Inhibition and inactivation of NADH–cytochrome *c* reductase activity of bovine heart submitochondrial particles by the iron(III)–adriamycin complex

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The NADH–cytochrome *c* reductase activity of bovine heart submitochondrial particles was found to be slowly (half-time of 16 min) and progressively lost upon incubation with the Fe^{2+} –adriamycin complex. In addition to this slow progressive inactivation seen on incubation, a reversible fast phase of inhibition was also seen. However, if EDTA was added to the incubation mixture within 15 s, the slow progressive loss in activity was largely preventable. Separate experiments indicated that EDTA removed about one-half of the iron from the Fe^{2+} –adriamycin complex in about 40 s. These results indicated the requirement for iron for the inactivation process. Since the V_{max} for the fast phase of inhibition was decreased by the inhibitor, the inhibition pattern was similar to that seen for uncompetitive or mixed-type inhibition. The direct binding of both Fe^{3+} –adriamycin and adriamycin to submitochondrial particles was also demonstrated, with the Fe^{3+} –adriamycin complex binding 8 times more strongly than adriamycin. Thus binding of Fe^{3+} –adriamycin to the enzyme or to the inner mitochondrial membrane with subsequent generation of oxy radicals *in situ* is a possible mechanism for the Fe^{3+} –adriamycin-induced inactivation of respiratory enzyme activity.

INTRODUCTION

Adriamycin is widely used as an anti-tumour drug; however, its use is severely limited by a unique dose-limiting cardiotoxicity. There is now considerable evidence to suggest that this toxicity may be due to an iron-based free radical oxidative stress on the relatively unprotected cardiac muscle (Gianni *et al.*, 1983; Halliwell & Gutteridge, 1985). Cardiac mitochondria are a prominent site of adriamycin-induced injury (Gianni *et al.*, 1983). Much of the damage to cellular components caused by adriamycin *in vitro* has been shown to be iron-dependent (Myers *et al.*, 1982; Demant, 1983; Demant & Jensen, 1983; Gianni *et al.*, 1983; Gutteridge, 1984; Nakano *et al.*, 1984; Mimnaugh *et al.*, 1985; Davies & Doroshov, 1986; Doroshov & Davies, 1986). The results of a recent randomized clinical trial with the cardioprotective agent ICRF-187 (Speyer *et al.*, 1988) have shown significant protection against adriamycin-induced cardiotoxicity. ICRF-187 probably exerts its action through its rings-opened hydrolysis product, which has a structure similar to EDTA and which, likewise, binds iron and copper very strongly. Adriamycin binds strongly to Fe^{3+} (Gianni *et al.*, 1983) forming a drug/ Fe^{3+} complex of 3:1. Thus ICRF-187 may be acting by competing for available iron or even by removing iron directly from any Fe^{3+} –adriamycin complex (Hasinoff, 1989*a,b,c*) formed *in vivo*.

Another factor in the cardiotoxicity of adriamycin may be the high affinity that both adriamycin (Goormaghtigh & Ruysschaert, 1984; Henry *et al.*, 1985; Hasinoff & Davey, 1988*a,b*) and Fe^{3+} –adriamycin (Demant, 1984*a*; Mimnaugh *et al.*, 1985) display for cardiolipin and cardiolipin-containing vesicles. Cardiolipin is most plentiful in the heart, where it is mainly found in the inner mitochondrial membrane. It comprises 25 % of total lipid phosphorus of the pig heart (Hostetler,

1982). The Fe^{3+} –adriamycin-induced degradation of cardiolipin in pig heart submitochondrial particles is accompanied by the simultaneous inactivation of several respiratory enzymes (Demant & Jensen, 1983; Demant, 1983). The heart muscle of adriamycin-treated mice displays significant decreases in both NADH–cytochrome *c* reductase and cytochrome *c* oxidase activity, concomitant with an increase in lipid peroxidation (Praet *et al.*, 1984). Adriamycin has also been reported to cause a 50 % inhibition of the NADH–cytochrome *c* reductase activity of bovine heart mitochondria at a concn. of 15 μM (Goormaghtigh *et al.*, 1986).

In previous studies, it was shown that Fe^{3+} –adriamycin caused a fast initial competitive inhibition of purified cytochrome *c* oxidase before its inactivation (Hasinoff & Davey, 1988*a,b*; Hasinoff *et al.*, 1989). In view of the fact that, *in vivo*, adriamycin causes the NADH–cytochrome *c* reductase activity of heart mitochondria to be reduced to 15 % of its original activity (compared with 40 % for cytochrome *c* oxidase) (Praet *et al.*, 1984), studies of the mechanism of the inhibition and inactivation of the reductase activity have also been carried out. This study also shows that both adriamycin and Fe^{3+} –adriamycin bind strongly to heart submitochondrial particles.

MATERIALS AND METHODS

Adriamycin was a gift from Adria Laboratories (Columbus, OH, U.S.A.), and the concentration of the aqueous stock solution was determined spectrophotometrically in methanol using an ϵ of 13050 $\text{M}^{-1}\cdot\text{cm}^{-1}$ at 477 nm (Arcamone, 1981). The Fe^{3+} –adriamycin complex was formed by adding microlitre amounts of 10 mM- $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ in 1 mM-HCl to stock adriamycin in water to give a drug/ Fe^{3+} ratio of 3:1 (Myers *et al.*, 1982). Bovine heart whole mitochondria were prepared by

differential centrifugation (Azzone *et al.*, 1979). The submitochondrial particles were prepared and collected by ultracentrifugation (Lee & Ernster, 1967) at 105 000 *g*. The NADH-cytochrome *c* reductase activity was assayed by monitoring the increase in absorbance in 1 cm cells (on a Shimadzu UV-260 spectrophotometer) which occurred upon the reduction of ferricytochrome *c* in the presence of 100 μM -NADH and 6 mM- NaN_3 (to inhibit the cytochrome *c* oxidase activity). The enzyme activity was both antimycin- and rotenone-sensitive. The initial rates were calculated using a $\Delta\epsilon$ (reduced-oxidized) of 21.1 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ at 550 nm (Van Gelder, 1962) or 40.3 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ at 412 nm (Margoliash & Walasek, 1967), and were obtained from first derivatives extrapolated back to time zero (Hasinoff & Davey, 1988b). For the studies of the fast phase of inhibition, the enzyme-catalysed reaction was initiated by adding the submitochondrial particle suspension last, and care was taken to measure the initial velocities over the first few seconds of the reaction before the slow Fe^{3+} -adriamycin-induced inactivation occurred. All reactions were carried out at 25 °C in pH 7.4 Tris (50 mM)/HCl/KCl (150 mM) buffer. Cytochrome *c* (horse heart, type IV), Tris, NADH, antimycin A, rotenone and polylysine (M_r 3700) were obtained from Sigma.

The binding of adriamycin and Fe^{3+} -adriamycin to bovine heart submitochondrial particles was determined by adding the drug or its iron complex to submitochondrial particles in Tris/HCl buffer at 0 °C. The particles were centrifuged at 105 000 *g* for 40 min at 4 °C and were visibly pink coloured due to the binding of adriamycin or Fe^{3+} -adriamycin. In the case of Fe^{3+} -adriamycin, the extent of binding was determined by measuring the absorbance of the supernatant at 480 nm and 600 nm (where the absorbance of the Fe^{3+} -adriamycin complex is much larger than that of free adriamycin) (Myers *et al.*, 1982). Millimolar absorption coefficients of 8.59 and 3.35 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ (on an adriamycin basis) at 480 and 600 nm respectively for the Fe^{3+} -adriamycin complex were used to determine the concentration of Fe^{3+} -adriamycin remaining in solution, and hence by difference the amount of Fe^{3+} -adriamycin bound to the particles. Since the binding of adriamycin to the particles was much less than for the Fe^{3+} -adriamycin complex, the amount of adriamycin bound was determined by measuring directly adriamycin bound to the particles. After centrifuging the adriamycin-treated particles as before, the particles were dissolved back up to their original volume of 1200 μl in a 50:50 (v/v) mixture of 10 mM-EDTA in 10% Triton X-100 and Tris/KCl buffer. The concentration of adriamycin in these solutions was determined by measuring the absorbance at 480 nm and using a molar absorption coefficient of 10.7 $\text{mM}^{-1}\cdot\text{cm}^{-1}$. The concentrations of adriamycin and of Fe^{3+} -adriamycin were always determined with respect to controls that were treated identically, but which contained no submitochondrial particles, in order to correct for any binding of drug or iron-drug complex to the centrifuge tubes or cells.

RESULTS

Slow inactivation of NADH-cytochrome *c* reductase activity

As found by Demant & Jensen (1983) and as Fig. 1 shows, the NADH-cytochrome *c* reductase activity of

Fig. 1. Inactivation of the NADH-cytochrome *c* reductase activity of bovine heart submitochondrial particles by Fe^{3+} -adriamycin

Submitochondrial particles (0.37 mg of protein) were incubated with Fe^{3+} -adriamycin (50 μM - Fe^{3+} /150 μM -adriamycin) in 250 μl of buffer. Duplicate activities were measured from absorbance changes that occurred at 550 nm after dilution of a 100 μl aliquot to a total volume of 1200 μl . EDTA (●) was added, or not (○), 15 s after Fe^{3+} -adriamycin was added to the submitochondrial particles. All activities were measured relative to controls treated identically, but in the absence of any added Fe^{3+} -adriamycin. An activity of 100% corresponds to a ferricytochrome *c* reduction rate of 33 $\mu\text{M}/\text{min}$.

the submitochondrial particles underwent a progressive slow inactivation in the presence of Fe^{3+} -adriamycin. Adriamycin (250 μM) on its own caused no detectable inactivation after 15 and 30 min incubations. When 1 mM-EDTA was added to submitochondrial particles after 3 min of incubation with Fe^{3+} -adriamycin (15 μM Fe^{3+} /45 μM -adriamycin) in the assay cell, EDTA offered no protection against the observed inactivation. However, when 1 mM-EDTA was added within 15 s of the addition of Fe^{3+} -adriamycin to the submitochondrial particles, there was a recovery to 90% of the original activity, compared with 18% activity remaining in the absence of any added EDTA. These results show that the inactivation is dependent upon the presence of Fe^{3+} and that the fast phase of inhibition can be largely reversed by the removal of Fe^{3+} from the complex. EDTA reacts quickly with Fe^{3+} -adriamycin; in the absence of any submitochondrial particles, 300 μM -EDTA removed 50% of the Fe^{3+} from Fe^{3+} -adriamycin (15 μM - Fe^{3+} /45 μM -adriamycin) in about 40 s, as measured from the decrease in absorbance that occurred at 600 nm. Thus some irreversible inactivation of the enzyme may be occurring, both during the time that Fe^{3+} and adriamycin remain in their complex and in the 15 s before the addition of EDTA.

Fast phase of inhibition of NADH-cytochrome *c* reductase activity by Fe^{3+} -adriamycin

The data of Fig. 1 display an initial fast drop in NADH-cytochrome *c* reductase activity that occurs before the slower progressive decrease in activity. In order to characterize further this fast phase of inhibition, initial velocities were measured in the presence and the

Fig. 2. Fast phase of inhibition of the NADH-cytochrome *c* reductase activity of bovine heart submitochondrial particles

The enzyme activity was measured from absorbance changes that occurred at 550 nm directly after the addition of submitochondrial particles (final protein concn. of 0.08 mg/ml) in the absence (○) or the presence (●) of Fe^{3+} -adriamycin ($10 \mu\text{M-Fe}^{3+}/30 \mu\text{M-adriamycin}$) to the assay mixture. The straight lines are weighted-linear least-squares calculated.

absence of Fe^{3+} -adriamycin (Fig. 2). The plots are almost parallel, suggesting uncompetitive or mixed-type inhibition (Segel, 1975). Thus whereas the slopes (K_m/V_{max}) of the plots are nearly constant ($0.36 \pm 0.04 \text{ min}$ in the presence of Fe^{3+} -adriamycin and $0.43 \pm 0.05 \text{ min}$ in its absence), the value of $V_{\text{max}}^{\text{cyt.c}}$ decreased from $17 \pm 1 \mu\text{M/min}$ to $11.8 \pm 0.8 \mu\text{M/min}$. The $K_m^{\text{cyt.c}}$ were determined to be 6.2 ± 0.8 and $5.2 \pm 0.7 \mu\text{M}$ in the absence and presence respectively of Fe^{3+} -adriamycin. Under the same conditions K_m^{NADH} was determined to be $8.6 \pm 0.9 \mu\text{M}$ in the presence of a nearly saturating ($60 \mu\text{M}$) ferricytochrome *c* concentration. Adriamycin in the absence of added Fe^{3+} , at concentrations of 45 and $100 \mu\text{M}$, caused no significant inhibition. However, $300 \mu\text{M-adriamycin}$ did decrease the activity by about 20%. Demant & Jensen (1983) also found that $100 \mu\text{M-adriamycin}$ only moderately inhibited the NADH oxidase activity of pig heart submitochondrial particles.

Initial velocities were measured as a function of the Fe^{3+} -adriamycin concentration when the ferricytochrome *c* concentration was nearly 10 times $K_m^{\text{cyt.c}}$ and when it was approximately equal to $K_m^{\text{cyt.c}}$ (Fig. 3). Under conditions where v is approximately equal to $V_{\text{max}}^{\text{cyt.c}}$, v displays a strong dependence on the inhibitor concentration. The enzyme activity was inhibited by approx. 50% at about $8 \mu\text{M-Fe}^{3+}$ -adriamycin (Fe^{3+} basis), independent of the submitochondrial protein concentration. At low ferricytochrome *c* concentrations, 50% inhibition occurred at about $6 \mu\text{M-Fe}^{3+}$ -adriamycin. The data of Fig. 1 also demonstrate that the fast phase of inhibition is reversible. In these experiments, the submitochondrial particles were rapidly diluted from a solution that contains Fe^{3+} -adriamycin at a concentration that is strongly inhibiting ($50 \mu\text{M-Fe}^{3+}$ -adriamycin; Fe^{3+} basis) and the activity was regained. The fact that EDTA also largely reverses the inhibition (Fig. 1) is

Fig. 3. Fast phase of inhibition of NADH-cytochrome *c* reductase activity of bovine heart submitochondrial particles by Fe^{3+} -adriamycin

The enzyme activity was measured from absorbance changes that occurred at 550 nm directly after the addition of submitochondrial particles to Fe^{3+} -adriamycin in the assay mixture, which contained, in addition to 6 mM- NaN_3 and $10 \mu\text{M-NADH}$, 0.12 mg of submitochondrial protein and $60 \mu\text{M-ferricytochrome c}$ (●), 0.06 mg of submitochondrial protein and $60 \mu\text{M-ferricytochrome c}$ (○), or 0.037 mg of submitochondrial protein and $6.2 \mu\text{M-ferricytochrome c}$ (◐).

Fig. 4. Fast phase of inhibition of NADH-cytochrome *c* reductase activity of bovine heart submitochondrial particles by Fe^{3+} -adriamycin: effect of protein concentration

The enzyme activity was measured at 412 nm directly after the addition of submitochondrial particles in the absence (●) and presence (○) of Fe^{3+} -adriamycin ($15 \mu\text{M-Fe}^{3+}/45 \mu\text{M-adriamycin}$). The assay conditions were as described in Fig. 3, except that the ferricytochrome *c* concentration was $5 \mu\text{M}$.

a further indication of the reversibility of the fast phase of inhibition. Dixon plots (Segel, 1975) of v^{-1} versus $[\text{Fe}^{3+}\text{-adriamycin}]$ of the data of Fig. 3 were, however, non-linear, indicating that the inhibition was not of the simple uncompetitive type.

Inhibition experiments were also conducted at a con-

Fig. 5. Binding of Fe^{3+} -adriamycin and adriamycin to bovine heart mitochondrial particles

The binding of Fe^{3+} -adriamycin (●) and adriamycin alone (○) was determined from the absorbance at 480 nm after removing the submitochondrial particles from solution by centrifugation as described in the Materials and methods section. The binding experiments were conducted in a total volume of 1200 μl that contained 0.12 mg of submitochondrial protein/ml. The straight lines are weighted-linear least-squares calculated, forced through the origin. The Fe^{3+} -adriamycin concentrations ranged from 5 to 22.5 μM (Fe^{3+} basis).

stant Fe^{3+} -adriamycin concentration of 15 μM , but at various submitochondrial protein concentrations (Fig. 4). The fact that, in the presence of inhibitor, the plot does not intersect the positive horizontal axis is further evidence for reversible inhibition (Segel, 1975).

Lack of protection against Fe^{3+} -adriamycin-induced inactivation of NADH-cytochrome *c* reductase activity by polylysine

Polylysine is known to be a relatively strong inhibitor of the NADH oxidase and succinate oxidase activity of submitochondrial particles, as it is for cytochrome *c* oxidase (Mochan *et al.*, 1973). This inhibition is of the competitive type due to the positively charged polylysine competing with the positively charged lysine-rich cytochrome *c* at the substrate-binding site. Experiments conducted at a ferricytochrome *c* concentration equal to $K_m^{\text{cyt. } c}$ (6.2 μM) displayed a 50% reduction in the fast phase of inhibition at about 40 μM -polylysine. Thus if the inactivation of NADH-cytochrome *c* reductase occurs due to binding of Fe^{3+} -adriamycin at the ferricytochrome *c* binding site, polylysine might be expected to prevent the slow inactivation phase shown in Fig. 1. Incubation experiments identical to those described in the caption to Fig. 1 in the absence of EDTA were carried out in the presence of 150 μM -polylysine. After 30 min of incubation, 10 μl of the incubation mixture was diluted into the assay cell, which resulted in a final polylysine concentration of 12.5 μM . The activity measured in this experiment was identical with that in an experiment when 150 μM -polylysine was eliminated from the incubation mixture, but which was assayed in the presence of the equivalent concentration (12.5 μM) of polylysine in the assay cell as a control. It can be concluded that polylysine does not protect against the slow inactivation of the NADH-cytochrome *c* reductase activity, and thus

inactivation does not occur upon binding of Fe^{3+} -adriamycin to the ferricytochrome *c* binding site.

Binding of Fe^{3+} -adriamycin and adriamycin to submitochondrial particles

As Fig. 5 shows, both Fe^{3+} -adriamycin and adriamycin bind strongly to submitochondrial particles. The amount bound is approximately a linear function of the amount of adriamycin added both for the Fe^{3+} complex and for uncomplexed adriamycin. It can be estimated from the slopes that $45.1 \pm 0.6\%$ and $5.5 \pm 0.3\%$ of the Fe^{3+} -adriamycin and adriamycin respectively bind to the submitochondrial particles. Thus the Fe^{3+} -adriamycin complex binds, on an adriamycin basis, 8.2 times as strongly as uncomplexed adriamycin. A determination of the percentage of Fe^{3+} -adriamycin bound, measured from the decrease in absorbance at 600 nm (where free adriamycin does not significantly absorb), gave a plot (results not shown) similar to that of Fig. 5 but with a slope that indicated that $56.2 \pm 1.3\%$ of the Fe^{3+} -adriamycin was bound to the submitochondrial particles. This increase might be an indication that a significant fraction of the complex may be binding as the Fe^{3+} -(adriamycin)₂ complex as well as the Fe^{3+} -(adriamycin)₃ complex. If the binding were very much stronger than the experimental concentrations employed (up to 22.5 μM - Fe^{3+}), the plot of Fig. 5 would be expected to level out at high adriamycin concentrations. The data of Fig. 5 suggest that relatively non-specific binding to multiple sites is occurring. The fact that, even at 5 μM - Fe^{3+} -adriamycin, 40% of the complex is bound is a measure of the strength of the binding to the submitochondrial particles.

DISCUSSION

The strong binding of Fe^{3+} -adriamycin to submitochondrial particles represents a mechanism whereby lipid peroxidation and consequent enzyme inactivation might be initiated. Myers *et al.* (1982) showed that oxidative destruction of erythrocyte membranes by Fe^{3+} -adriamycin is accompanied by binding. Demant (1983, 1984a) has also demonstrated the binding of Fe^{3+} -adriamycin to pig heart submitochondrial particles as well as to sonicated phospholipids and mixed phospholipid liposomes. The binding was accompanied by lipid peroxidation as well as by inactivation of respiratory enzyme activities. A previous study (Hasinoff & Davey, 1988a) demonstrated the high affinity of adriamycin for cardiolipin, which is present in relatively large amounts in the inner mitochondrial membrane. The Fe^{3+} -adriamycin complex, likewise, has a strong affinity for cardiolipin (Demant, 1984a), as it has been shown to bind to aqueous cardiolipin dispersions and also to partition (in the presence of ADP) into a hexane phase corresponding to 1.8 ± 0.2 mol of adriamycin and 0.6 ± 0.2 mol of Fe^{3+} /mol of cardiolipin. Assuming that both the protein and cardiolipin compositions of bovine heart Keilin-Hartree particles (Vinogradov & King, 1979) are the same as in our submitochondrial particles, it can be estimated that the solutions of Fig. 5 each contained approx. 6 nmol of cardiolipin. Thus assuming the Fe^{3+} -adriamycin complex binds to the cardiolipin of the submitochondrial particles, using Demant's (1984a) ratio for binding of the Fe^{3+} -adriamycin complex, the cardiolipin could account for approx. 10 nmol of the adriamycin observed to bind

in Fig. 5, which is, however, not sufficient to account for all of the Fe^{3+} -adriamycin binding observed. Complexes I and III have been shown to have an absolute requirement for cardiolipin (Fry & Green, 1981), similar to that found for cytochrome *c* oxidase (Fry & Green, 1980). Thus binding of Fe^{3+} -adriamycin to the cardiolipin-rich inner mitochondrial membrane provides a mechanism whereby the mitochondrial membranes might be inactivated through generation of oxy radicals *in situ*.

Although Fe^{3+} -adriamycin was shown to be a strong competitive inhibitor (K_i 12 μM) of cytochrome *c* oxidase (Hasinoff & Davey, 1988b), Fe^{3+} -adriamycin is not a competitive inhibitor of NADH-cytochrome *c* reductase activity. Whereas ferricytochrome *c* offered partial protection against the inactivation of cytochrome *c* oxidase (Hasinoff *et al.*, 1989), polylysine, which presumably also competitively inhibits NADH-cytochrome *c* reductase (Mochan *et al.*, 1973), demonstrated no protection against the Fe^{3+} -adriamycin-induced inactivation. This result is a further indication that Fe^{3+} -adriamycin does not inactivate NADH-cytochrome *c* reductase through binding at the ferricytochrome *c* binding site, as in the case with cytochrome *c* oxidase.

Whereas adriamycin is known to be reductively activated to its semiquinone free radical form (Gianni *et al.*, 1983; Davies & Doroshaw, 1986; Doroshaw & Davies, 1986) by a variety of NAD(P)H dehydrogenases, the inactivation of the enzymes demonstrated in this and previous studies (Hasinoff & Davey, 1988a; Hasinoff *et al.*, 1989) is occurring in the absence of any added NAD(P)H, suggesting that a self-reductive activation is occurring (Gutteridge, 1984; Nakano *et al.*, 1984). The demonstration that Fe^{3+} -adriamycin binds to submitochondrial particles and reversibly inhibits their NADH-cytochrome *c* reductase activity suggests a mechanism whereby the progressive slow inactivation may occur. In this proposed site-specific type of mechanism (Chevion, 1988) Fe^{3+} -adriamycin first reversibly binds to the submitochondrial particles. Then, while Fe^{3+} -adriamycin is bound to the particles, self-reductive activation of the Fe^{3+} -adriamycin complex occurs which leads to formation of damaging oxy radical species *in situ*. Thus destruction of the enzyme may be occurring through a mechanism involving some of the following reactions that occur when Fe^{3+} -adriamycin (ADM) is bound to the submitochondrial particle:

It has been proposed by Zweier *et al.* (1986) that the α -ketol group ($-\text{COCH}_2\text{OH}$) of adriamycin reduces the Fe^{3+} to Fe^{2+} (reaction 1), while it is itself oxidized to an α -oxoaldehyde ($-\text{COCHO}$). Subsequent reoxidation of Fe^{2+} by O_2 would result in superoxide ($\text{O}_2^{\bullet-}$) formation (reaction 3), with the possibility of iron-based Fenton-type $\text{OH}^{\bullet}$ radical production (reactions 3–5). Alternatively, formation of the strongly oxidizing perferryl ion (Aust & Svingen, 1982) (reaction 6) might occur. Either of these strongly oxidizing species could initiate lipid

peroxidation with a consequent inactivation of the membrane-bound respiratory enzymes.

Adriamycin may be able to form the damaging Fe^{3+} -adriamycin complex *in vivo*, as it has been demonstrated that the iron-storage protein ferritin slowly releases its iron to adriamycin (Demant, 1984b), and that even the strongly bound iron of the iron-transport protein transferrin can, at acidic pH, give up its iron to adriamycin (Demant & Norskov-Lauritsen, 1986). Thomas & Aust (1986) have also demonstrated that in the presence of NADPH-cytochrome *P*-450 reductase, adriamycin mediates a slow reductive release of iron from ferritin.

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