

# Oxidative and non-oxidative mechanisms in the inactivation of cardiac mitochondrial electron transport chain components by doxorubicin

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The quinonoid anthracycline, doxorubicin (Adriamycin) is a potent anti-neoplastic agent whose clinical use is limited by severe cardiotoxicity. Mitochondrial damage is a major component of this cardiotoxicity, and rival oxidative and non-oxidative mechanisms for inactivation of the electron transport chain have been proposed. Using bovine heart submitochondrial preparations (SMP) we have now found that both oxidative and non-oxidative mechanisms occur *in vitro*, depending solely on the concentration of doxorubicin employed. Redox cycling of doxorubicin by Complex I of the respiratory chain (which generates doxorubicin semiquinone radicals,  $O_2^-$ ,  $H_2O_2$ , and  $\cdot OH$ ) caused a 70% decrease in the  $V_{max}$  for NADH dehydrogenase during 15 min incubation of SMP, and an 80% decrease in NADH oxidase activity after 2 h incubation. This inactivation required only 25–50  $\mu M$ -doxorubicin and represents true oxidative damage, since both NADH (for doxorubicin redox cycling) and oxygen were obligatory participants. The damage appears localized between the NADH dehydrogenase flavin (site of doxorubicin reduction) and iron-sulphur centre N-1. Succinate dehydrogenase, succinate oxidase, and cytochrome *c* oxidase activities were strongly inhibited by higher doxorubicin concentrations, but this phenomenon did not involve doxorubicin redox cycling (no NADH or oxygen requirement). Doxorubicin concentrations of 0.5 mM were required for 50% decreases in these activities, except for cytochrome *c* oxidase which was only 30% inhibited following incubation with even 1.0 mM-doxorubicin. Our results indicate that low concentrations of doxorubicin (50  $\mu M$  or less) can catalyse a site-specific oxidative damage to the NADH oxidation pathway. In contrast, ten-fold higher doxorubicin concentrations (or more) are required for non-oxidative inactivation of the electron transport chain; probably via binding to cardiolipin and/or generalized membrane chaotropic effects. The development of agents to block doxorubicin toxicity *in vivo* will clearly require detailed clinical studies of doxorubicin uptake in the heart.

## INTRODUCTION

Doxorubicin (Adriamycin) is an anthracycline antibiotic widely used in the treatment of various human tumours. The usefulness of this drug is, however, limited by significant toxicity; including a potentially lethal cardiomyopathy [1]. At the cellular level, doxorubicin treatment induces distortion and disruption of mitochondrial and sarcoplasmic reticulum membranes [2]. The effects of doxorubicin and related drugs on mitochondrial properties have been extensively studied *in vivo* and *in vitro*, although some of the results remain controversial [3–30]. Studies with cultured cardiac cells have shown that doxorubicin affects both ATP and phosphocreatine levels [9]. At the mitochondrial level, impairment of several bioenergetic functions has been reported [3–5, 12–14, 26, 28].

Doxorubicin has been demonstrated to be able to form an electrostatic complex with cardiolipin, a phospholipid of the mitochondrial inner membrane [12, 24, 26, 29]. Possible mechanisms for toxicity have been proposed on the basis of this interaction, since Complexes I, III and

IV are known to require cardiolipin in their environment in order to maintain maximal activity [31]. It has also been shown that doxorubicin can undergo a one-electron reduction leading to the formation of a semiquinone radical species, this reduction being catalysed by the mitochondrial Complex I [6, 18–22]. The semiquinone radical species can be re-oxidized, by a one-electron transfer to molecular oxygen, to form superoxide anion. Interaction with the NADH dehydrogenase of Complex I may, thus, allow Adriamycin to produce superoxide, hydrogen peroxide and hydroxyl radicals [18–22]. Such activated oxygen species have been recognized as agents which can damage lipids, nucleic acids and carbohydrates [32–34]. Recent work has shown that proteins are also targets for oxidative damage, and that oxidatively denatured proteins undergo rapid proteolytic degradation in mitochondria [35, 36] and various cells [35–42].

We felt it was important to gain a more complete biochemical understanding of electron transport chain inactivation by doxorubicin. Such studies could have relevance for the reactions of a wide series of quinonoid compounds with mitochondrial enzymes. Of more

Abbreviations used: SMP, submitochondrial preparations; DCPIP, dichlorophenolindophenol; PMS, phenazine methosulphate; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine; TTFA, thenoyltrifluoroacetone.

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immediate importance is the problem of doxorubicin clinical toxicity. Several compounds (vitamins E and C,  $\beta$ -carotene, etc.) are being tested as protective agents *in vivo*, yet we do not know whether oxidative damage (by oxygen radicals and  $H_2O_2$ ) and non-oxidative damage (cardiolipin binding, membrane chaotropic effects) occur independently or synergistically. To begin to answer such questions (the topic is clearly too large to be solved with a single paper) we have attempted to discriminate oxidative damage and non-oxidative damage to cardiac mitochondrial electron transport chains, caused by doxorubicin. Damage has been assessed both in terms of dehydrogenase ( $V_{max}$  and  $K_m$ ) and overall oxidase activities. As previously [18–22], bovine heart submitochondrial preparations (SMP) have been used because these preparations contain the entire electron transport chain without the complications of outer membrane, inter-membrane space and matrix components. Since oxidative damage requires both a reductant for the electron transport chain (to 'fuel' doxorubicin redox cycling) and oxygen, we have used incubations in the presence or absence of NADH or succinate, under both aerobic and anaerobic conditions, to discriminate between oxidative damage and non-oxidative damage.

## EXPERIMENTAL

### Submitochondrial preparations (SMP)

Bovine heart mitochondria were isolated by the following modification to the method of Smith, procedure 1 [43]. Bovine hearts were sliced, minced and then homogenized in a buffer consisting of 0.25 M-sucrose, 0.001 M-EDTA, and 0.01 M-Hepes (pH 7.6). The homogenates (20% w/v) were centrifuged at 1600 g and the supernatants were carefully decanted and filtered through a double layer of gauze. The 1600 g pellets were resuspended, centrifuged again, and the supernatants were decanted and filtered through gauze. The two 1600 g filtered supernatants were combined and then centrifuged at 6000 g to produce a mitochondrial pellet. Mitochondrial pellets were washed three times (by centrifugation) and stored overnight at  $-80^\circ\text{C}$ . Before sonication, the mitochondria were suspended in 50 mM-potassium phosphate buffer (pH 7.6) and centrifuged at 6000 g. The pellets were resuspended in 50 mM-potassium phosphate buffer (pH 8.6) containing 2 mM-EDTA [44], at 20 mg of Biuret protein per ml, and sonicated ( $4 \times 40$  s bursts) under an argon atmosphere [19,20]. The unbroken mitochondria were removed by centrifugation at 8000 g. The supernatant thus obtained was then centrifuged at 100000 g for 30 min. The pellet was resuspended in the same volume of 50 mM-potassium phosphate buffer (pH 7.4), resonicated, and the SMP were recovered by centrifugation at 100000 g for 30 min. The final SMP pellet was resuspended at 20 mg of Biuret protein per ml in the same buffer.

### Incubations

SMP were incubated for various periods of time, with various concentrations of doxorubicin, in the presence or absence of oxidizable substrate (NADH or succinate). All the incubations were performed at  $30^\circ\text{C}$  in 50 mM-potassium phosphate buffer (pH 7.4) with 0.1 mg of SMP protein/ml. Phosphate buffer was chosen because

of its low reactivity with activated oxygen species [45]. Small volumes of doxorubicin were added from freshly prepared solutions in water. For anaerobic incubations, the medium was purged with argon, and the sample vials were filled and closed under an argon atmosphere.

### Enzyme assays

NADH dehydrogenase activity was assayed spectrophotometrically by the rate of NADH-dependent ferricyanide reduction at 420 nm, as described by Singer [46]. Five ferricyanide concentrations (0.5–1.0 mM) were used to determine each  $V_{max}$  or  $K_m$  data point. In one set of experiments (Table 1), NADH dehydrogenase was assayed by the alternate method of 2,6-dichlorophenol-indophenol (DCPIP) reduction [46]. Five DCPIP concentrations (20–80  $\mu\text{M}$ ) were used to determine each  $V_{max}$  or  $K_m$  data point.

Succinate dehydrogenase activity was measured spectrophotometrically [46] by the rate of succinate-dependent DCPIP reduction, in the presence of various concentrations of phenazine methosulphate (PMS). Five PMS concentrations (60–600  $\mu\text{M}$ ) were used to determine each  $V_{max}$  or  $K_m$  data point, by reduction of 37  $\mu\text{M}$ -DCPIP. In certain experiments (Fig. 3) the effects of incubation with NADH ( $\pm$  doxorubicin) on succinate dehydrogenase activity were measured. Such measurements required the removal of residual NADH prior to determination of succinate dehydrogenase, since NADH can reduce PMS directly. In such experiments, NADH remaining after incubation was oxidized to  $\text{NAD}^+$  by reaction with 2.0 mM-pyruvate and 2.0 i.u. of lactate dehydrogenase.

In each NADH dehydrogenase or succinate dehydrogenase assay, enzymatic velocities were extrapolated to infinite ferricyanide, DCPIP or PMS concentrations (as appropriate) by least-squares linear regression analyses of Lineweaver–Burk plots. Rates of ferricyanide reduction by NADH dehydrogenase (Figs. 1 and 2) were divided by two to obtain  $V_{max}$  results for NADH oxidation (1 NADH = 2 ferricyanide). Rates of DCPIP reduction by NADH dehydrogenase (Table 1) were not converted to  $V_{max}$  values for NADH oxidation because DCPIP does not measure the full activity of the enzyme.

NADH oxidase, succinate oxidase and cytochrome *c* oxidase activities were measured polarographically [19], using a Gilson 'Oxygraph' fitted with a Clark electrode (Rank Bros.). All measurements were performed at  $30^\circ\text{C}$  in 50 mM-potassium phosphate buffer (pH 7.4). Substrate concentrations, tested to produce maximal oxidase activities, were: 0.2 mM-NADH; 10 mM-succinate; or 5 mM-ascorbate plus 0.5 mM *NNN'*-tetramethyl-*p*-phenylenediamine (TMPD). Following incubation of SMP with NADH, succinate oxidase activity was measured in the presence of 0.2  $\mu\text{M}$ -rotenone, and cytochrome *c* oxidase activity was measured in the presence of rotenone plus 0.4  $\mu\text{M}$ -antimycin A. Following incubation of SMP with succinate, NADH oxidase activity was measured in the presence of 1.0  $\mu\text{M}$ -thenoyltrifluoroacetone (TTFA), and cytochrome *c* oxidase activity was measured in the presence of TTFA and 0.4  $\mu\text{M}$ -antimycin A.

The results of enzyme assays presented in all Figures and Tables are the means  $\pm$  S.E.M. of between three and six independent experiments with different preparations.

## RESULTS

### NADH dehydrogenase activity following incubation with doxorubicin

Previous work with cardiac SMP [18–22] demonstrated that doxorubicin undergoes redox cycling with mitochondrial Complex I, probably at the flavin moiety of NADH dehydrogenase. Such redox cycling generates  $O_2^-$  and other reactive oxygen species. Studies of  $O_2^-$  production revealed an apparent  $K_m$  for doxorubicin of 50–60  $\mu M$  [20]. To determine if NADH dehydrogenase could be oxidatively damaged by the redox cycling of doxorubicin, we incubated SMP in the presence or absence of 50  $\mu M$ -doxorubicin. Fig. 1 shows NADH dehydrogenase activity, as measured by the ferricyanide reduction assay [46], following, 0, 5, 10, or 15 min of incubation.

In the absence of doxorubicin, NADH dehydrogenase activity was relatively stable for 15 min of incubation without oxidizable substrate, or with succinate as a reductant for the electron transport chain (Fig. 1a). NADH dehydrogenase activity was also stable in the presence of NADH under an atmosphere of argon (Fig. 1a). Prolonged NADH oxidation in an air atmosphere, however, induced a small but consistent decrease in NADH dehydrogenase activity (Fig. 1a). Addition of 50  $\mu M$ -doxorubicin had little or no effect on NADH dehydrogenase activity during 15 min incubation with succinate, or without oxidizable substrate (Fig. 1b). In

contrast, incubation with both NADH and doxorubicin caused marked decreases in NADH dehydrogenase activity; 30% loss of activity after 5 min incubation and 70% loss of activity after 15 min incubation. This NADH and doxorubicin-dependent loss of activity was completely prevented by using an atmosphere of argon for incubation (Fig. 1b). The results of Fig. 1 demonstrate the kind of synergism between NADH (as electron donor) and doxorubicin (as the redox cycling agent) which one might expect, if damage to the NADH dehydrogenase is caused by active oxygen species. Succinate (and succinate dehydrogenase) do not reduce doxorubicin [18–22], and no redox cycling can occur in the absence of an oxidizable substrate for the electron transport chain, or under anaerobic conditions (argon atmosphere).

To further characterize the inactivation of NADH dehydrogenase by doxorubicin and NADH, we tested the effects of various doxorubicin concentrations after 15 min incubations, or with no incubation. No significant decrease in NADH dehydrogenase activity was observed, at any doxorubicin concentration, if SMP were not incubated (Fig. 2). Following 15 min incubation, the loss of NADH dehydrogenase activity was maximal at doxorubicin concentrations of 25–50  $\mu M$  (Fig. 2) in good agreement with the  $K_m$  for  $O_2^-$  generation (50–60  $\mu M$ -doxorubicin) previously reported [20]. In Fig. 2 the initial difference between the NADH dehydrogenase activities in the absence of doxorubicin was due to incubation with

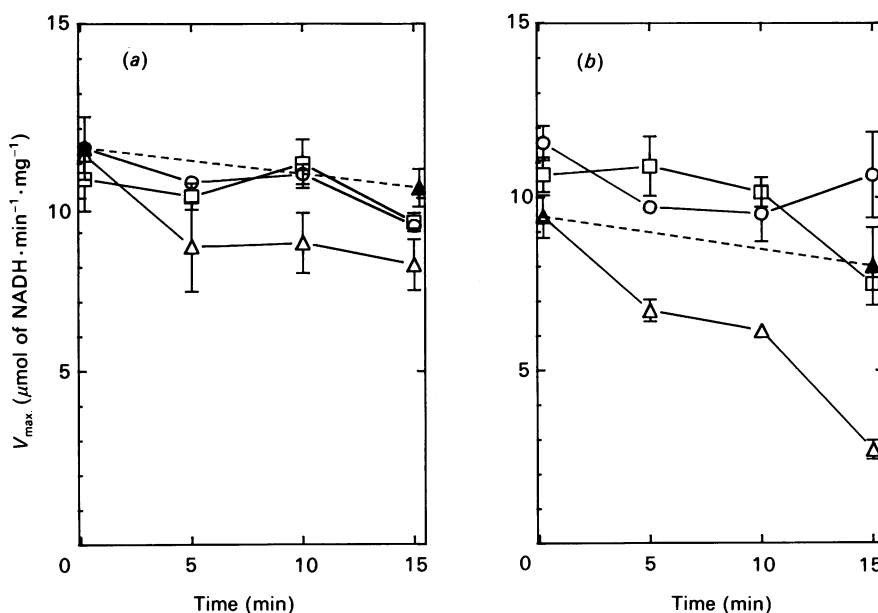
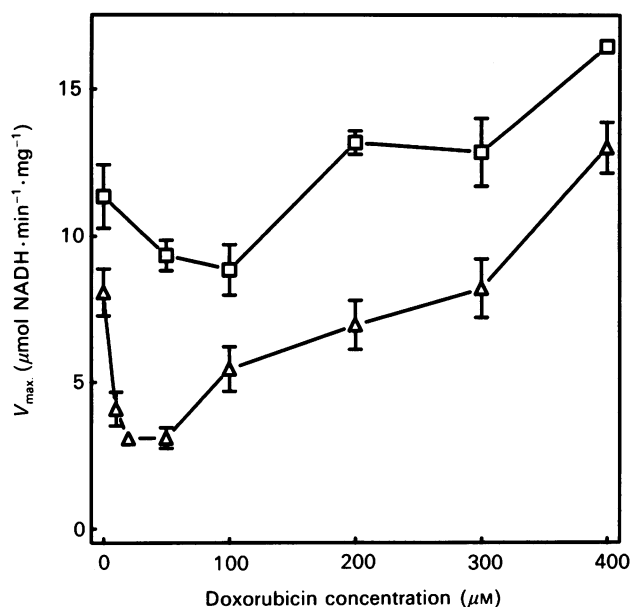


Fig. 1. Time course of NADH dehydrogenase inhibition by doxorubicin

SMP were incubated for up to 15 min at 30 °C, in a shaking water bath (see Experimental section). The SMP samples described in (a) were incubated without doxorubicin, whereas those described in (b) were incubated in the presence of 50  $\mu M$ -doxorubicin. In both panels, the other conditions were as follows: □, no oxidizable substrate for the electron transport chain; △, NADH as substrate; ○, succinate as substrate, and ▲, NADH as substrate under an anaerobic atmosphere of argon. Where indicated, NADH was added to a final concentration of 0.8 mM for 5 min incubations, 1.6 mM for 10 min incubations and 2.5 mM for 15 min incubations. Succinate was added to a final concentration of 7 mM for 5 min incubations, 14 mM for 10 min incubations and 20 mM for 15 min incubations. NADH dehydrogenase  $V_{max}$  activities were measured by the ferricyanide reduction assay [46] as described in the Experimental section. The presence of residual succinate had no effect on ferricyanide reduction. The concentrations of NADH added were chosen (on the basis of preliminary experiments) to produce a final NADH/NAD<sup>+</sup> ratio of 2.1–2.3 (in all cases), as judged by absorbance at 340 nm. This residual NADH was too low to affect NADH dehydrogenase measurements, due to the 50-fold dilution of SMP in the assay.



**Fig. 2. Effects of doxorubicin concentration on NADH dehydrogenase activity**

SMP were mixed with 2.5 mM-NADH and various concentrations of doxorubicin. Some samples were then incubated for 15 min at 30 °C (Δ) while others were immediately assayed for NADH dehydrogenase activity (□). The NADH dehydrogenase activity of the incubated samples was measured after 15 min. Incubation conditions and the ferricyanide assay for NADH dehydrogenase activity [46] were as described in the Experimental section and in the legend to Fig. 1.

NADH (see Fig. 1a). At higher doxorubicin concentrations, the extrapolated  $V_{\max}$  for ferricyanide reduction increased markedly. This may have been due to further modification of the enzyme, with production of a secondary site for ferricyanide reduction. Since the activity increased with doxorubicin concentration in a similar manner, with or without incubation, such further modification cannot be attributed to damage by active oxygen species but must be a direct effect of doxorubicin on the enzyme or its membrane environment.

$K_m$  values for NADH dehydrogenase were also determined for the experiments shown in both Figs. 1 and 2 (results not shown), but no statistically significant changes were observed following any of the treatments described. The ferricyanide reduction assay for NADH dehydrogenase used in Figs. 1 and 2 is the preferred method for determining both  $V_{\max}$  and  $K_m$  [46]. It should be noted, however, that ferricyanide accepts electrons from an iron-sulphur cluster (probably Fe-S<sub>N-1</sub>) within Complex I [46,47]. Thus, although the activity of the entire enzyme is measured, the  $K_m$  obtained is that of an artificial acceptor (ferricyanide) for an artificial site.

The reduction of DCPIP can serve as an alternative assay for NADH dehydrogenase, but this is less favoured because DCPIP does not measure the full activity of the entire enzyme [46]. Following 15 min incubation with NADH alone, both the  $V_{\max}$  and  $K_m$  for DCPIP were greatly increased (Table 1). This effect is probably related to the decline in ferricyanide reduction which occurs after incubation with NADH (Figs. 1a and 2). Decreased

**Table 1. NADH dehydrogenase activity measured by the DCPIP reduction assay**

SMP were incubated for 15 min in air, in the presence or absence of 2.5 mM-NADH and 50 μM-doxorubicin, or were not incubated (or given additions) at all. The DCPIP reduction assay for NADH dehydrogenase [46] was then conducted as described in the Experimental section. Values are means for which S.E.M. values were less than 10%.

Treatment/additions	$V_{\max}$ * †	$K_m$ †
No incubation or additions	166	53
15 min incubation with NADH	377	80
15 min incubation with NADH + doxorubicin	397	71

\* nmol of DCPIP reduced · min<sup>-1</sup> · mg SMP protein<sup>-1</sup>  
 † DCPIP concentration (mM) for  $\frac{1}{2} V_{\max}$  activity

ferricyanide reduction, and the appearance of new reductase activities, are observed with NADH incubation of the solubilized enzyme [48] and appear to reflect altered subunit interactions in the region of the flavin. Addition of doxorubicin had no effect on either  $V_{\max}$  or  $K_m$  in the DCPIP assay (Table 1). Since doxorubicin appears to be reduced by the NADH dehydrogenase flavin [18,19], it is possible that DCPIP may also be reduced by the flavin or a nearby site. This would imply that doxorubicin may not affect the ability of the NADH dehydrogenase flavin to accept or donate electrons. In any event, the site which is damaged by doxorubicin redox cycling must lie between the DCPIP reduction site and the ferricyanide reduction site, Fe-S<sub>N-1</sub> [46,47].

From the results of Figs. 1 and 2 and Table 1, it would appear that low concentrations of doxorubicin (50 μM or less) induce an NADH-, oxygen- and time-dependent damage to NADH dehydrogenase. Active oxygen species (O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, ·OH, or others) generated by redox cycling of doxorubicin [18–22] are the presumed cause of such damage. Neither the DCPIP reduction site nor the ferricyanide reduction site appear to be the actual targets of this damage. Higher concentrations of doxorubicin induce an apparent increase in NADH dehydrogenase activity, probably by directly modifying the enzyme or its membrane environment. Alternatively, the effects of high doxorubicin concentrations may be explained by an artificial ‘shuttling’ of electrons between the flavin and ferricyanide.

#### Succinate dehydrogenase activity following incubation with doxorubicin

We wondered if the active oxygen species generated by NADH dehydrogenase-dependent redox cycling of doxorubicin would also cause damage to succinate dehydrogenase. To test this possibility, we incubated SMP for 15 min in the presence of doxorubicin alone, or doxorubicin plus NADH, and measured succinate dehydrogenase activity.  $V_{\max}$  values for succinate dehydrogenase declined with doxorubicin concentration in an almost linear manner (Fig. 3a), but the presence or absence of NADH had no effect. Thus, inhibition or inactivation of succinate dehydrogenase by doxorubicin is a direct or physical effect of the drug (on the enzyme or its environment), and does not depend on oxidative

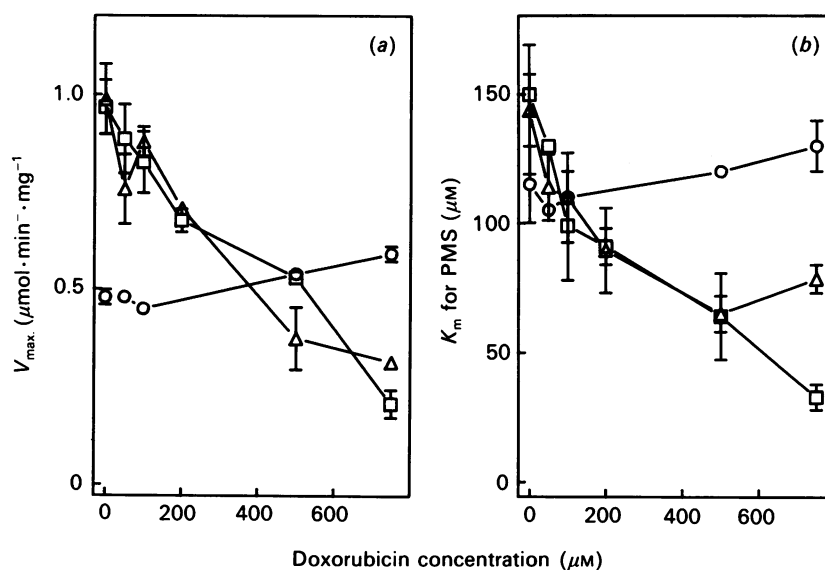


Fig. 3. Effects of doxorubicin concentration on succinate dehydrogenase activity

SMP were incubated for 15 min at 30 °C with doxorubicin alone ( $\square$ ), or with doxorubicin plus 2.5 mM-NADH ( $\Delta$ ). Succinate dehydrogenase  $V_{\text{max}}$  (a) and  $K_m$  (b) activities were measured by the PMS-DCPIP assay [46] as described in the Experimental section.  $V_{\text{max}}$  activities are given as  $\mu\text{mol}$  of DCPIP reduced  $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ , where 1  $\mu\text{mol}$  of DCPIP reduced is equivalent to 1  $\mu\text{mol}$  of succinate oxidized. Since NADH can directly reduce PMS, samples which had been incubated were first treated with 2 mM-pyruvate and 2 i.u. of lactate dehydrogenase. This procedure effectively oxidized the remaining NADH (1.52 mM as measured by absorbance at 340 nm), as also evidenced by a lack of PMS reduction in samples tested for background reactivity. The pH of the buffered SMP suspensions was not altered by reaction of lactate dehydrogenase with pyruvate and NADH. Also shown in both panels are the results obtained immediately after mixing doxorubicin with SMP ( $\circ$ ), i.e. no NADH and no incubation. All incubation conditions were described in the Experimental section.

damage. The same conclusion is reached from analysis of  $K_m$  data, measured under identical conditions (Fig. 3b).  $K_m$  values for PMS (in the succinate-PMS-DCPIP assay [46]) declined with increasing doxorubicin concentration, whether or not NADH was present.

Figs. 3(a) and 3(b) also include  $V_{\text{max}}$  and  $K_m$  data which were obtained immediately upon mixing of SMP with doxorubicin (no NADH was added and samples were not incubated). These data reveal that the direct effects of doxorubicin on succinate dehydrogenase activity are not immediate; some incubation time (approximately 15 min) is required for the effect to be seen. It will also be noted that  $V_{\text{max}}$  and  $K_m$  values for non-incubated samples (with or without doxorubicin) were significantly lower than those obtained for incubated SMP. This difference is due to the well-known 'inactivation' of succinate dehydrogenase during isolation of SMP in the absence of succinate [49]. The enzyme is readily re-activated by incubation with succinate [49] or by incubation with NADH ([48], also Figs. 3a and 3b). Interestingly, succinate dehydrogenase activity was also re-activated during 15 min incubation with doxorubicin alone (Figs. 3a and 3b). This phenomenon is probably related to a breakdown of endogenous lipids which can (via the electron-transferring flavoprotein dehydrogenase) provide electrons to re-activate the enzyme. Support for this hypothesis has been gained from ESR studies which demonstrate the gradual appearance (over 15–20 min) of a succinate dehydrogenase-dependent (but succinate-independent) ubisemiquinone species (K. J. A. Davies, unpublished results).

#### Electron transport activities following incubation with 50 $\mu\text{M}$ -doxorubicin

Since low concentrations of doxorubicin, in the presence of NADH, caused damage to NADH dehydrogenase but not succinate dehydrogenase, it was important to determine if overall rates of electron transport would be affected. NADH oxidase activity was relatively stable for 120 min in the absence of NADH or doxorubicin (Fig. 4). Anaerobic incubation (100% argon) of SMP with NADH and 50  $\mu\text{M}$ -doxorubicin also caused little or no decrease in NADH oxidase activity; however, aerobic incubation decreased NADH oxidase activity by approximately 80% in 120 min (Fig. 4). Although aerobic incubation with NADH alone caused a small (20%) loss of NADH oxidase activity (results not shown), the decline was markedly greater after aerobic incubation with both NADH and doxorubicin than with either agent alone. The slight decrease in NADH oxidase activity induced by aerobic incubation with NADH alone may reflect the small decrease in NADH dehydrogenase activity seen in Fig. 1(a). Whether this decline is due to oxygen radicals generated by the electron transport chain [50,51], or whether it reflects a direct modification of NADH dehydrogenase by NADH [48], is not known. The activity of NADH-ubiquinone reductase is, however, thought to be quite sensitive to oxidative inactivation [52].

Fig. 4 clearly indicates an oxidative inactivation of NADH oxidase activity with low concentrations of doxorubicin (+NADH and  $\text{O}_2$ ). Under similar conditions, succinate oxidase activity increased during

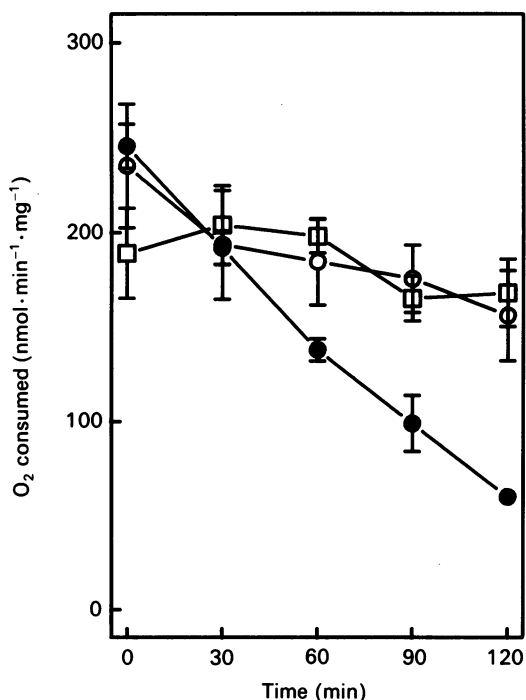


Fig. 4. Aerobic and anaerobic effects of 50  $\mu\text{M}$ -doxorubicin on NADH oxidase activity

SMP were incubated (as described in the Experimental section) for up to 120 min at 30 °C. The symbols for different incubation conditions are as follows: (□), no additions; (○), NADH plus doxorubicin under an atmosphere of 100% argon; and (●), NADH plus doxorubicin under an air atmosphere. Where used, NADH concentration was 4.5 mM for 30-min incubations, 9 mM for 60-min incubations, 14 mM for 90-min incubations and 20 mM for 120-min incubations; doxorubicin concentration was 50  $\mu\text{M}$  when used. Following incubation, samples were diluted 1:1 with 50 mM-potassium phosphate buffer, pH 7.4, 0.2 mM-NADH was added, and oxygen consumption was measured in the oxygen electrode (see Experimental section).

the first 5 min (reflecting the expected re-activation of this activity [49]) and remained stable thereafter. Cytochrome oxidase activity was completely stable to aerobic incubation with NADH and 50  $\mu\text{M}$ -doxorubicin (results not shown). These results indicate that only the NADH oxidation pathway is sensitive to oxidative inactivation by doxorubicin redox cycling.

#### Electron transport activities following incubation with high concentrations of doxorubicin

Although doxorubicin did not appear to damage succinate or cytochrome *c* oxidase activities by redox cycling, the results of Figs. 2 and 3 indicated that dehydrogenase activities could be affected by high concentrations of the drug (by a non-oxidative mechanism). We next tested the effects of higher doxorubicin concentrations (up to 1.0 mM) on oxidase activities.

NADH oxidase activity declined sharply at doxorubicin concentrations greater than 0.2 mM (Fig. 5). Results with and without NADH were essentially the same, indicating direct (non-oxidative) effects of doxo-

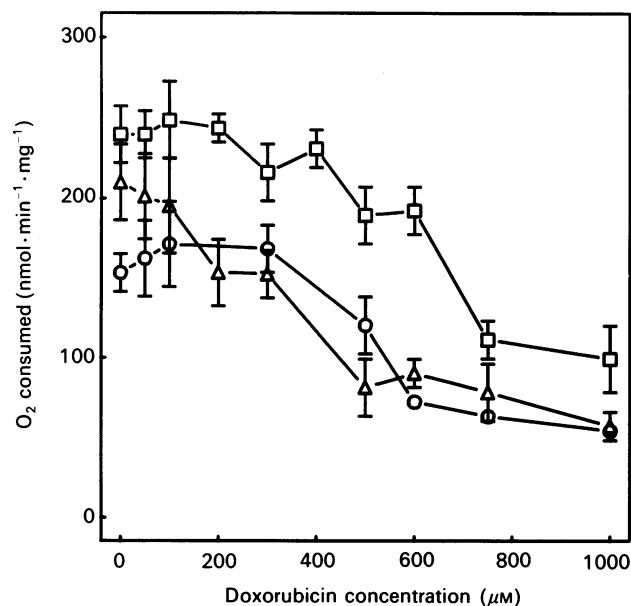


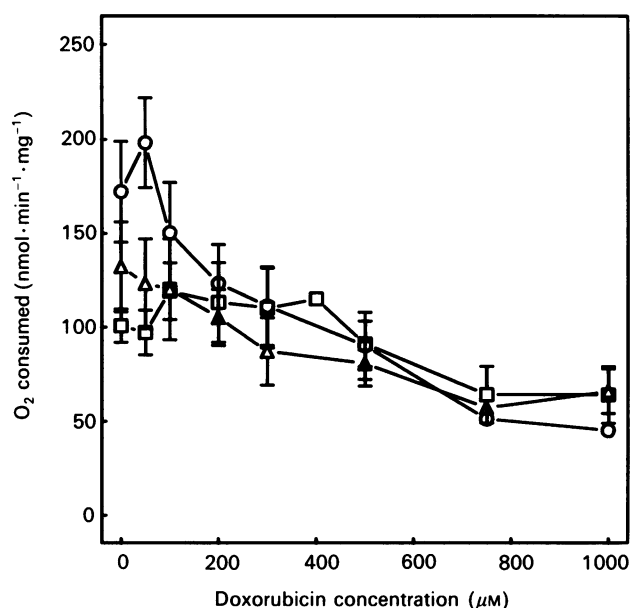
Fig. 5. Effect of doxorubicin concentration on NADH oxidase activity

NADH oxidase activity after various treatments was measured polarographically, as described in the Experimental section. The various treatments are indicated as follows: (○), activity measured after 15 min incubation with 2.5 mM-NADH; (Δ), activity measured after 15 min incubation without NADH; (□), activity measured immediately after mixing SMP with doxorubicin (no NADH added). Where indicated, incubations were conducted at 30 °C, as described in the Experimental section.

rubin on components of the respiratory chain (or its lipid environment). More than a 70% loss of NADH oxidase activity was observed with 1.0 mM-doxorubicin. Interestingly, the decline in oxidase activity required time (15 min incubation) at doxorubicin concentrations below 0.4–0.6 mM, but appeared to be an immediate effect at higher drug concentrations.

Succinate oxidase activity underwent re-activation during incubation with NADH, but declined with increasing doxorubicin concentration (Fig. 6). In the absence of NADH, doxorubicin still exerted its inhibitory effects on succinate oxidase activity, although a period of incubation (15 min) appeared necessary at drug concentrations below 0.4–0.5 mM (Fig. 6). In contrast with succinate dehydrogenase activity (Fig. 3), succinate oxidase exhibited only limited re-activation following incubation with doxorubicin alone (Fig. 6). Thus, the re-activation of the entire oxidase pathway appears to involve more than just the re-activation of the dehydrogenase. At a concentration of 1.0 mM, doxorubicin caused an apparent 75% decrease in maximal succinate oxidase activity, regardless of the addition of NADH or the use of an incubation period.

The experiments of Fig. 6 were next repeated in the presence and absence of succinate as electron source (instead of NADH). The results of these experiments were essentially indistinguishable from those presented in Fig. 6. At 100  $\mu\text{M}$ -doxorubicin, for example, succinate oxidase activity ( $\text{nmol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ) was 120 after incubation without succinate, and 126 after incubation



**Fig. 6. Effects of doxorubicin concentration on succinate oxidase activity**

This experiment is an exact parallel of the study reported in Fig. 5. Succinate oxidase activity after various treatments was measured polarographically, as described in the Experimental section. The various treatments are indicated as follows: (○), activity measured after 15 min incubation with 2.5 mM-NADH; (△), activity measured after 15 min incubation without NADH; (□), activity measured immediately after mixing SMP with doxorubicin (no NADH added). Where indicated, incubations were conducted at 30 °C, as described in the Experimental section.

with succinate. At 300 μM-doxorubicin, the activity was 87 without succinate and 83 with succinate. At 1.0 mM-doxorubicin, the activity was 65 without succinate and 72 with succinate. Since succinate dehydrogenase does not support the redox cycling of doxorubicin [18–22] these results were not surprising (see also Fig. 1). These data do, however, provide an important verification that high concentrations of doxorubicin directly affect electron transport chain activities (by modifying enzymes or their environment), regardless of electron source or the lack of an electron source.

As measured by ascorbate + TMPD-induced oxygen consumption, cytochrome *c* oxidase activity ( $416 \pm 30$  nmol  $O_2 \cdot \text{min}^{-1} \cdot \text{mg}$  of SMP $^{-1}$ ) appeared to increase (by as much as 62% at 1.0 mM-doxorubicin) with increasing doxorubicin concentration (results not shown). This apparent increase in activity ( $\pm$  NADH and incubation period) was quickly found to be an artifact, however, since ascorbate + TMPD were able to reduce doxorubicin (in the absence of SMP) and induce oxygen consumption by redox cycling of the drug. Redox cycling of doxorubicin by ascorbate was an unexpected finding which has not, to our knowledge, been reported previously. This reaction would not be predicted from the redox potentials of ascorbate and doxorubicin. Whether the redox cycling of doxorubicin by ascorbate may cause oxidative damage *in vivo* is not known. Interestingly, ascorbate plus doxorubicin alone (no SMP) caused a measurable rate of oxygen consumption ( $7.0 \pm 0.1$  nmol/

min at 1.0 mM-doxorubicin). When corrected for these artifactual sources of oxygen consumption, cytochrome *c* oxidase activity was actually found to decrease with increasing doxorubicin concentrations. The maximal loss of activity observed was 38% at 1.0 mM-doxorubicin ( $416 \pm 30$ , decreased to  $259 \pm 32$  nmol  $O_2 \cdot \text{min}^{-1} \cdot \text{mg}$  of SMP $^{-1}$ ), and the use of an incubation period or the presence of NADH had little or no effect (results not shown).

## DISCUSSION

Doxorubicin cardiotoxicity clearly involves damage to heart mitochondria [2,53] and two main causes for this damage have been proposed: (1) oxidative damage by oxygen radicals and  $H_2O_2$ , generated by redox cycling of doxorubicin with mitochondrial NADH dehydrogenase [6,15,18–22,54,55]; and (2) non-oxidative inactivation of the electron transport chain by formation of electrostatic complexes between doxorubicin and cardiolipin [12,24,26,29]. Our results indicate that oxidative damage requires less than 50 μM-doxorubicin, and is localized to the site of doxorubicin reduction, the NADH dehydrogenase. Such damage leads to a specific decrease in the overall NADH oxidation capacity of the electron transport chain. In contrast, non-oxidative inactivation requires higher doxorubicin concentrations, and affects several components of the electron transport chain.

As measured by the ferricyanide reduction assay [45], NADH dehydrogenase activity decreased by 70% following a 15 min incubation of SMP with NADH and 50 μM-doxorubicin. NADH oxidase activity decreased by 80% following aerobic incubation with NADH + 50 μM-doxorubicin for 120 min. These activity losses were dependent upon both NADH (succinate was not effective) and oxygen, and thus qualify as true oxidative damage. Importantly, succinate dehydrogenase, succinate oxidase and cytochrome *c* oxidase activities were unaffected even by prolonged incubation with doxorubicin at concentrations of 50 μM or less.

At concentrations greater than 50 μM, doxorubicin caused an NADH-independent and oxygen-independent apparent increase in NADH dehydrogenase activity (ferricyanide assay). This effect indicates a non-oxidative modification of the enzyme or its environment, resulting in improved ferricyanide accessibility (or an additional ferricyanide reduction site). Incubation with more than 50 μM-doxorubicin also caused significant inactivation of NADH oxidase, succinate dehydrogenase, succinate oxidase and cytochrome *c* oxidase by a non-oxidative mechanism (no dependence on NADH or succinate). A doxorubicin concentration of approximately 500 μM was required for 50% inactivation of NADH oxidase, succinate dehydrogenase and succinate oxidase, but cytochrome *c* oxidase activity was only 38% diminished even with 1.0 mM-doxorubicin. Since doxorubicin binds strongly to cardiolipin [12,24,26,29], and since cardiolipin is required for normal Complex I, III and IV activities [31], the NADH oxidase and succinate oxidase impairments may be the result of cardiolipin sequestration by doxorubicin. Alternatively, enzymatic impairments observed at high doxorubicin concentrations may be the result of a generalized chaotropic effect of the drug in the inner mitochondrial membrane [29]. The loss of succinate dehydrogenase (no cardiolipin requirement but severe inactivation) and cytochrome *c* oxidase



(cardiolipin requirement but only mild inactivation) activities appear to be good candidates for a generalized chaotropic effect.

Importantly, the concentrations of doxorubicin which we now show cause non-oxidative inactivation of electron transport in SMP are similar to those which have been shown to cause inhibition of oxidase activities in intact mitochondria [3,5,25,26]. Lower concentrations of doxorubicin effectively inhibit succinate oxidation in intact mitochondria [13,14,26] but this cannot be due to inactivation of the succinate oxidase pathway itself, since no effect is observed in broken mitochondria or SMP [13]. Unfortunately, we have no explanation for the high doxorubicin sensitivity of cytochrome *c* oxidase reported by Goormaghtigh *et al.* [12]. It should be noted, however, that Goormaghtigh *et al.* [12] measured the oxidation of cytochrome *c* rather than actual oxygen consumption.

Johnson *et al.* [56] have reported that heart cells in culture readily accumulate doxorubicin. Treatment of myocytes with 10  $\mu\text{M}$ -doxorubicin resulted in intracellular concentrations of 2 mM following 30 min incubation. Approximately 50% of the accumulated drug appeared to be associated with cell membranes, since it was extractable with ethanol [56]. Mitochondrial membranes, which appear to be major targets of doxorubicin toxicity [2,53], may achieve particularly high doxorubicin concentrations under such conditions because of the affinity of doxorubicin for cardiolipin [12,24,26,29]. The experiments of Johnson *et al.* [56] are particularly significant because the concentration of doxorubicin used, 10  $\mu\text{M}$ , closely matches peak plasma concentrations observed in clinical use of the drug [55].

The results presented indicate that doxorubicin can cause both oxidative damage and non-oxidative damage to heart mitochondria. The relative proportions of each form of damage are determined by the concentration of doxorubicin employed. The cardiac mitochondrial toxicity of doxorubicin *in vivo* probably involves initial oxidative damage to NADH dehydrogenase, with gradual loss of NADH oxidase activity during prolonged exposure. Other mitochondrial activities may also be diminished by oxidative damage [52] during prolonged and/or repeated exposures to doxorubicin. Reports [57,58] that vitamin E protects cultured myocytes against doxorubicin toxicity lend further credence to the 'oxidative damage' hypothesis. If experiments with cultured heart cells [56] mirror events *in vivo*, however (i.e. if myocardial cells can accumulate doxorubicin from the circulation), we must also consider the non-oxidative inactivation of the electron transport chain by doxorubicin. Clinical studies of doxorubicin uptake and elimination in the heart (which may now be performed by non-invasive n.m.r. techniques) will be critical steps in the application of biochemical understanding to human toxicity. The combination of biochemical studies *in vitro* and pharmacology *in vivo* should also permit the development of a rational approach to the amelioration or prevention of doxorubicin toxicity.

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