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REDUCTION OF OXYGEN BY NADH/NADH DEHYDROGENASE IN THE PRESENCE OF ADRIAMYCIN

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Cardiac mitochondrial NADH dehydrogenase (Cytochrome *c* reductase, EC1.6.99.3) catalyses the reduction of *ferri*cytochrome *c* to *ferro*cytochrome *c* by NADH. In the presence of the anthracycline anti-tumour drug, adriamycin, electron transfer from NADH is subverted to dioxygen. Using the electron spin resonance technique of spin trapping with the spin trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) adriamycin was found to stimulate the formation of superoxide and hydroxyl radicals in the NADH/NADH dehydrogenase reaction. Hydroxyl radical formation is dependent on the availability of trace amounts of redox active metal ions – particularly ferric ions. Trace amounts of ferric ions catalyse the formation of hydroxyl radicals by both superoxide-dependent and adriamycin-dependent one electron reduction of hydrogen peroxide.

The metabolism of adriamycin by cardiac mitochondrial NADH dehydrogenase may be an important etiological factor in adriamycin-induced cardiotoxicity. It may be therapeutically beneficial to keep non-essential ferric/ferrous ions in the myocardium at minimum levels with siderophoric iron chelators – providing the anti-tumour activity of adriamycin is not impaired.

KEY WORDS: NADH/NADH Dehydrogenase, Adriamycin, Superoxide, Hydroxyl Radicals, Metal Ions, Spin-trapping.

INTRODUCTION

Adriamycin (doxorubicin) is currently one of the most widely used therapeutic agents in the treatment of cancer.[1] It achieves high remission rates against a broad range of tumours.[2] The clinical use of adriamycin is limited, however, by cumulative adverse effects, of which congestive cardiomyopathy is the most life-threatening.[3] At the biochemical level, this cardiomyopathy is initially characterised by the swelling and calcification of myocrdial cell mitochondria,[4] decreased cellular levels of reduced glutathione,[5] and lipid peroxidation.[6] These sequelae of adriamycin-induced cardiotoxicity may arise from oxidative damage caused by enhanced formation of superoxide, hydrogen peroxide and hydroxyl radicals from the metabolism of adriamycin in the myocardium.[7]

Cardiac "low-molecular weight" NADH dehydrogenase (cytochrome *c* reductase, EC 1.6.99.3), a flavin-containing iron-sulphur protein devoid of ubiquinone reductase activity,[8] has been shown to catalyse the transfer of reducing equivalent from NADH to adriamycin.[9] The formation of superoxide (measured as superoxide dismutase-inhibitable reduction of ferricytochrome *c*) and the formation of hydroxyl radicals

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(measured as methane formation from dimethylsuphoxide) was shown to be stimulated by adriamycin in the NADH/NADH dehydrogenase reaction. In this report, we describe the use of electron spin resonance (ESR) technique of the spin trapping[10] to demonstrate the formation of superoxide and hydroxyl radicals arising from the metabolism of adriamycin by cardiac NADH dehydrogenase. The results obtained clearly demonstrate a profound influence of traces of ferric/ferrous ions on the formation of hydroxyl radicals in this system.

MATERIALS AND METHODS

Adriamycin hydrochloride, the metal ion chelating agent diethylenetriaminepentaacetic (DETAPAC), diphosphopyridine nucleotides – oxidised from NAD^+ (Grade V) and reduced form NADH (Grade III), porcine cardiac NADH dehydrogenase (cytochrome *c* reductase, NADH: [acceptor] oxidoreductase, EC 1.6.99.3) with an activity of 1.35 U/mg protein (one unit will reduce 1 μmole of oxidised cytochrome *c* per minute at pH 8.5 and 25°C), and bovine hepatic catalase with an activity of 5000 U/mg protein (one unit will decompose 1.0 μmole of H_2O_2 per minute at pH 7.0 and 25°C), were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, UK. Human erythrocyte superoxide dismutase was prepared and purified from outdated blood and had an activity of 3000 U/mg protein.[11] Desferal (desferrioxamine B) was purchased from Ciba Laboratories, Horsham, Sussex, UK.

The spin trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich Chem. Co. Ltd., Gillingham, Dorset, UK. and purified by published methods.[12]

The formation of free radical intermediates from NADH/NADH dehydrogenase catalysed by adriamycin was investigated by the electron spin resonance (ESR) technique of spin trapping using the spin trap DMPO. ESR spectra were recorded on as Varian E104 ESR spectrometer with a E900-3 data acquisition system. Typical incubations contained 100 μM NADH, 200 mU/ml NADH dehydrogenase, 100 mM DMPO, 10 mM potassium phosphate buffer, pH 7.4 and 10–100 μM adriamycin. ESR spectra reported are the average of 4 × 60 sec scans starting from 60 sec into the reaction time. ESR spectra illustrated are typical of four independent determinations.

RESULTS AND DISCUSSION

Mitochondrial "low molecular weight" NADH dehydrogenase (cytochrome *c* reducatase) catalyses the reduction of ferricytochome *c* to the ferrocytochrome by NADH and has no detectable ubiquinone reductase activity. It is usually considered to be located on the outer leaflet of the mitochondrial membrane and therefore may be accessible to xenobiotic substrates which bind to but do not readily cross mitochondrial membranes. One such substrate is adriamycin. Adriamycin is known to bind to mitochondrial membranes and stimulate NADH dehydrogenase activity.[9,13]

When NADH (100μM) is incubated with NADH dehydrogenase (200 mU/ml) in 10 mM potassium phosphate buffer pH 7.4 at 25°C in the presence of the spin trap DMPO (100 mM), no ESR signal is detected (Figure 1a). When 100 μM adriamycin is added to this system, an ESR spectrum is observed (Figure 1b). This spectrum can be assigned to a composite of the ESR spectra of two DMPO-derived spin adducts:

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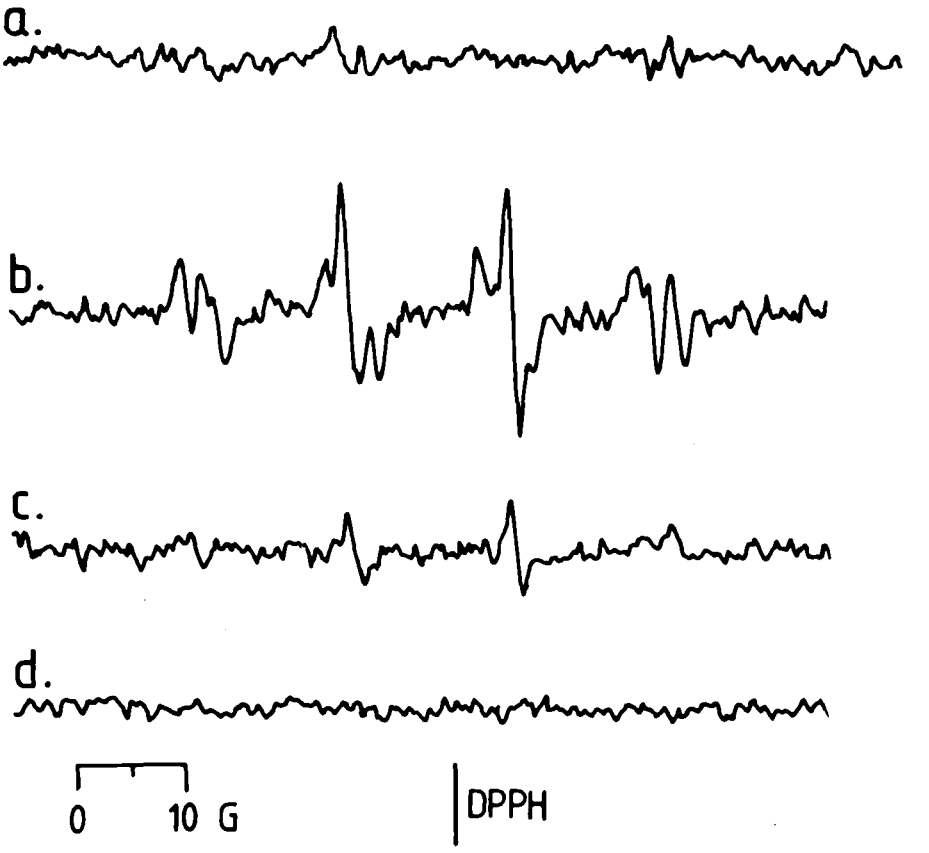

FIGURE 1 Spin trapped intermediates in the NADH/NADH dehydrogenase system in the presence of adriamycin. All incubations contained 10 mM potassium phosphate buffer, pH 7.4 with 100 μM NADH, 200 mU/ml NADH dehydrogenase and 100 mM DMPO. ESR spectra are an average of 4 × 60 s scans after an initial delay time of 60 s. Field set 3385 G, field scan 100 G, modulation amplitude 1.0 G, modulation frequency 100 kHz, microwave power 10 mW, microwave frequency 9.462 GHz, sample temperature 25°C. (a) control, (b) + 100 μM adriamycin, (c) + 165 U/ml superoxide dismutase, and (d) + 250 U/ml catalase + 165 U/ml superoxide dismutase.

i) the hydroxyl radical spin adduct of DMPO, 5,5-dimethyl-2-hydroxy-pyrrolidino-1-oxyl (DMPO–OH) characterised by $9 = 2.0050$, $a_N = a_H^\beta = 14.9$G,[14] and

ii) the superoxide spin adduct of DMPO, 5,5-dimethyl-2-hydroperoxy-pyrrolidino-1-oxyl (DMPO–OOH) characterised by $g = 2.0061$, $a_N = 14.3$G, $a_H^\beta = 11.7$G and $a_H^\gamma = 1.25$G (Figure 2).

This assignment is confirmed by the inhibition of the DMPO–OOH component when superoxide dismutase (165 U/ml) is added to the incubation mixture (Figure 1c), and the inhibition of both DMPO–OOH and DMPO–OH components when superoxide dismutase and catalase (250 U/ml) are added to the incubation mixture (Figure 1d). Hydrogen peroxide, which is removed by catalase, is considered to be the precursor to hydroxyl radical and consequently DMPO–OH formation.

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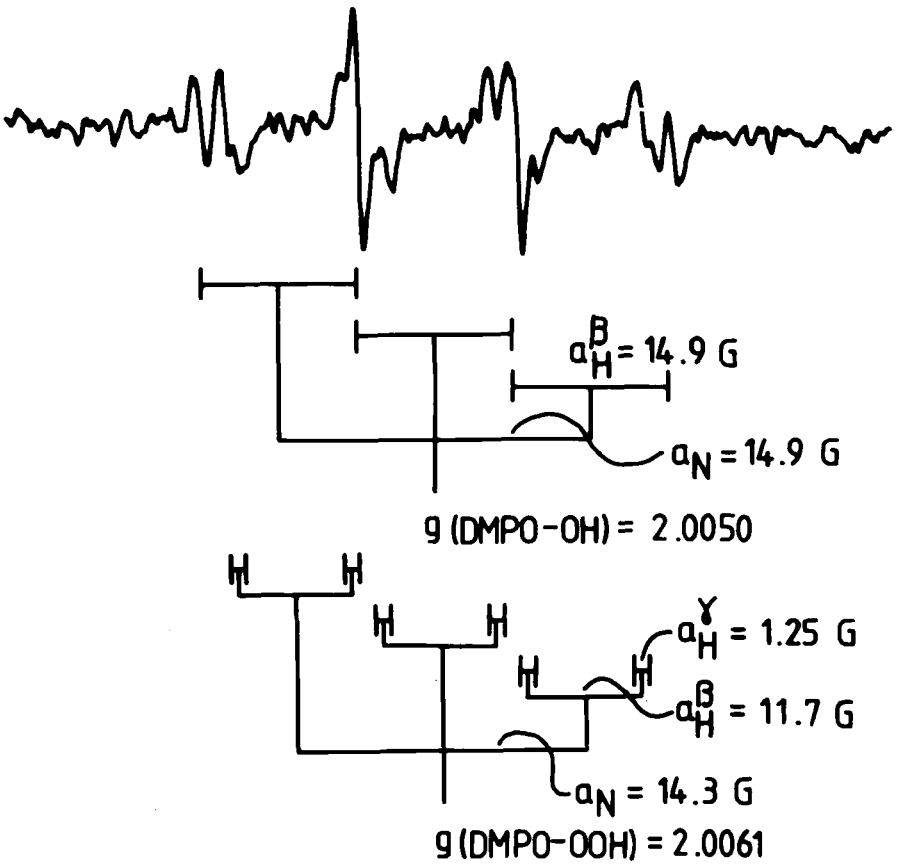

FIGURE 2 Assignment of component ESR spectra in the composite spectrum from the NADH/NADH dehydrogenase/adriamycin incubation. The experimental spectrum from Figure 1b is presented with stick diagrams to show assignment of hyperfine structure to the superoxide and hydroxyl spin adducts of DMPO: DMPO–OOH and DMPO–OH.

Metal ion chelators, which avidly bind certain metal ions and make them essentially kinetically inert, have been found to have an effect on spin adduct formation in the adriamycin/NADH/NADH dehydrogenase system. Addition of 1 mM DETAPAC, which chelates many redox active metal ions – particularly ferrous, ferric and cupric ions,[16] decrease DMPO–OH formation and increases DMPO–OOH formation (Figures 3a and b). Addition of 1 mM Desferal, which binds only ferric redox active metal ions inhibits the formation of DMPO–OH and stimulates the formation of DMPO–OOH (Figures 3a and c). This suggests that hydroxyl radical formation is catalyzed by traces of ferric ions.

Ferric complexes are known to catalyse the Haber-Weiss reaction, the superoxide-dependent reduction of hydrogen peroxide to hydroxyl radicals:

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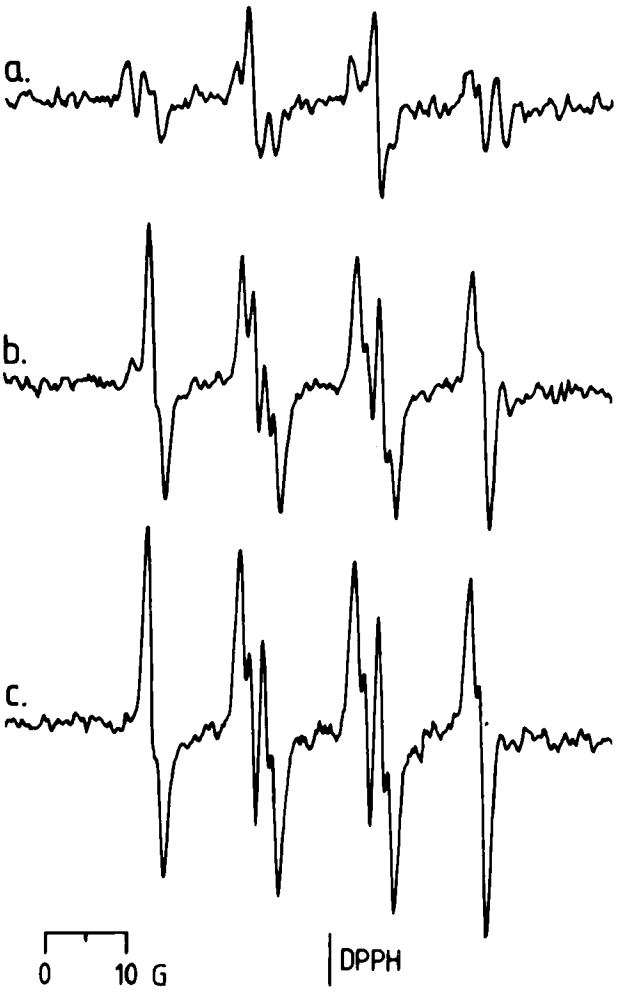

FIGURE 3 The effect of metal ion chelators on spin adduct formation in the NADH/NADH dehydrogenase system in the presence of adriamycin. All incubations contained: 10 mM potassium phosphate buffer, pH 7.4, 100 μM NADH, 200 mU/ml NADH dehydrogenase, 100 mM DMPO and 100 μM adriamycin. ESR spectra and instrumental details are as for Figure 1. (a) no added chelator, (b) + 1 mM DETAPAC, and (c) + 1mM Desferal.

$$O_2 + Fe(III) \longrightarrow Fe(II) + O_2$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^- + \cdot OH \qquad (1)$$

$$\text{Overall: } O_2 + H_2O_2 \xrightarrow{Fe(II/III)} O_2 + OH^- + \cdot OH$$

Ferric ions also bind to adriamycin[19] and may catalyse the reduction of hydrogen peroxide to hydroxyl radicals by adriamycin semiquinone:

$$SQ^{\cdot} - Fe(III) \longrightarrow \ - Fe(II) \xrightarrow{H_2O_2} Q - Fe(III) + OH^- + \cdot OH \qquad (2)$$

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TABLE I
Logarithmic association constants for iron (III) complexes with adriamycin, DETAPAC and Desferal

Ligan, L	log K_{FeL}	log K_{FeL2}	log K_{FeL3}	Ref.
Adriamycin	18	29	33.4	16
DETAPAC	27.5	–	–	17
Desferal	30.6			19

Inspection of Figures 1b, and c suggests there is both superoxide-dependent and superoxide-independent routes to hydroxyl radical formation (Eqs. 1 and 2) in the adriamycin/NADH/NADH dehydrogenase system. Comparison of this result with the effect of Desferal (Figure 3c) suggests that ferric ions are involved in the catalysis of both superoxide-dependent and superoxide-independent (adriamycin and semiquinone-mediated) routes to hydroxyl radical formation.

Association constant data for ferric adriamycin, ferri-DETAPAC and ferrioxamine complexes (Table I) indicate that Desferal is the most effective competitor, with adriamycin, for complexing ferric ions; indeed this also indicates the relatively strong association of the ferric trisadriamycin complex. A solution of adriamycin will therefore contain a trace amount of ferric trisadriamycin complex. On addition of a 10 fold excess of Desferal over adriamycin, trace ferric ions will be sequestered by Desferal. The resulting ferrioxamine complex is relatively kinetically inert to redox reactions. It is not a good catalyst for the Haber Weiss reaction[21] or, indeed for redox reactions of adriamycin semiquinone.[22] Rather, in the presence of Desferal, the favoured reaction of adriamycin semiquinone is the reduction of dioxygen to superoxide – with a very much slower residual reduction of hydrogen peroxide to hydroxyl radicals.

This argument is supported by the relative magnitude of the bimolecular rate constants for the reaction of adriamycin semiquinone with dioxygen, hydrogen peroxide and ferric-DETAPAC and ferrioxamine (Table II). The rate constant for the reaction of adriamycin semiquinone with dioxygen is of the same order of magnitude as the rate constant for the reaction of adriamycin semiquinone with ferric-DETAPAC but is at least four orders of magnitude greater than the rate constant for the reaction of adriamycin with ferrioxamine. This indicates that the formation of superoxide from adriamycin semiquinone in aerobic systems is a kinetically favourable process. In the absence of Desferal, trace amounts of ferric ions may catalyse both superoxide-dependent (Haber–Weiss) and superoxide-independent (adriamycin semiquinone mediated) formation of hydroxyl radicals by the one electron reduction of hydrogen peroxide (formed by the spontaneous dismutation of superoxide). The

TABLE II
Biomolecular rate constants for the reaction of adriamycin semiquinone with oxygen, hydrogen peroxide, ferric-DETAPAC and ferrioxamine at 298 K

Substrate	$k/M\text{-}1_s\text{-}1$	Ref.
O_2	3×10^8	22
H_2O_2	10^4-10^5	20
Ferric-DETAPAC	7×10^8	22
Ferrioxamine	$< 6 \times 10^4$	22

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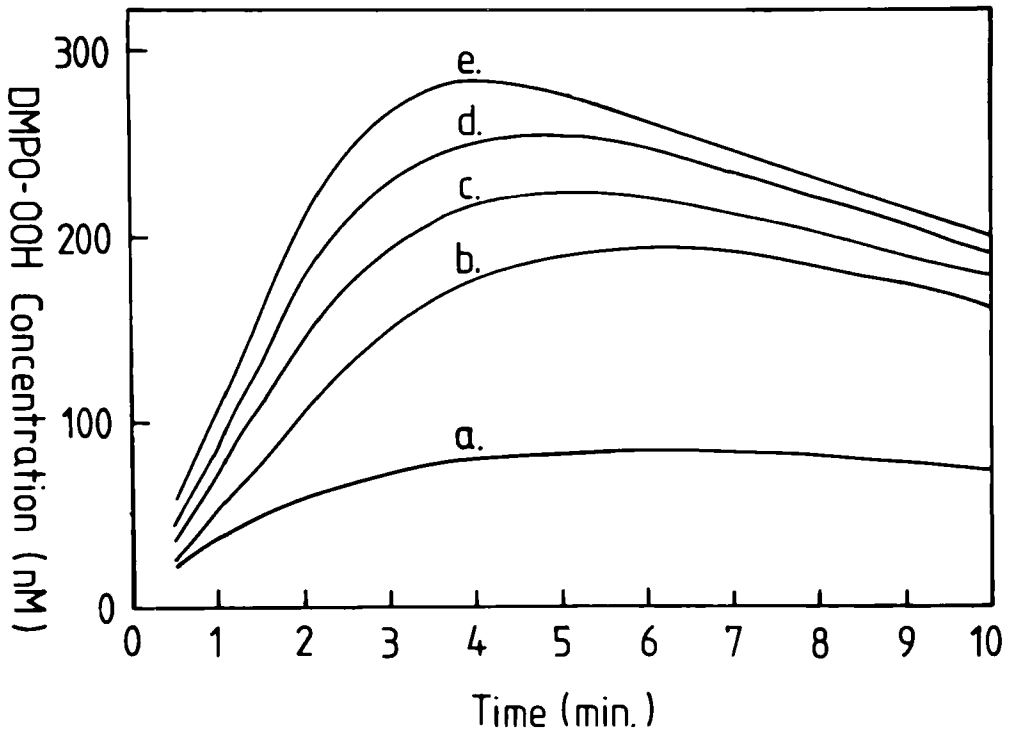

FIGURE 4 Superoxide spin adduct formation by the NADH/NADH dehydrogenase/adriamycin system in the presence of desferal. All incubations contained: 10 mM potassium phosphate buffer, pH 7.4, 100 μM NADH, 200 mU/ml NADH dehydrogenase, 100 mM DMPO and 1 mM Desferal. Instrumental details are as for Figure 1 except the field sweep is zero and the field is set for the top of the down field peak in the ESR spectrum of DMPO–OOH, and the signal intensity was followed with time. Spin adduct concentrations were calculated from a calibrated second integral. Incubations contain varying concentrations of adriamycin: (a) 100 μM, (b) 765 μM, (c) 50 μM, and (d) 10 μM.

hydroxyl radicals formed may initiate lipid peroxidation, in active enzymes and denature protein in the mitrochondrial membrane, and thereby trigger the sequelae of adriamycin-induced cardiotoxity.

In the presence of Desferal, trace amounts of ferric ions are effectively removed from partaking in the redox cycling reactions of adriamycin and superoxide formation only is favoured. In mitochondria, this poses a far less severe oxidative threat (superoxide is rapidly metabolised by superoxide dismutase) than the formation of hydroxyl radicals.

The myocardium is particularly sensitive to drug-induced oxidative stress. Enzymes which rapidly metabolise-superoxide (superoxide dismutase) and hydrogen peroxide (catalase and glutathione peroxidase) have low specific activities in the myocardium compared to those in other tissue where adriamycin is also extensively metabolised such as the liver.[23] Adriamycin has also been shown by ESR spin trapping to stimulate the formation of superoxide and hydroxyl radical in rat cardiac mitochondria[24,25] and sarcoplasmic reticulum.[26] The multiple site of metabolism of adriamycin in the

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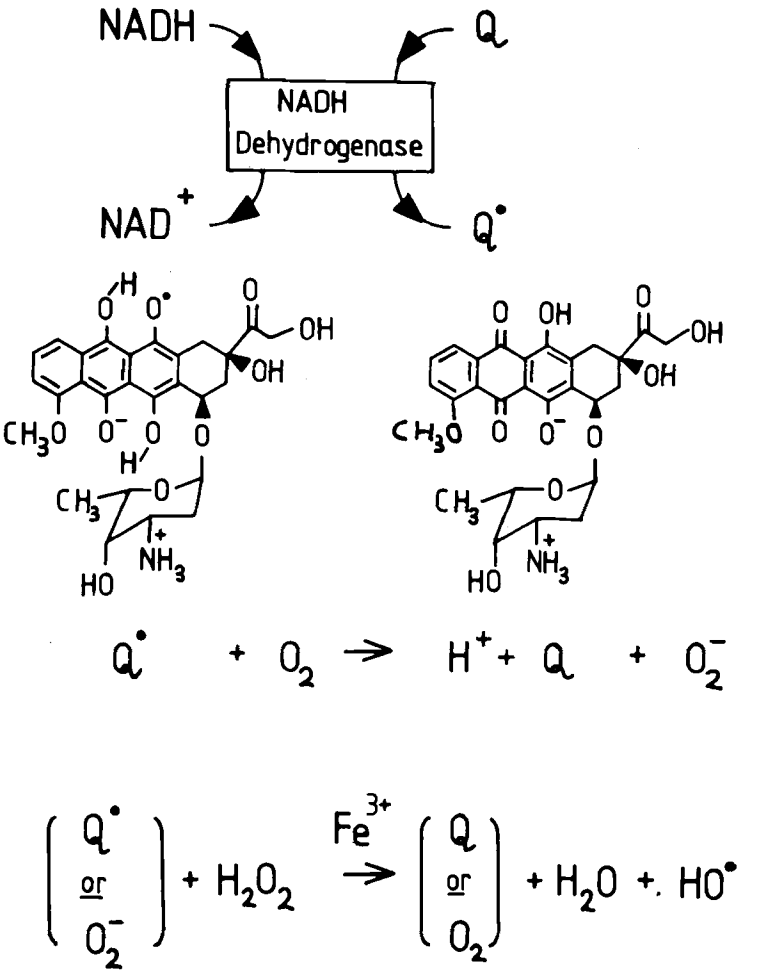

FIGURE 5 A mechanistic scheme showing the formation of superoxide and hydroxy radicals and the reduction of adriamycin by NADH/NADH dehydrogeanse.

myocardium combined with the low native enzymatic anti-oxidant protection of the myocardium may predispose the myocardium to oxidative damage induced by adriamycin.[23,24] From this study we conclude that adriamycin catalyses the formation of superoxide and hydroxyl radicals from NADH/cardiac "low molecular weight" NADH dehydrogenase. Hydroxyl radical formation is enhanced by traces of ferric ions (Figure 5). If a similar reaction occurs during adriamycin therapy, adriamycin-induced cardiotoxicity may be suppressed by therapy with siderophoric ion chelators. This deserves further clinical investigation — although there is some recent evidence[27–29] to suggest that such a regime may also suppress the toxicity of adriamycin to tumour tissue, which could negate the prospect of suppressing the adverse effects of adriamycin therapy only with iron chelators.

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