*Biochimica et Biophysica Acta* 892 (1987) 320–330
Elsevier

BBA 42571

# Effects of adriamycin on respiratory chain activities in mitochondria from rat liver, rat heart and bovine heart. Evidence for a preferential inhibition of complex III and IV

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(Received 3 February 1987)

Key words: Adriamycin; Mitochondrial respiratory chain; Anthracycline antibiotic; Cardiolipin; Cardiotoxicity; (Rat mitochondria)

**The inhibition of respiratory chain activities in rat liver, rat heart and bovine heart mitochondria by the anthracycline antibiotic adriamycin was measured in order to determine the adriamycin-sensitive sites. It appeared that complex III and IV are efficiently affected such that their activities were reduced to 50% of control values at 175 ± 25 μM adriamycin. Complex I displayed a minor sensitivity to the drug. Of the complex-I-related activities tested, only duroquinone oxidation appeared sensitive (50% inhibition at approx. 450 μM adriamycin). Electron-transfer activities catalyzed by complex II remained essentially unaltered up to high drug concentrations. Of the activities measured for this complex, only duroquinone oxidation was significantly affected. However, the adriamycin concentration required to reduce this activity to 50% exceeded 1 mM. Mitochondria isolated from rat liver, rat heart and bovine heart behaved essentially identical in their response to adriamycin. These data support the conclusion that, in these three mitochondrial systems, the major drug-sensitive sites lie in complex III and IV. Cytochrome *c* oxidase and succinate oxidase activity in whole mitochondria exhibited a similar sensitivity towards adriamycin, as inner membrane ghosts, suggesting that the drug has direct access to its inner membrane target sites irrespective of the presence of the outer membrane. By measuring NADH and succinate oxidase activities in the presence of exogenously added cytochrome *c*, it appeared that adriamycin was less inhibitory under these conditions. This suggests that adriamycin competes with cytochrome *c* for binding to the same site on the inner membrane, presumably cardiolipin.**

## Introduction

The anthracycline antibiotic adriamycin is widely employed to treat a broad range of malignancies [1]. The clinically most important restriction to long-term treatment with adriamycin is the development of a specific cardiotoxicity which is cumulative and total dose-dependent [2]. The mechanism by which adriamycin interferes with heart performance has been extensively investigated. Although there is no conclusive evidence as to the site where adriamycin exerts its major effects, it seems that mitochondria represent an important target [3,4].

The early development of impaired myocardial function is characterized by changes in the mor-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Q-1, ubiquinone-1; DCIP, dichlorophenolindophenol; $P_i$, inorganic phosphate.

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phology and function of heart mitochondria [5–7], and the onset of changes in the electrocardiogram shows a good correlation with the onset of depressed mitochondrial function [5]. Moreover, we have recently demonstrated by cytofluorescence microscopy that, in the isolated rat heart perfused with drug-containing medium, nuclei and mitochondria are the exclusive sites of adriamycin localization [4]. The interference with energy metabolism which occurred under these conditions most probably is due to adriamycin-mitochondria interactions, since acute effects due to the nuclear localization are hard to envisage.

Several mechanisms have been suggested to be responsible for the inhibition of mitochondrial function. These include: (i) inhibition of respiration [8–10]; (ii) inhibition of enzymes involved in oxidative phosphorylation [11], e.g., the inorganic phosphate carrier [12,13]; (iii) lipid peroxidation [14,15]; (iv) dissociation of the mitochondrial isozyme of creatine kinase from the inner membrane leading to impaired energy metabolism [16,17]. It is important to note that all four mechanisms involve the action of adriamycin at the level of the mitochondrial inner membrane. This also holds for drug-induced lipid peroxidation since: (i) in the heart cell mitochondria are the major organelles responsible for free radical production [10,18,19]; (ii) the peroxidation process is initiated through reduction of adriamycin by NADH dehydrogenase [20,21]; (iii) only inner-membrane-bound adriamycin induces lipid peroxidation [14].

The above considerations imply that, in the mitochondrion, the inner membrane represents the major target for adriamycin. There is considerable evidence to suggest that, within the inner membrane, adriamycin exerts its effects by forming a highly stable and specific complex with the negatively charged phospholipid cardiolipin [3,9,22–24]. In normal tissue, the inner mitochondrial membrane is the exclusive source of cardiolipin.

In the present report the effects of adriamycin on a variety of respiratory chain activities in mitochondria isolated from rat liver, rat heart and bovine heart are described. The major objective of this study was twofold. Firstly, to carry out an extensive and complete investigation on the adriamycin sensitivity of the various electron-transport components. Such a study had not been carried out sofar and is also of value because it may shed light on the mechanism by which the drug interferes with respiratory chain functions. Secondly, to compare mitochondria from rat liver, rat heart and bovine heart in their response to adriamycin. Apart from giving a potential contribution to the mechanistic aspects of adriamycin action on mitochondria, such a comparison is of interest because of the specific heart toxicity of adriamycin observed in vivo and because of suggestions in the literature that mitochondria from different tissues display a differential response to adriamycin in vitro. The data presented here demonstrate that, in particular, the activity of complex III and IV is progressively inhibited by increasing concentrations of adriamycin. Complex III and IV inactivation may be partly due to dissociation of cytochrome *c* from the outer surface of the inner membrane, since exogenously added cytochrome *c* shifted their inhibition curves to higher adriamycin concentrations. Mitochondria from the three sources studied were essentially indistinguishable in their response to adriamycin. These results will be discussed in the light of the proposed role of adriamycin-cardiolipin interactions in drug-induced mitochondrial toxicity.

## Materials and Methods

*Chemicals.* Adriamycin (NSC123127), duroquinone, horse-heart cytochrome *c* (type VI), NADH (grade III) and 2,6-dichlorophenolindophenol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Coenzyme Q-1 was a gift from Hoffmann-La Roche (Basel, Switzerland). All other chemicals were of analytical grade and obtained from regular commercial sources.

*Mitochondria.* Mitochondria were isolated from the liver and heart of male Wistar rats, and from bovine heart as described in Refs. 9, 25 and 26, respectively. Mitochondrial suspensions were stored at $-80\,^{\circ}C$. Freezing and thawing the mitochondria leads to (partial) loss of the permeability barrier of their inner membrane towards NADH, thereby explaining the efficient oxidation of this coenzyme through NADH dehydrogenase-linked activities. Inner membrane ghosts were prepared from rat-liver mitochondria as detailed before [9].

*Cytochrome c oxidase.* Cytochrome *c* oxidase was purified from bovine heart according to Fowler et al. [27], as modified by Hartzell et al. [28]. The enzyme preparation was stored in liquid nitrogen in 50 mM Tris/$H_2SO_4$ (pH 8.0). Its activity was determined at 30 °C in 100 mM NaCl, 10 mM Hepes (pH 7.4), 1 mM EDTA and 1% (v/v) Tween-20.

*Enzymatic assays.* Spectrophotometric assays of respiratory chain activities were measured on a Varian model 635 double-beam spectrophotometer. Rates of oxygen consumption were determined polarographically as detailed earlier [9]. All activities were measured at 30 °C.

Reduced cytochrome *c* was prepared by dissolving 10 μmol cytochrome *c* and 200 μmol ascorbic acid in 2 ml 10 mM Tris/HCl (pH 7.4) at 4 °C. After 2 h incubation, the reduced cytochrome *c* was separated from its reductant by passing the above mixture through a Sephadex G-15 column, eluted with $N_2$-saturated 10 mM Tris/HCl (pH 7.4). The concentration of cytochrome *c* was determined using a difference in molar extinction coefficient at 550 nm between reduced and oxidized forms of 18 500 $M^{-1} \cdot cm^{-1}$.

Reduced forms of coenzyme Q-1 and duroquinone were prepared according to Rieske [29]. Concentrations of stock solutions of reduced and oxidized forms were determined as described by Lenaz et al. [30].

All mitochondrial activities were measured in the presence of the uncoupler CCCP in order to avoid potential complications due to concomitant adriamycin-induced reduction of respiratory rates through inhibition of the enzymes involved and stimulation of these rates through drug-induced uncoupling [9]. NADH oxidase activity was measured at 340 nm in 50 mM KCl, 50 mM Hepes (pH 7.4), 2 μM CCCP and 100 μM NADH. NADH-cytochrome *c* reductase activity was measured as above, except that the buffer also contained 2 mM KCN and 60 μM cytochrome *c*. NADH-Q-1 and NADH-duroquinone reductase activities were assayed as described for NADH oxidase, except that the buffer also included 2 mM KCN, and 40 μM Q-1 and 0.2 mM duroquinone, respectively, as the substrates.

The following enzyme activities were assessed by measuring the reduction of cytochrome *c* at 550 nm: (i) NADH-cytochrome *c* reductase; (ii) succinate-cytochrome *c* reductase; (iii) ubiquinol ($Q_1H_2$)-cytochrome *c* reductase; (iv) duroquinol-cytochrome *c* reductase. Substrate was either 100 μM NADH, 10 mM potassium succinate plus 5 μM rotenone, 25 μM $Q_1H_2$ or 40 μM duroquinol, respectively. The remaining reaction medium was 50 mM KCl, 50 mM Hepes (pH 7.4), 2 mM KCN and 2 μM CCCP. The reactions were initiated by adding 60 μM cytochrome *c*.

Succinate oxidase activity was measured polarographically with a Clark oxygen electrode in a medium containing 50 mM KCl, 50 mM Hepes (pH 7.4), 5 μM CCCP, 5 μM rotenone and 10 mM potassium succinate.

Cytochrome *c* oxidase activity was measured from the rate of oxidation of reduced cytochrome *c* at 550 nm. The medium contained 50 mM KCl, 50 mM Hepes (pH 7.4), 2 μM CCCP, 5 μM rotenone and 25 μM reduced cytochrome *c*. In some cases, the effects of the detergents Tween-20 and Triton X-100, both added to 1% (v/v), on the above activity was measured. Before initiating the reaction with cytochrome *c*, mitochondria were preincubated for 5 min with the detergent.

NADH-ferricyanide reductase was measured at 420 nm in the same medium as used to determine NADH oxidase activity, except that 2 mM KCN and 0.5 mM potassium ferricyanide were added.

The following enzyme activities were assessed by measuring the rate of dichlorophenol-indophenol (DCIP) reduction at 600 nm: (i) succinate-DCIP reductase; (ii) succinate-Q-1 reductase; (iii) succinate-duroquinone reductase. Substrate was 10 mM succinate, 10 mM succinate plus 40 μM Q-1, or 10 mM succinate plus 40 μM duroquinone, respectively. The remaining medium contained 50 mM KCl, 50 mM Hepes (pH 7.4), 2 μM CCCP, 2 mM KCN, 5 μM rotenone, and 0.1 mM DCIP.

Adriamycin was added from a freshly prepared 20 mM stock solution in 50 mM Hepes (pH 7.4). Unless otherwise indicated, adriamycin was added to the mitochondrial suspension immediately prior to addition of the respiratory substrates. No preincubation period of mitochondria with drug was employed.

In all enzyme tests, reaction rates were determined from the initial linear parts of the ab-

sorbance vs. time curves. Except for cytochrome *c* oxidase activities which, due to the exponential decay in reduced cytochrome *c* concentration, were arrived at by plotting log (absorbance) vs. time.

*Protein determination.* Protein was measured according to Peterson [31] with bovine serum albumin as the standard. The protein concentrations used in the spectrophotometric and oxygraph experiments amounted to 100–200 and to 200–500 μg/ml, respectively.

## Results

The present paper deals with the possible inhibitory effects of adriamycin on respiratory chain activities in isolated mitochondria. Firstly, observations made on activities confined to one respiratory complex will be described. Secondly, dose-response curves of adriamycin towards activities spanning two electron-transport complexes are treated. Finally, the effects of adriamycin on NADH oxidase and succinate oxidase activity (i.e., electron transport from NADH or succinate to oxygen) are presented.

*Complex I (NADH dehydrogenase)*

Three different artificial water-soluble electron acceptors were used to probe the action of adriamycin at the level of complex I. Fig. 1 depicts dose-response curves of adriamycin action towards NADH oxidation by rat liver mitochondria using potassium ferricyanide, coenzyme Q-1 and duroquinone, respectively, as electron acceptors. The reduction of duroquinone was significantly inhibited in the range of drug concentrations tested, such that at approx. 450 μM 50% of the original activity is lost. The reduction of the other two electron acceptors remained largely unaffected up to 1 mM adriamycin.

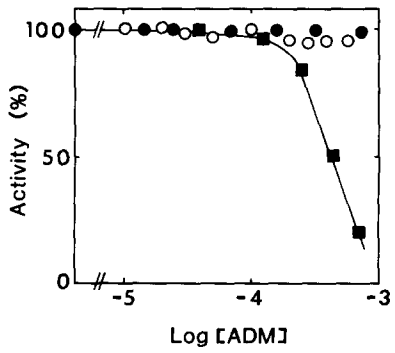


Fig. 1. Effects of adriamycin on complex I activity in rat-liver mitochondria. In all cases, NADH (100 μM) served as electron donor. The following electron acceptors were used: potassium ferricyanide (○); coenzyme Q-1 (●); duroquinone (■). Rotenone (5 μM) inhibited the various activities to 0%, 85% and 90%, respectively.

It is interesting to note that the dose-response curve for duroquinone reduction by complex I is slightly shifted to higher drug concentrations when increasing the duroquinone concentration (data not shown). This suggests that the inhibition by adriamycin of NADH-duroquinone oxidoreductase activity is partly due to a weak competition between drug and electron acceptor for sites on the inner membrane involved in duroquinone reduction.

The above activities were also measured in rat-heart and bovine-heart mitochondria (see Table I). The response to adriamycin was essentially identical as for the rat-liver system described above.

*Complex II (succinate dehydrogenase)*

As indicated in Table I, reduction of dichlorophenol-indophenol (DCIP) and of coenzyme Q-1 through succinate dehydrogenase activity was completely insensitive to adriamycin up to 1 mM. Furthermore, the adriamycin concentration required to inhibit duroquinone reduction to 50% exceeded 1 mM. The above holds for all three types of mitochondria tested.

*Complex III (ubiquinol-cytochrome c oxidoreductase)*

The influence of adriamycin on the oxidation of reduced coenzyme Q-1 and of reduced duroquinone has been studied to probe its effect on complex III activity (Table I). Both activities were inhibited to 50% around 170 μM adriamycin in mitochondria from rat liver, rat heart and bovine heart.

*Complex IV (cytochrome c oxidase)*

The activity of the terminal enzyme complex of the respiratory chain (i.e., cytochrome *c* oxidase) appeared to be also sensitive to adriamycin. The dose-response curves of this enzyme in rat liver

TABLE I

INHIBITION OF RESPIRATORY CHAIN ACTIVITIES BY ADRIAMYCIN IN RAT LIVER, RAT HEART AND BOVINE HEART MITOCHONDRIA

n.e., no significant effect up to 1 mM adriamycin; n.d., not determined.

| Oxidoreductase activity | Adriamycin concentration at 50% inhibition (μM) | | |
|---|---|---|---|
| | rat liver | rat heart | bovine heart |
| NADH-$Fe(CN)_6^{3-}$ | n.e. | n.e. | n.e. |
| NADH-Q-1 | n.e. | n.e. | n.e. |
| NADH-duroquinone | 450 | 400 | 425 |
| Succinate-DCIP | n.e. | n.e. | n.e. |
| Succinate-Q-1 | n.e. | n.e. | n.e. |
| Succinate-duroquinone | >1000 | >1000 | >1000 |
| Q-1 $H_2$-cytochrome *c* | 175 | 185 | 170 |
| Duroquinol-cytochrome *c* | 180 | 180 | 165 |
| Cytochrome *c*-$O_2$ | 175 [a] | 165 | 170 |
| NADH-cytochrome *c* | 240 | 250 | 240 |
| Succinate-cytochrome *c* | 175 | 165 | 180 |
| NADH-$O_2$ | 65 [b] | 60 | 55 |
| Succinate-$O_2$ | 100 [c] | n.d. | n.d. |

[a] When assayed in the presence of 1% (v/v) Triton X-100 or Tween-20, these numbers amount to 230 and 60 μM, respectively. For rat-liver inner-membrane ghosts in control medium it is 175 μM.

[b] When 15 μM cytochrome *c* is added prior to adriamycin, the 50% inhibitory concentration becomes 320 μM. Upon reversal of the order of addition of cytochrome *c* and drug, the corresponding concentration was found to be 200 μM adriamycin.

[c] Addition of both 7.7 and 15.4 μM cytochrome *c* prior to adriamycin addition displaces this concentration to 200 μM. For inner membrane ghosts assayed in control buffer (i.e., without exogenous cytochrome *c*) the corresponding number was 80 μM adriamycin.

mitochondria and in rat liver inner mitochondrial membrane ghosts are shown in Fig. 2. Clearly, both preparations are equally affected by the drug (50% inhibition at 175 ± 25 μM adriamycin), thereby suggesting that removal of the outer membrane does not alter the inhibitory potency of adriamycin (see below).

When assayed under identical conditions as used above, bovine-heart mitochondrial cytochrome *c* oxidase displayed a nearly identical response (Fig. 3, Table I). The 50% inhibitory drug concentration was 170 μM. Goormaghtigh et

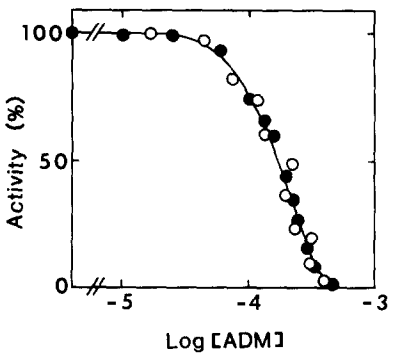


Fig. 2. Effects of adriamycin on complex IV activity in rat liver mitochondria (●) and in rat-liver inner membrane ghosts (○). Reduced cytochrome *c* (25 μM) was employed as electron donor.

al. [3] have previously reported that, under their conditions, a considerably lower concentration (approx. 3 μM) was sufficient to reduce the bovine heart mitochondrial terminal oxidase activity by 50%. Since the discrepancy might be caused by the fact that these authors carried out their experiments in 1% (v/v) Triton X-100, we have also measured a dose-response curve in the presence of this detergent (Fig. 3). However, the 50% inhibitory drug concentration increased to 230 μM instead of decreasing. Only when measuring the

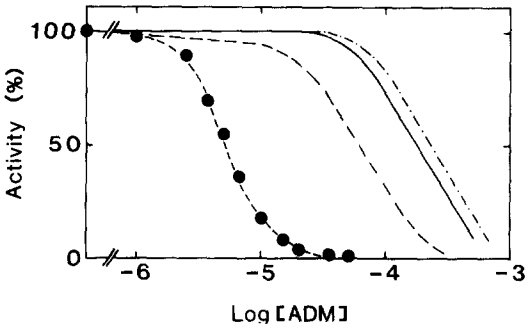


Fig. 3. Effects of adriamycin on complex IV activity in bovine heart mitochondria and on the activity of purified bovine heart mitochondrial complex IV. Bovine heart mitochondria were assayed in buffer (———), in buffer plus 1% (v/v) Triton X-100 (·–·–·), or in buffer plus 1% (v/v) Tween-20 (— — —). Purified cytochrome *c* oxidase (final concentration, 10 nM) was measured in 1% Tween-20, as indicated in Materials and Methods (●— — —●). Reduced cytochrome *c* (final concentration, 25 μM) served as electron donor. For simplicity, experimental points are only shown for the isolated enzyme.

activity of purified bovine heart cytochrome *c* oxidase in isolated form in the presence of 1% Tween-20 (i.e., the detergent employed to keep the enzyme in solution), did we observe a sensitivity towards adriamycin (50% loss in activity at $5 \pm 1$ μM (Fig. 3)) comparable to that reported by Goormaghtigh et al. [3]. Cytochrome *c* oxidase activity in bovine-heart mitochondria when assayed in the presence of 1% (v/v) of this detergent Tween-20 was inhibited to 50% of control values at 60 μM adriamycin (Fig. 3).

The data in Fig. 3 demonstrate that the apparent inhibitory efficiency of adriamycin towards cytochrome *c* oxidase greatly depends on the composition of the assay medium, which may be due to variations in the number and the accessibility of the target sites. However, when measured under identical conditions rat liver (Fig. 2), bovine-heart (Fig. 3) and rat-heart mitochondria (Table I) behave virtually identical in the drug-induced inhibition of their terminal oxidase activities.

*Complex I and III (NADH-cytochrome c oxidoreductase)*

We have also measured the action of adriamycin on combined complex I and III activity in mitochondria from the three sources used here. Fig. 4A contains the dose-response curves of complex I and III for rat-liver and bovine-heart mitochondria, measured as cytochrome *c* reduction. Both mitochondrial systems are equally affected, such that 50% reduction in activity occurs at 240 μM. A similar result was obtained for rat-heart mitochondria: 250 μM (Table I).

It has been suggested from several studies that adriamycin itself may participate in electron transport [20,23]. This involves reversible reduction and oxidation at a redox potential level comparable to that of ubiquinone [20,21]. The reduced semiquinone radical form of adriamycin has been proposed to be capable of reacting with molecular oxygen leading to reoxidation of the drug and the formation of superoxide anion [21], a process believed to be of importance for the initiation of adriamycin-induced lipid peroxidation [32]. If activation of adriamycin to its semi-quinone radical, followed by reaction with oxygen, would occur at significant rates under our experimental conditions, it might be expected that, in the presence of adriamycin, the rate of NADH oxidation would be higher than that of cytochrome *c* reduction. To study this possibility, complex I–III activity in rat liver mitochondria was determined both from NADH oxidation (at 340 nm) and from cytochrome *c* reduction (at 550 nm). The results, as shown in Fig. 4B, demonstrate that both approaches yield comparable electron-transfer rates, thereby implying that most electrons donated by NADH are transferred to cytochrome *c*. However, in the concentration range from 50 to 200 μM adriamycin the rate of NADH oxidation is consistently higher than the rate of cytochrome *c*

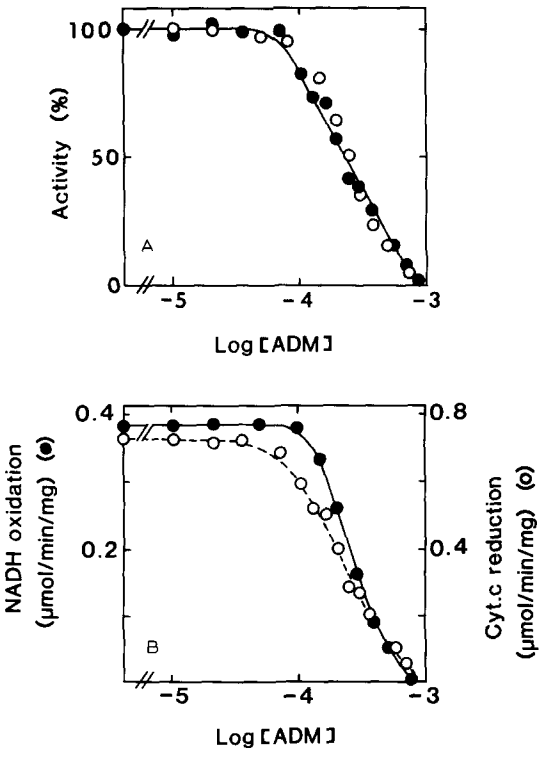


Fig. 4. Effects of adriamycin on complex I-III activity in rat-liver and bovine-heart mitochondria. (A) Activity (as percentage of control) in rat-liver (●) and bovine-heart mitochondria (○), measured as cytochrome *c* reduction; (B) specific activity (in μmol/min per mg protein) in rat-liver mitochondria, measured as NADH oxidation (●) or cytochrome *c* reduction (○). Since 1 mol NADH donates 2 mol electrons and 1 mole cytochrome *c* accepts 1 mol electrons, the scale for NADH oxidation is presented as half that of cytochrome *c* reduction.

reduction. This might be due to loss of electrons from the electron-transport chain through oxidation of reduced adriamycin by oxygen.

It has recently been demonstrated that the above adriamycin-mediated electron transport may lead to the formation of a covalent complex between the drug and cardiolipin [33]. However, the formation of this compound appeared to be a rather slow process. Since the present experiments did not involve preincubation of the mitochondrial systems in the presence of both drug and electron donor, the drug-induced effects presented here are most likely due to unmodified adriamycin itself.

*Complex II-III (succinate-cytochrome c oxidoreductase)*

Complex II-III activity, measured as the rate of cytochrome *c* reduction with succinate as electron donor, was also inhibited by adriamycin. Inhibition of the activity to 50% of control values occurred around 170 μM adriamycin in the three mitochondrial systems (Table I).

*Complex I-III-IV (NADH oxidase)*

In order to probe the efficiency of adriamycin in inhibiting the entire respiratory chain, its effects on the oxidation of NADH to oxygen (i.e., on NADH oxidase activity) were measured. Fig. 5 demonstrates that for rat-liver mitochondria 50% loss in activity is reached at 65 μM of the drug. Bovine-heart and rat-heart mitochondrial NADH oxidase activities showed an almost identical inhibition pattern: 50% inhibition at 55 and 60 μM adriamycin, respectively (Table I).

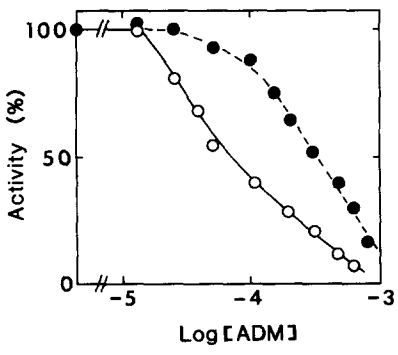


Fig. 5. Effects of adriamycin on NADH oxidase activity in rat liver mitochondria. The experiment was carried out in the absence (○) and in the presence of 15 μM exogenous cytochrome *c* (●) which was added 1 min prior to adriamycin.

Apart from measuring the effect of adriamycin on NADH oxidase activity in the presence of NADH and oxygen as the only exogenous respiratory co-factors, we have also measured the influence of addition of exogenous cytochrome *c* on the modulation of this activity by adriamycin. This experiment was prompted by the observation of Goormaghtigh et al. [34] that adriamycin effectively interferes with the binding of cytochrome *c* to cardiolipin-containing liposomes. Moreover, it has been suggested that: (i) cytochrome *c* mediates electron transfer between complex III and IV by free diffusion along the external surface of the inner membrane [35,36] while bound to cardiolipin molecules [35]; (ii) cardiolipin is the major binding site for adriamycin in mitochondria [9,37]. This potential role of competition between adriamycin and cytochrome *c* for binding to inner membrane cardiolipin in drug-induced enzyme inactivation was studied by measuring dose-response curves in the presence of exogenously added cytochrome *c*. In the absence of adriamycin, NADH oxidase activity was stimulated in a saturatable fashion upon addition of increasing concentrations of cytochrome *c*. Stimulation was saturated at 15 and 5 μM and was found to be 2.3- and 2.4-fold in rat-liver and bovine-heart mitochondria, respectively (data not shown). It appeared that NADH oxidase was less sensitive to adriamycin in the presence of exogenous cytochrome *c*. As an example, Fig. 5 shows the results of a dose-response curve for rat liver mitochondrial NADH oxidase measured in the presence of 15 μM cytochrome *c*. In this experiment, in which the mitochondria were preincubated with cytochrome *c* during 1 min prior to addition of adriamycin, the 50% inhibitory drug concentration amounted to 320 μM, which is considerably higher than the corresponding concentration (i.e., 65 μM) obtained without adding exogenous cytochrome *c*. When adriamycin was added before cytochrome *c*, this reduction of the inhibitory efficiency of adriamycin was partly reversed. Using this protocol and 15 μM exogenous cytochrome *c*, 50% inhibition of NADH oxidase occurred at 200 μM adriamycin (Table I).

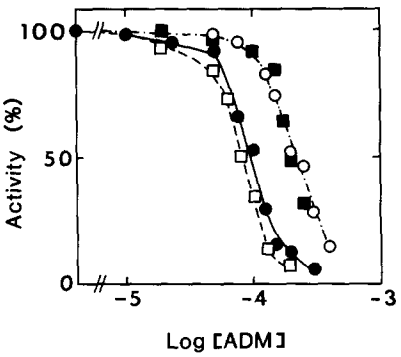


Fig. 6. Effects of adriamycin on succinate oxidase activity in rat liver mitochondria (●, ■, ○) and in rat liver inner membrane ghosts (□). The activity was measured in the absence (●, □) and in the presence of 7.7 (■) or 15.4 μM (○) exogenous cytochrome *c* which was added 1 min prior to adriamycin.

*Complex II-III-IV (succinate oxidase)*

The action of adriamycin on succinate oxidase activity, measured polarographically as the rate of oxygen consumption, has been measured for rat-liver mitochondria and rat-liver inner-membrane ghosts (Fig. 6). Uncoupled respiration was inhibited to 50% of control values at 100 and 80 μM for mitochondria and inner membrane ghosts, respectively.

As for NADH oxidation by rat liver mitochondria, it appeared that exogenously added cytochrome *c* displaces the dose-response curve of succinate oxidation to higher drug concentrations (Fig. 6). In the absence of drug, cytochrome *c* slightly stimulates respiration on succinate: 1.1-fold at 7.7 and 1.3-fold at 15.4 μM cytochrome *c*, respectively (not shown). Interestingly, the 50% inhibitory adriamycin concentration is approx. 200 μM at both cytochrome *c* concentrations (Fig. 6).

## Discussion

The present report confirms earlier studies (e.g., Refs. 8–10) that in vitro incubation of isolated mitochondria with adriamycin results in the inhibition of respiration. This study adds a number of relevant new elements to these observations. Firstly, sensitivity to adriamycin is specific for certain activities of the electron-transport chain. Secondly, it appeared that, as far as the activities are measured here, mitochondria from heart and liver are indistinguishable in their acute in vitro response to adriamycin.

The data as summarized in Table I demonstrate that all activities of complex III and IV measured here were inhibited by 50% between 150 and 200 μM adriamycin. Complex I-catalyzed NADH-duroquinone oxidoreductase activity was inhibited by 50% around 425 μM adriamycin, while ferricyanide and coenzyme Q-1 reduction by this complex were essentially unaffected up to 1 mM. It should be realized that artificial water-soluble electron acceptors were used to measure complex I. These might deviate in their response from the behavior of the intrinsic acceptor ubiquinone-10 [30,38]. The different acceptors employed are reduced at different sites in both complex I and II (see also Ref. 39), as is evident from the different sensitivity of their reduction to adriamycin. Duroquinone reductase activity is located very close to the oxygen side of NADH dehydrogenase [39] and has been reported to require endogenous ubiquinone-10 [40]. This leads us to conclude that there is a minor adriamycin-sensitive site in the acceptor side of complex I. Taken together the data seem to imply that complex III and IV and, to a minor extent, complex I possess adriamycin-sensitive sites while the drug is without significant effect upon complex II.

A possible mechanistic explanation of the adriamycin-induced enzyme inactivations will have to address the following points. (i) Are their effects caused by direct drug-enzyme interactions or are they indirectly mediated through drug-lipid interactions? (ii) Why do rat liver, rat-heart and bovine-heart mitochondria display an identical response to the drug? (iii) Why are complex III and IV preferentially inhibited by adriamycin? These three items will be discussed below.

There is considerable evidence that the adriamycin-induced inhibition of inner membrane enzymes is due to effects of the drug exerted at the level of the lipid matrix and not to direct drug-enzyme interactions. Evidence for this comes from studies on cytochrome *c* oxidase [3,14,41,42], NADH-cytochrome *c* oxidoreductase [24], the inorganic phosphate carrier [12,13] and the carnitine-acylcarnitine translocase [43]. Moreover, the above studies strongly suggest that the enzyme

inactivations are due to adriamycin binding to the negatively charged phospholipid cardiolipin. Thus, when employing adriamycin and related drugs, a linear relationship was found between the logarithm of the drug concentration required to inhibit cytochrome *c* oxidase [42] and NADH-cytochrome *c* oxidoreductase [24] activity by 50% and the logarithm of the association constant of the antibiotic-cardiolipin complex. Carofoli and coworkers [12,13] have presented evidence that: (i) the $P_i$ carrier of the inner membrane requires cardiolipin for its activity; (ii) cardiolipin activation of carrier activity is counteracted by adriamycin; (iii) the latter phenomenon is due to adriamycin-cardiolipin complex formation.

Obviously, cardiolipin palys a special role in the activity of several enzymes localized in the inner mitochondrial membrane (for a review, see Ref. 44), including complex I, III and IV of the respiratory chain. It has been demonstrated that for the above enzyme systems full reactivation after extensive delipidation requires the presence of cardiolipin in the reconstitution medium [45,46]. Therefore, we propose that the effects of adriamycin exerted on the four complexes of the respiratory chain as described here can be explained in terms of adriamycin-cardiolipin interactions. Binding of the drug to cardiolipin molecules which are essential for enzymatic activity causes inactivation of complex III and IV, and is also responsible for inhibition of duroquinone reduction by complex I. In this model the essentially complete resistance of complex II activity towards adriamycin would then be explained by the fact that this enzyme lacks an essential requirement for cardiolipin.

The remarkable similarity of mitochondria from heart and liver in their response to adriamycin can also be explained in terms of a primary role of cardiolipin as a drug target. Both mitochondria from rat liver [9,37] and rat heart [37] bind adriamycin to a level equivalent to their cardiolipin content. The identical response to adriamycin of bovine heart mitochondria as compared to the rat-heart and rat-liver systems can be accounted for by their similar cardiolipin content [9,37,47]. It is interesting to note that the drug-binding capacity of rat liver [9,37] and rat heart [37] mitochondria is essentially saturated around 250 μM adriamycin, i.e., in the concentration range where most of the activities reported here have been reduced by at least 50% (Table I).

An important point to discuss next is the mechanism by which adriamycin-cardiolipin complex formation leads to inactivation of enzymes having an absolute requirement for this lipid. Although this mechanism is not fully understood as yet, a number of factors which are most probably of importance have been recognized. Firstly, Ruysschaert and coworkers [24,41] have recently proposed that efficient drug-induced inactivation not only requires high-affinity binding to cardiolipin, but also segregation of drug cardiolipin complexes in a phase separated from the surface of the enzyme. This phase segregation phenomenon, as induced by adriamycin in model membranes containing negatively charged phospholipids, has been demonstrated using differential scanning calorimetry [24,41] and $^{31}$P-NMR [22]. This process, which is driven by ring-stacking interaction between adriamycin chromophores, would disable the close contact between cardiolipin and enzyme as required for the latter to be active. Secondly, adriamycin has been demonstrated by $^{31}$P-NMR to eliminate efficiently non-bilayer lipid structures in model membranes [22,34]. Moreover, the drug completely removes the strong isotropic component, presumably originating from lipids organized in non-bilayer configurations, from $^{31}$P-NMR spectra of rat liver inner-membrane ghosts [22]. Of special interest for the present discussion is the observation that the non-bilayer lipid organization as induced by cytochrome *c* in cardiolipin liposomes is completely eliminated by subsequent addition of adriamycin [34]. Thirdly, this study presents evidence that competition between cytochrome *c* and adriamycin for binding to a common site in the inner membrane (presumably cardiolipin) may play a role in explaining complex III and IV inactivation. This evidence primarily comes from the observation that exogenous cytochrome *c* efficiently antagonizes adriamycin-induced inactivation of NADH oxidase (Fig. 5) and succinate oxidase (Fig. 6) activity in rat-liver mitochondria. Furthermore, Goormaghtigh et al. [34] have reported that cytochrome *c* bound to cardiolipin liposomes is dissociated from this system for 50% at drug concentrations around 200

μM, i.e., in the range where we have observed pronounced effects on cytochrome *c*-linked respiratory activities.

The outer mitochondrial membrane does not seem to play a role in the adriamycin-induced inhibition of respiratory function. Thus, cytochrome *c* oxidase activity was equally susceptible in whole mitochondria as in inner membrane ghosts, devoid of outer membrane (Fig. 2). In principle, this experiment might be complicated by the fact that, in whole mitochondria reduced cytochrome *c* can only reach its oxidase in those mitochondria having a damaged outer membrane allowing free permeation of the molecule to the external surface of the inner membrane [48]. For this reason, the drug-sensitivity comparison was also made for succinate-oxidase activity (Fig. 6). This activity is measured with substrates which are freely permeable through the outer membrane. Also in this case, whole mitochondria and inner membrane ghosts displayed virtually identical dose-response curves. Therefore, it seems appropriate to conclude that adriamycin has direct access to all its inner membrane target sites irrespective of the presence of the outer membrane. Our observations are at variance with those of Mannella et al. [49] who reported that they observed a correlation between outer membrane lysis and susceptibility of rat liver mitochondrial succinate oxidase to adriamycin.

In summary, adriamycin preferentially inhibits the complexes III and IV of the mitochondrial respiratory chain. The inactivation may be explained by binding of the drug to the inner membrane phospholipid cardiolipin, the latter being essential for proper functioning of the complexes. Interference with enzyme activity by adriamycin-cardiolipin complex formation may arise from drug-induced phase segregation and elimination of functionally important non-bilayer lipid structures. Furthermore, the inhibition of cytochrome *c*-linked respiratory activities most probably partly arises from competition between adriamycin and cytochrome *c* for binding to sites on the inner membrane which play a role in the catalytic activity.

**Acknowledgements**

Hoffmann-La Roche is gratefully acknowledged for a generous gift of coenzyme Q-1. We are indebted to Prof. Karl Folkers (Austin, TX, U.S.A.) for stimulating discussions. The purified cytochrome *c* oxidase was a kind gift of Dr. A. Rietveld and Dr. A.O. Muysers. The authors are grateful to Dr. J.A. Berden (University of Amsterdam) for reading the manuscript. This research was supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

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