO and mannitol toward hydroxyl radicals [19, 20]. Similar results were observed for the addition of 50 mM N-acetylcysteine to incubations—Table 1.

Addition of superoxide dismutase 330 units/ml to the incubations gave a small but not statistically significant decrease in DMPO–OH formation (curves *a* and *b*). Addition of catalase (500 units/ml) to the incubation, however, decreased the formation of DMPO–OH by *ca.* 90%. This suggests that most of the DMPO–OH production was hydrogen peroxide-dependent. Moreover, Table 1 shows that formation of hydroxyl radicals was not influenced by chelation of iron with either DETAPAC or desferrioxamine.

The dependence of the initial rate of DMPO–OH formation on NADPH in sarcosomal fractions, treated with 75 μM adriamycin is shown in Fig. 3. The kinetic curve suggests two reaction kinetic phases: (i) at low NADPH concentrations (< 100 μM), there is a large incremental increase in the initial rate of DMPO–OH production with increasing NADPH; and (ii) at high NADPH concentrations (> 100 μM), there is a relatively small

Table 1. Maximal DMPO-OH formation from rat cardiac sarcosomes in the presence of adriamycin

Incubation	Sample No.	DMPO-OH Maximal peak intensity (arbitary units)
Control	6	1.02 ± 0.23
+ 100μM adriamycin	6	2.83 ± 0.28
+ 100μM adriamycin and 0.1 mg/ml superoxide dismutase	6	2.62 ± 0.35
+ 100μM adriamycin and 500 U/ml catalase	6	0.18 ± 0.10
+ 100μM adriamycin and 100mM mannitol	6	0.45 ± 0.31
+ 100μM adriamycin and 100mM N-acetyl cysteine	6	0.29 ± 0.16
+ 100μM adriamycin and 1mM Desferrioxamine	6	2.75 ± 0.20

All reaction mixtures contain: 1 mg/ml sarcosomal protein, 1 mM NADPH, 100 mM DMPO and 1 mM DETAPAC (except where replaced by desferrioxamine) in 150 mM potassium phosphate, pH 7.4. DMPO–OH production was maximised after 15–20 min incubation time in all cases.

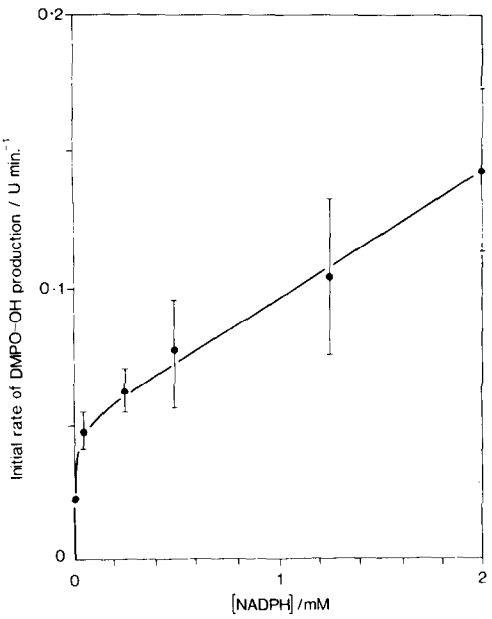

Fig. 3. The dependence of the rate of formation of DMPO–OH on NADPH concentration. Reaction mixtures contain 1 mg/ml sarcosomal protein, 100 μM adriamycin, 100 mM DMPO and 1 mM DETAPAC in 150 mM potassium phosphate, pH 7.4. Further addition was NADPH at the concentration indicated. Initial rates of formation of DMPO–OH shown are the means ± S.D. of six determinations, at 22°.

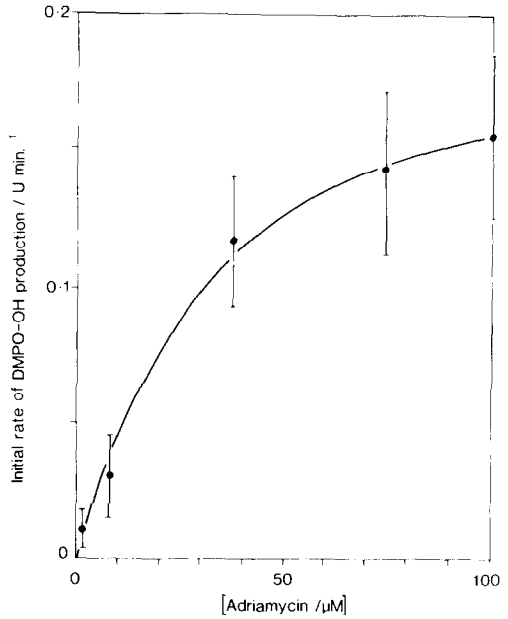

Fig. 4. The dependence of the rate of formation of DMPO–OH on adriamycin concentration. Reaction mixtures contain 1 mg/ml sarcosomal protein, 1 mM NADPH, 100 mM DMPO, 1 mM DETAPAC in 100 mM sodium phosphate pH 7.4, plus the concentration of adriamycin indicated. Initial rates of formation of DMPO–OH were measured and data shown are the means ± S.D. of six determinations.

incremental increase in the initial rate of DMPO–OH production with increasing NADPH.

The dependence of the stimulation of the initial rate of DMPO–OH formation in sarcosomal fractions on adriamycin concentration is shown in Fig. 4 (rates of DMPO–OH production shown are the experimentally observed rates less the mean initial rate of DMPO–OH formation in sarcosomal fractions in the absence of adriamycin). This kinetic curve suggests the stimulation of the rate of formation of DMPO–OH by adriamycin approaches a maximum value at relatively high concentrations (75–100 μM) of adriamycin. This saturation effect could not be due to the quenching of hydroxyl radicals by adriamycin as the biomolecular rate constant for reaction of hydroxyl radicals with DMPO is high ($2 \times 10^9 M^{-1}sec^{-1}$) [19], and the DMPO concentration is in a 1000 fold excess over adriamycin. Rather, the saturation kinetics may reflect the saturation of a reductive binding site for adriamycin in the sarcoplasmic reticular membrane.

2.2 *Oxygen consumption.* Oxygen consumption by rat heart sarcosomal fractions, with NADPH, was measured on a Clark-type oxygen electrode in the absence and presence of adriamycin. The initial rates of oxygen consumption by rat heart sarcosomal incubations at 37°, are given in Table 2. Adriamycin stimulates oxygen consumption in rat heart sarcosomal incubations with NADPH, as expected and is consistent with the observed hydroxyl radical pro-

Scheme 1. A mechanism for hydroxyl production from adriamycin-treated rat heart sarcosomes

1. Quinone metabolic reduction:

$$NADPH + 2Q \xrightarrow{\text{Quinone reductase}} NADP^+ + 2Q^{\cdot} + H^+$$

2. Superoxide production:

$$Q^{\cdot} + O_2 \rightarrow Q + O_2^{\cdot}$$

3. Superoxide dismutation:

$$2O_2^{\cdot} + 2H^+ \rightarrow O_2 + H_2O_2$$

4. Hydroxyl radical production:

$$Q^{\cdot} + H_2O_2 \rightarrow Q + HO^- + {}^{\cdot}OH$$

or

$$Fe^{3+}\text{–}X + Q^{\cdot} \rightarrow Fe^{2+}\text{–}X + Q$$
$$Fe^{2+}\text{–}X + H_2O_2 \rightarrow Fe^{3+}\text{–}X + HO^- + {}^{\cdot}OH$$

5. Semiquinone dismutation:

$$2Q^{\cdot} + 2H^+ \rightarrow QH_2 + Q.$$

Table 2. Initial rates of oxygen consumption by rat heart sarcosomal fractions

Incubation	Sample No.	$-d[O_2]/dt/\mu M\ mg^{-1}$ protein min^{-1}
Control	6	0.98 ± 0.18
+ 0.1 mM adriamycin	6	1.95 ± 0.36

Incubations contain: 1 mg/ml sarcosomal protein, 1 mM DETAPAC, 1 mM NADPH in 150 mM potassium phosphate, pH 7.4. Incubations were at 37°.

duction arising from the reduction of molecular oxygen.

3. DISCUSSION

The cardiotoxicity arising as a side effect of chronic adriamycin therapy appears to be a clinical manifestation of gross changes in myocardial cell structural and functional morphology [2, 3]. The observation of enhanced peroxidation of lipids in myocardial membrane of adriamycin-treated animals [21] and the ability of sulphydryl reagents to inhibit adriamycin-induced cardiotoxicity [11, 22] suggested that the cardiac myopathy may be an oxidative pathology involving the production of reactive intermediates of oxygen reduction: superoxide, hydrogen peroxide and hydroxyl radicals [23].

The sarcoplasmic reticulum is an important site of adriamycin metabolism in the myocardium [3] and adriamycin stimulates oxygen uptake. Superoxide radicals and hydrogen peroxide (resulting from the dismutation of the former) are not intrinsically highly damaging to the myocardial cells, but their production is of far greater concern if they are precursors of the much more highly oxidising and cellular damaging hydroxyl radical [23].

In this report, the production of hydroxyl radicals by rat heart sarcosomes, treated with adriamycin and NADPH, has been demonstrated. Adriamycin semiquinone and superoxide are believed to be formed as intermediates, superoxide being a precursor of hydrogen peroxide. Consequently hydroxyl radical production was not significantly affected by addition of superoxide dismutase, but was greatly inhibited by catalase. Hydroxyl radical production occurred in the presence of the metal ion chelators DETAPAC or desferrioxamine. This suggests that a Haber–Weiss mechanism is not involved [24], although a recent report [25] has indicated that Fe^{III} chelated by DETAPAC can be reduced by radicals such as the paraquat radical and can then catalyze the decomposition of hydrogen peroxide to hydroxyl radicals (Fenton reaction). Adriamycin semiquinone may similarly reduce Fe^{III}-DETAPAC or some other iron complex present in the biological system. The reactions thought to occur are summarized in the scheme.

Adriamycin appears to be reduced to the semiquinone by an NADPH-dependent adriamycin reductase, probably similar to that reported by Goodman and Hochstein [26]. The semiquinone was not observed by ESR in the sarcosomal incubations, probably because of its rapid reaction with oxygen in such aerobic and physiologically functioning biological systems. This probably also prevented the formation of the fully reduced form of adriamycin (QH_2).

ESR failed to detect a spin adduct of superoxide, although superoxide production has been implicated in the reaction mechanism. The superoxide scavenging efficiency of 100 mM DMPO is *ca.* 10 times less efficient than 50 μM cytochrome *c* (as used by Doroshow [5]); the bimolecular rate constants for the reaction of superoxide with DMPO and ferricytochrome *c* at pH 7.4 and 25° are $10\ M^{-1}sec^{-1}$ and approx. $2 \times 10^5 M^{-1}sec^{-1}$ respectively [19, 27]. Moreover, the superoxide spin adduct of DMPO is relatively unstable ($t_{\frac{1}{2}}$ 90 sec) [28]. These two features of superoxide detection with DMPO, compared to those of ferricytochrome *c* reduction probably explain the lack of detection of a superoxide spin adduct in the sarcosomal incubations.

In contrast, the detection of hydroxyl radicals by spin trapping with DMPO is much more sensitive than for superoxide. The bimolecular rate constant for the reaction of hydroxyl radicals with DMPO is *ca.* $2 \times 10^9 M^{-1}sec^{-1}$ at 298 K and the half life of DMPO–OH is *ca.* 1 hr at 298 K [19]. The longer half life of DMPO–OH probably is most important feature for high sensitivity in hydroxyl detection by spin trapping with DMPO, as although hydroxyl radicals react very rapidly with DMPO, they also react rapidly with virtually every cellular component.

The oxidative events stimulated by adriamycin in rat heart sarcosomes are seen here to be a result of an enhancement of normal spontaneous oxygen radical production by 2–3-fold in the sarcoplasmic reticulum. Adriamycin appears to stimulate NADPH oxidase-like activity in the rat heart sarcoplasmic reticulum and thereby induce oxidative stress in the myocardium (together with minor contributions from adriamycin metabolism in other subcellular fractions [11]). The rat myocardium appears singularly ill-equipped to detoxify reactive oxygen intermediates: catalase and superoxide dismutase activities in the rat heart are 140 and 4 times lower than in the rat hepatic tissue, respectively, and cardiac glutathione peroxidase is inhibited by adriamycin [29]. The dearth of these enzyme activities, important in the detoxification of superoxide and hydrogen peroxide, is thought to make the myocardium particularly sensitive to oxidative stress. Moreover, similar spin trapping studies with adriamycin-treated rat liver microsomes gave only the superoxide spin adduct of DMPO—the hydroxyl spin adduct could only be formed by addition of H_2O_2 to the medium [30, 31]. This suggests that the heart sarcoplasmic reticulum is apparently better equipped to promote the reduction of hydrogen peroxide to hydroxyl radicals than is the endoplasmic reticulum of the liver

(at least for the rat). The critical role of reduced flavoprotein and/or iron proteins is anticipated.

Preventive measures to avoid oxidative damage may be seen to be (i) the development of adriamycin anaolgues which do not stimulate hydroxyl radical production of the heart sarcoplasmic reticulum, and (ii) the administration of antioxidants, particularly hydroxyl radical scavengers, e.g. N-acetyl cysteine [11], and (iii) avoidance of the loss of glutathione peroxidase activity.

Acknowledgements—The work was supported by the Cancer Research Campaign.

The authors thank Drs. J. Butler and A. J. S. Swallow for helpful discussion.

RESFERENCES

1. F. Arcamone, *Doxorubicin Anticancer Antibiotics*, Medicinal Chemistry Monographs 17, 360 pp. (1981).
2. L. Lenzas and A. Page, *Cancer Treat. Rev.* **3**, 111 (1976).
3. V. J. Ferrans, *Cancer Treat. Rep.* **62**, 955 (1978).
4. N. R. Bachur, S. L. Gordon, M. V. Gee and H. Kon, *Proc. natn. Acad. Sci. U.S.A.* **76**, 954 (1979).
5. J. H. Doroshow, *Cancer Res.* **49**, 460 (1983).
6. E. J. Land, T. Mukherjee, A. J. Swallow and M. J. Bruce, *Arch. biochem. Biophys.* **225**, 116 (1983).
7. C. C. Winterbourn, *Arch. biochem. Biophys.* **209**, 159 (1981).
8. D. A. Bates and C. C. Winterbourn, *Biochem. J.* **203**, 159 (1982).
9. C. C. Winterbourn, *FEBS Lett.* **136**, 89 (1981).
10. T. Komiyama, T. Kikuchi and Y. Sugiura, *Biochem. Pharmac.* **31**, 3651 (1982).
11. J. J. Doroshow, G. Y. Locker, I. Ifrim and C. E. Myers, *J. clin. Invest.* **68**, 1053 (1981).
12. M. J. Perkins, *Adv. phys. org. Chem.* **17**, 1 (1980).
13. P. J. Thornalley and N. J. F. Dodd, manuscript in preparation.
14. F. P. Mettler, D. M. Young and J. M. Ward, *Cancer Res.* **37**, 2705 (1977).
15. J. M. McCord and I. Fridovich, *J. biol. Chem.* **244**, 6049 (1960).
16. A. Martonosi, *J. biol. Chem.* **243**, 71 (1968).
17. E. Finkelstein, G. M. Rosen and E. J. Rauckman, *Arch. biochem. Biophys.* **200**, 1 (1980).
18. E. Finkelstein, G. M. Rosen and E. J. Rauckman, *Mol. Pharmac.* **21**, 262 (1982).
19. E. Finkelstein, G. M. Rosen and E. J. Rauckman, *J. Am. chem. Soc.* **102**, 4994 (1980).
20. J. S. Moore, K. G. Kemsley, J. V. Davies and G. O. Phillips, *Proc. Assoc. Radiat. Res. Meeting*, p. 99. Elsevier–North Holland, New York (1979).
21. C. E. Myers, W. P. McGuire, R. M. Liss, I. Ifrim, K. Grotzinger and R. C. Young, *Science* **197**, 165 (1977).
22. R. W. Freeman, J. S. MacDonald, R. D. Olson, R. C. Boerth, J. A. Oates and R. P. Harbison, *Toxic. appl. Pharmac.* **54**, 164 (1980).
23. J. V. Bannister, W. H. Bannister, H. A. O. Hill and P. J. Thornalley, *Life Chem. Reports* **1**, 1 (1983).
24. G. R. Buettner, L. W. Oberley and S. W. H. C. Leuthauser, *Photochem. Photobiol.* **28**, 693 (1978).
25. H. S. Sutton and C. C. Winterbourn, in: *Oxygen Radicals in Chemistry and Biology* (Eds. W. Bors, M. Saran and D. Taid), p. 177. Walter de Gruyter, Berlin (1984).
26. J. Goodman and P. Hochstein, *Biochem. biophys. Res. Commun.* **77**, 797 (1977).
27. J. Butler, W. H. Koppenol and E. Margoliash, *J. biol. Chem.* **257**, 10747 (1982).
28. G. R. Beuttner and L. W. Oberley, *Biochem. biophys. Res. Commun.* **83**, 69 (1978).
29. J. H. Doroshow, G. Y. Locker and C. E. Myers, *J. clin. Invest.* **65**, 128 (1980).
30. B. Kalyanaraman, E. Perez-Reyes and R. P. Mason, *Biochim. biophys. Acta.* **680**, 119 (1980).
31. H. Nohl and W. Jordan, *Biochem. biophys. Res. Commun.* **114**, 197 (1983).