Biochem. J. (1988) 250, 827–834 (Printed in Great Britain)

The iron(III)–adriamycin complex inhibits cytochrome *c* oxidase before its inactivation

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Cytochrome *c* oxidase was found to be competitively inhibited by a complex formed between Fe^{3+} and the cardiotoxic antitumour drug adriamycin (doxorubicin) with an inhibition constant, K_i, of 12 μM. This competitive inhibition precedes the slower Fe^{3+}–adriamycin induced inactivation of cytochrome *c* oxidase. In strong contrast with this result, free adriamycin was not observed to either inhibit or inactivate cytochrome *c* oxidase ($K_i > 3$ mM). Since, typically, polycations are known to inhibit cytochrome *c* oxidase, the competitive inhibition displayed by the Fe^{3+}–adriamycin complex may also result from its polycationic character. Cytochrome *c* oxidase was also inhibited by pentan-1-ol (K_i 13 mM), and kinetic studies carried out in the presence of both inhibitors demonstrated that the Fe^{3+}–adriamycin complex and pentan-1-ol are mutually exclusive inhibitors of cytochrome *c* oxidase. The inhibitor pentan-1-ol was also effective in preventing the slow inactivation of cytochrome *c* oxidase induced by Fe^{3+}–adriamycin, presumably by blocking its binding to the enzyme. It is postulated that the slow inactivation of cytochrome *c* oxidase occurs when reactive radical species are produced while the Fe^{3+}–adriamycin is complexed to cytochrome *c* oxidase in an enzyme–inhibitor complex. The Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase may be, in part, responsible for the cardiotoxicity of adriamycin.

INTRODUCTION

Adriamycin (doxorubicin) (Fig. 1) is an anthracycline quinone antibiotic with a wide range of antitumour activities. Unfortunately, the clinical use of adriamycin is limited by its cumulative dose-dependent cardiotoxicity (Kantrowitz & Bristow, 1984; Gianni *et al.*, 1983). The mechanism or mechanisms of the cardiotoxicity are not understood, but it may involve free-radical oxidative stress on the heart muscle. The heart muscle is relatively unprotected, as it contains much less of the protective enzymes catalase, glutathione peroxidase and superoxide dismutase than does, for example, the liver (Halliwell & Gutteridge, 1985). Another factor in the cardiotoxicity of adriamycin may be its high affinity for the phospholipid cardiolipin (diphosphatidylglycerol) (Goormaghtigh & Ruysschaert, 1984; Goormaghtigh *et al.*, 1986). The positively charged adriamycin forms a 2:1 adriamycin–cardiolipin complex with the doubly negatively charged cardiolipin with a dissociation constant of about 1 μM. Likewise the Fe^{3+}–adriamycin complex has also been demonstrated to have a strong affinity for cardiolipin (Demant, 1984). Cardiolipin is most plentiful in the heart muscle, where it is located mainly in the mitochondrial inner membrane. In pig heart it represents 25% of the total lipid phosphorus (Hostetler, 1982) of the mitochondrial inner membrane. Adriamycin causes lipid peroxidation, and there is now ample evidence that this oxygen-dependent reaction depends upon the presence of iron or copper (Gianni *et al.*, 1983; Demant & Jensen, 1983; Demant, 1983; Nakano *et al.*, 1984; Gutteridge, 1984; Mimnaugh *et al.*, 1985). The cardiolipin in pig heart submitochondrial particles is degraded by the Fe^{3+}–adriamycin complex. This occurs simultaneously with the inactivation of cytochrome *c* oxidase and several other respiratory enzymes (Demant & Jensen, 1983; Demant, 1983).

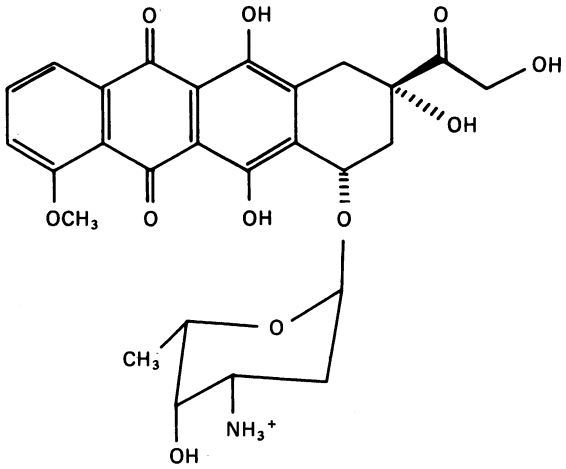

Fig. 1. Adriamycin (doxorubicin)

Abbreviations used: ADM, adriamycin; $ADM^{\cdot -}$, the adriamycin semiquinone free radical; cyt^{2+} (or cyt) and cyt^{3+}, ferro- and ferri-cytochrome *c* respectively; $S_{red.}$ and $S_{ox.}$, a reductant of adriamycin and the oxidized species respectively; $K_i^{Fe^{3+}\text{-ADM}}$ and $K_i^{pentanol}$, inhibition constants for the Fe^{3+}–adriamycin inhibition and pentan-1-ol inhibition of cytochrome *c* oxidase respectively; K_m^{app}, apparent Michaelis constant in the presence of inhibitor.

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Cytochrome *c* oxidase, the terminal respiratory enzyme, is inactivated by adriamycin both *in vivo* (Arena *et al.*, 1975) and *in vitro* (Goormaghtigh *et al.*, 1982; Demant & Jensen, 1983; Demant, 1983; Goormaghtigh & Ruysschaert, 1984). The adriamycin-induced inactivation of cytochrome *c* oxidase is EDTA-inhibitible and requires the presence of small amounts of iron in the incubation mixture (Demant & Jensen, 1983). Bovine heart cytochrome *c* oxidase requires cardiolipin for optimal activity (Vik *et al.*, 1981; Robinson *et al.*, 1980), and thus the phospholipid associated with cytochrome *c* oxidase might be damaged or removed by the Fe^{3+}–adriamycin complex or by reactive species produced by the complex.

In a previous study (Hasinoff & Davey, 1987) it was found that cytochrome *c* oxidase was inhibited at a hydrophobic binding site by a variety of alcohols. The presence of a hydrophobic inhibitor site has also been shown by the inhibition of cytochrome *c* oxidase by a number of hydrophobic amine local anaesthetics (Singer, 1980; Casanovas *et al.*, 1983) and other compounds (Casanovas *et al.*, 1985; Igisu & Nakamura, 1986). Likewise, adriamycin or its Fe^{3+} complex might be capable of forming a complex with cytochrome *c* oxidase in a first step before inactivation of the enzyme. In the present study it is shown that free adriamycin in fact does not inhibit (or inactivate) cytochrome *c* oxidase but that the Fe^{3+}–adriamycin complex is a strong competitive inhibitor of the enzyme.

Adriamycin (ADM) binds strongly to Fe^{3+} (Myers *et al.*, 1982):

$$Fe^{3+} + 3ADM \rightleftharpoons Fe^{3+}(ADM)_3$$

with an overall formation constant of $10^{33}\ M^{-3}$ and stepwise formation constants of $10^{18}\ M^{-1}$, $10^{11}\ M^{-1}$ and $10^{4.4}\ M^{-1}$ (May *et al.*, 1980). However, other reports (Martin, 1985; Beraldo *et al.*, 1985) have suggested that the overall formation constant of $10^{33}\ M^{-3}$ has been overestimated, as an overall formation constant of $10^{28.4}\ M^{-3}$ has also been measured.

It is the goal of the present study to understand the basic mechanisms of adriamycin's cardiotoxicity. This should allow for a more rational basis on which to synthesize new effective non-cardiotoxic derivatives and to select co-administered protective agents.

MATERIALS AND METHODS

Bovine heart cytochrome *c* oxidase (Sigma Chemical Co.) was dissolved in 10 mM-sodium phosphate buffer, pH 7.4, containing 0.4% Tween 80. The enzyme used was reportedly prepared according to the method of Yonetani (1967), which involves a cholic acid extraction and results in a preparation that is about 10% lipid. The enzyme concentration was determined spectrophotometrically by using a millimolar absorption coefficient (reduced minus oxidized) of $24.0\ mM^{-1}\cdot cm^{-1}$ (Van Gelder, 1966) and is expressed in terms of the concentration of a haem aa_3 unit. The activity was assayed spectrophotometrically by the method of Yonetani & Ray (1965) under identical conditions of pH, buffer, ionic strength and temperature and was found to have a turnover number calculated from $V_{max.}/[E]$ of $59\ s^{-1}$. Yonetani & Ray (1965) by comparison reported a turnover number of $60\ s^{-1}$. Turnover numbers in the range $30–300\ s^{-1}$ have been reported (Vik & Capaldi, 1980) for cytochrome *c* oxidase. These are, however, highly variable, as they depend upon a number of factors including the preparation, the detergent, the presence of added lipids, pH, buffer, temperature and even the method of measurement (whether spectrophotometric or polarographic). The substrate, ferrocytochrome *c* (Sigma type VI from horse heart) was reduced with dithionite and passed down a Sephadex G-25 column (all under N_2) to remove excess dithionite. Adriamycin, as the hydrochloride, admixed with lactose, was obtained from Adria Laboratories (Columbus, OH, U.S.A.). The Fe^{3+}–adriamycin complex was formed by adding stock $FeCl_3,6H_2O$ (99% pure) dissolved in 1 mM-HCl [to prevent iron(III) hydroxide polymer formation] to adriamycin dissolved in water giving a final ratio of $Fe^{3+}$to adriamycin of 1:3. The reactions were studied in 0.10 *I* phosphate buffer, pH 7.0, at 25 °C. At this high ionic strength cytochrome *c* oxidase exhibits only simple linear Lineweaver–Burk kinetics (Yonetani & Ray, 1965; Malmström & Andréasson, 1985) consistent with a single kinetically significant K_m of 2.1 μM. The phosphate buffer was purified of adventitious metal ions by slowly passing it down a 1 cm × 40 cm Dowex 50W cation-exchange column, which lowered the iron and copper to below detectable concentrations (< 0.5 μM by atomic absorption analysis) and resulted in a buffer in which no adriamycin-induced inactivation of cytochrome *c* oxidase occurred. The addition of comparable amounts of Fe^{3+} alone to the reaction mixture also caused no detectable inactivation of cytochrome *c* oxidase.

The initial velocities, *v*, of the cytochrome *c* oxidase-catalysed aerobic oxidation of ferrocytochrome *c* were measured at 550 nm on a Shimadzu UV-260 spectrophotometer by using the following millimolar absorption coefficients (Margoliash, 1954): $\epsilon_{red.}$ $27.7\ mM^{-1}\cdot cm^{-1}$ and $\Delta(\epsilon_{red.} - \epsilon_{ox.})$ $18.5\ mM^{-1}\cdot cm^{-1}$. The initial velocities, *v*, were estimated from the in-built computer-calculated first derivatives extrapolated back to time zero. The reaction was started by adding the stock enzyme solution, deposited on the end of a plunger (Uvonic Instruments), into the buffer in the cell containing ferrocytochrome *c* and the inhibitors as indicated. When the Fe^{3+}–adriamycin complex was added to the solution containing enzyme a slow (half-time approx. 4 min) inactivation of cytochrome *c* oxidase was observed to occur, as has been reported previously (Demant, 1983; Demant & Jensen, 1983; Goormaghtigh *et al.*, 1982). Typically, Fe^{3+}–adriamycin (20 μM-Fe^{3+}, 60 μM-adriamycin) inactivated cytochrome *c* oxidase to about 50% of its original activity after a 15 min incubation at room temperature. For the competitive inhibition studies the reaction was initiated by adding enzyme last, and care was taken to measure the initial velocities over the first few seconds of the reaction before any significant slow Fe^{3+}–adriamycin-induced enzyme inactivation could occur. Thus the initial velocities are a measure of only the fast prior inhibition that is occurring. The Michaelis parameters were analysed in Lineweaver–Burk plots by weighted (assuming 5% error in *v*) linear least-squares analyses. Standard deviations thus obtained on these parameters were then used as weights in the weighted least-squares analyses of the secondary inhibitor plots. Fe^{3+}–adriamycin by itself caused no detectable oxidation of ferrocytochrome *c*.

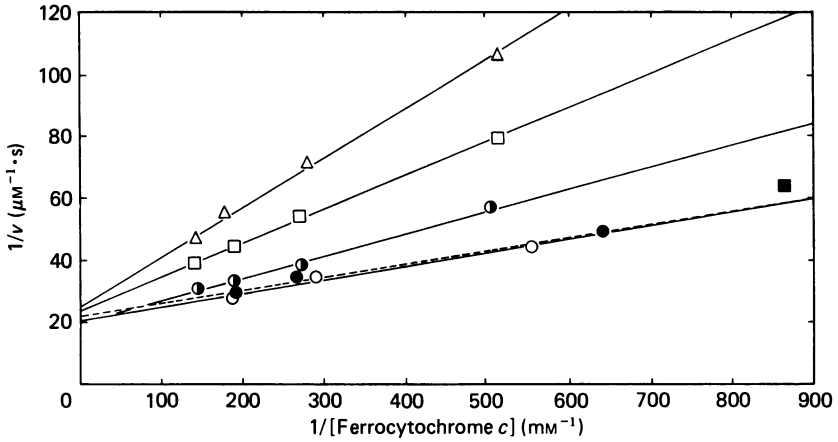


Fig. 2. Lineweaver–Burk plots for the aerobic oxidation of ferrocytochrome *c* catalysed by cytochrome *c* oxidase in the presence of the Fe^{3+}–adriamycin complex and adriamycin in 0.10 *I* phosphate buffer, pH 7.0, at 25 °C

The enzyme concentration was 0.8 nM on a haem aa_3 unit concentration basis. The linear least-squares fits are given by the continuous straight lines. The Fe^{3+}–adriamycin concentrations are: △, 30 μM; □, 20 μM; ◑, 10 μM; ○, 0 μM (Fe^{3+} basis). The [adriamycin]/[Fe^{3+}] ratio was maintained at a constant 3:1 ratio. The linear least-squares fit given by the broken line (-----) was obtained at an adriamycin concentration of 100 μM (●) in the absence of any added Fe^{3+}. The point at 300 μM-adriamycin (■) was not included in the regression. The straight lines beyond the last experimental points are extrapolations.

RESULTS

Inhibition of cytochrome *c* oxidase by Fe^{3+}–adriamycin

As Fig. 2 shows, the Fe^{3+}–adriamycin complex is a strong competitive inhibitor of cytochrome *c* oxidase. The initial velocities were measured as described in the Materials and methods section at times that preceded the slow Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase. The tight intercepts of the plots are consistent with pure competitive inhibition, for which:

$$v = \frac{V_{max.}[cyt]}{K_m\left(1+\frac{[Fe^{3+}\text{–ADM}]}{K_i^{Fe^{3+}\text{-ADM}}}\right)+[cyt]}$$

$$\frac{K_m^{app.}}{V_{max.}} = \frac{K_m}{V_{max.}}\left(1+\frac{[Fe^{3+}\text{–ADM}]}{K_i^{Fe^{3+}\text{-ADM}}}\right) \quad (1)$$

The Michaelis parameters are given in Table 1. The $V_{max.}$ values are constant within the sums of their respective standard deviations indicating pure competitive inhibition. The parameter $K_m^{app.}/V_{max.}$ is plotted in Fig. 3 versus $[Fe^{3+}\text{–adriamycin}_3]_\Sigma$, the total concentration of all iron(III) complexed species, and in accord with eqn. (1) yields an apparent $K_i^{Fe^{3+}\text{-ADM}}$ of 12±4 μM (on an Fe^{3+} basis) from the slope and intercept. The linearity of the data of Fig. 3 is again indicative of pure competitive inhibition. Initial velocities and Michaelis parameters (data of Fig. 2 and Table 1) obtained in the presence of 100 μM-adriamycin and no added Fe^{3+} yield Michaelis parameters almost identical with those in the absence of any adriamycin. Thus free adriamycin causes no detectable inhibition of cytochrome *c* oxidase, in strong contrast with the Fe^{3+}–adriamycin complex. With these results it is possible to place a lower limit on $K_i^{ADM} > 3000$ μM.

Inhibition of cytochrome *c* oxidase by pentan-1-ol and pentan-1-ol together with Fe^{3+}–adriamycin

In a previous study (Hasinoff & Davey, 1987) it was shown that several alcohols were observed to inhibit cytochrome *c* oxidase. Fig. 4 demonstrates the inhibition of cytochrome *c* oxidase by pentan-1-ol, and Table 1 gives the Michaelis parameters obtained from these

Table 1. Michaelis parameters for cytochrome *c* oxidase in the presence of Fe^{3+}–adriamycin, adriamycin or pentan-1-ol

The standard deviations shown are from weighted linear least-squares analyses only. The data for pentan-1-ol have been normalized to the same enzyme concentration as the other data. The reactions were studied in 0.10 *I* phosphate buffer, pH 7.0, at 25 °C.

[Fe^{3+}] (μM)	[Adriamycin] (μM)	[Pentan-1-ol] (mM)	$V_{max.}$ (nM·s^{-1})	$K_m^{app.}$ (μM)	$K_m^{app.}/V_{max.}$ (s)
0	0	0	48±5	2.1±0.4	44±7
0	100	0	45±4	1.9±0.3	43±6
10	30	0	50±5	3.5±0.6	71±9
20	60	0	42±5	4.6±0.7	109±12
30	90	0	39±5	5.9±1.0	150±15
0	0	10	46±3	3.2±0.4	69±7
0	0	40	38±5	6.5±1.1	173±16

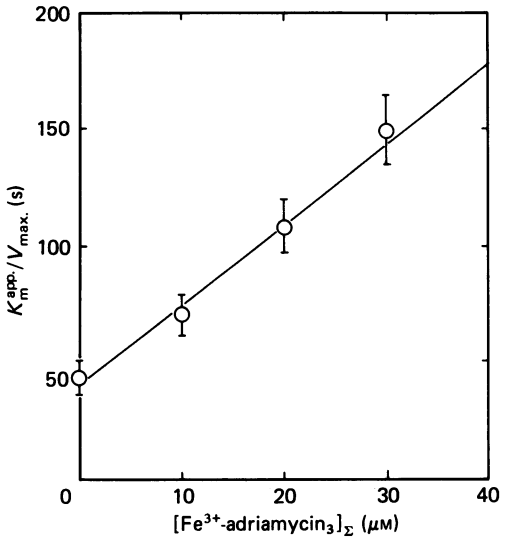


Fig. 3. Secondary plot of the slopes ($K_m^{app.}/V_{max.}$) of Fig. 2 versus $[Fe^{3+}\text{–adriamycin}_3]_\Sigma$, the total concentration of all iron(III) complexed species, showing competitive inhibition of cytochrome *c* oxidase

The slope and the intercept combined yield $K_i^{Fe^{3+}\text{-ADM}} = 12 \pm 4$ μM from eqn. (1). The straight line is weighted least-squares-calculated.

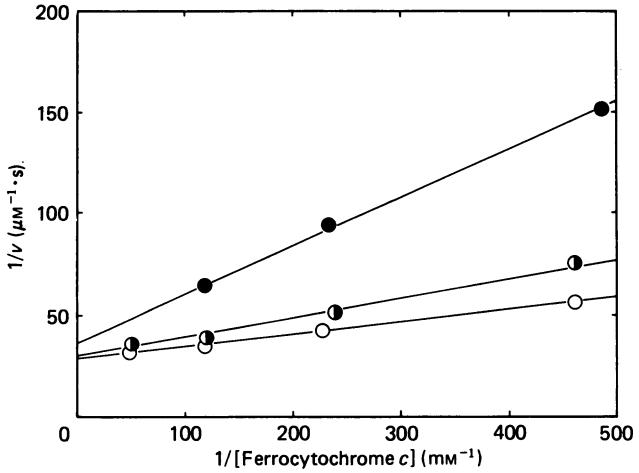


Fig. 4. Lineweaver–Burk plots for the aerobic oxidation of ferrocytochrome *c* catalysed by cytochrome *c* oxidase in the presence of pentan-1-ol in 0.10 *I* phosphate buffer, pH 7.0, at 25 °C

The data were weighted least-squares fit and were obtained at pentan-1-ol concentrations of (starting from the uppermost line): ●, 40 mM; ◑, 10 mM; ○, 0 mM.

plots. The $V_{max.}$ values do decrease slightly with increasing pentan-1-ol concentration, indicating that the inhibition may be of the more general linear mixed inhibition type (Segel, 1975), where the inhibitor can also bind to the E·cyt^{2+} complex as well as E. However, since the $V_{max.}$ values over the whole inhibitor concentration range are just within the sums of their standard deviations (Table 1), this indicates that in the experimental concentration range the kinetics can be adequately described by competitive kinetics, which is, in any event, a

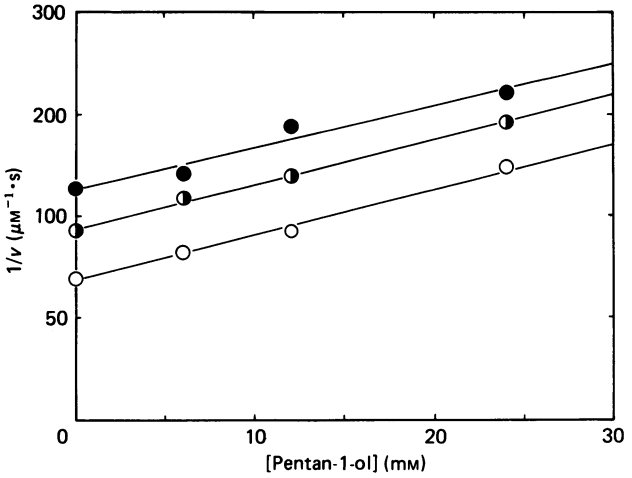
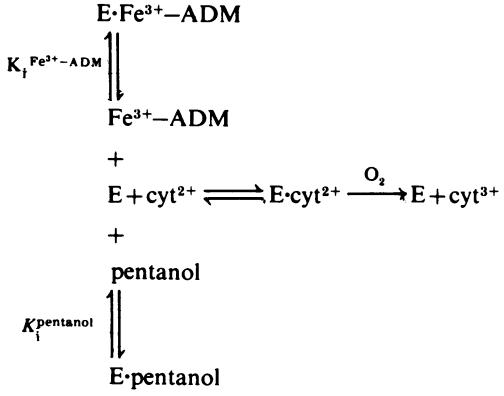


Fig. 5. Plot of 1/*v* versus [pentan-1-ol] for the aerobic oxidation of ferrocytochrome *c* catalysed by cytochrome *c* oxidase in the presence of Fe^{3+}–adriamycin in 0.10 *I* phosphate buffer, pH 7.0, at 25 °C

The data were weighted linear-least-square fit and were obtained at a constant Fe^{3+}–adriamycin concentration of (starting from the top): ●, 24 mM; ◑, 12 mM; ○, 0 mM (Fe^{3+} basis). The ferrocytochrome *c* concentration was maintained constant at 2.2 μM throughout.

limiting special case of linear mixed inhibition (Segel, 1975). A plot of $K_m^{app.}/V_{max.}$ versus [pentan-1-ol] (plot not shown) in accord with an equation similar to eqn. (1) gave $K_i^{pentanol}$ 13 ± 3 mM. In addition to the five alcohols that were previously shown (Hasinoff & Davey, 1987) to inhibit cytochrome *c* oxidase, two other longer-chain alcohols, octan-1-ol (K_i 1.2 mM) and decan-1-ol (K_i 0.15 mM), were also determined to be inhibitors. Neither pentan-1-ol (24 mM) nor adriamycin (100 μM) changed the spectrum of ferrocytochrome *c*. Thus there is no spectroscopic evidence for binding of these substances to the substrate.

Inhibition studies on cytochrome *c* oxidase were also conducted in which the two inhibitors, Fe^{3+}–adriamycin and pentan-1-ol, were present simultaneously. These experiments can potentially show whether these two inhibitors bind to the same site and whether the binding of one excludes the other (mutually exclusive inhibition). These results are shown in Fig. 5. These results were also obtained at a time preceding the slow Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase. For pure competitive inhibition by two different exclusive inhibitors



Scheme 1.

Table 2. Slopes and intercepts from the plots of Fig. 5

The average of the $slope_1$ values is 2200 ± 100 mM^{-2}·s.

$[Fe^{3+}]$ (μM)	[Adriamycin] (μM)	$Slope_1$ (mM^{-2}·s)	$Intercept_1$ (mM^{-1}·s)
0	0	2230 ± 270	$6.8 \times 10^4 \pm 0.3 \times 10^4$
12	36	2270 ± 330	$9.3 \times 10^4 \pm 0.4 \times 10^4$
24	72	2070 ± 380	$11.3 \times 10^4 \pm 0.4 \times 10^4$

in the simplified rapid-equilibrium scheme (Segel, 1975) shown in Scheme 1:

$$v = \frac{\frac{V_{max.}[cyt]}{K_m}}{1 + \frac{[cyt]}{K_m} + \frac{[pentanol]}{K_i^{pentanol}} + \frac{[Fe^{3+}\text{–ADM}]}{K_i^{Fe^{3+}\text{–ADM}}}} \quad (2)$$

$$\frac{1}{v} = \frac{K_m}{[cyt]\,V_{max.}\,K_i^{pentanol}}[pentanol] + \frac{1}{V_{max.}}\left(1 + \frac{K_m}{[cyt]} + \frac{K_m[Fe^{3+}\text{–ADM}]}{K_i^{Fe^{3+}\text{–ADM}}[cyt]}\right) \quad (3)$$

Eqn. 3 predicts that at a fixed $[Fe^{3+}$–ADM] and [cyt] a plot of $1/v$ versus [pentanol] is linear with the following slope ($slope_1$) and intercept ($intercept_1$):

$$Slope_1 = \frac{K_m}{[cyt]\,V_{max.}\,K_i^{pentanol}} \quad (4)$$

$$Intercept_1 = \frac{1}{V_{max.}}\left(1 + \frac{K_m}{[cyt]} + \frac{K_m[Fe^{3+}\text{–ADM}]}{K_i^{Fe^{3+}\text{–ADM}}[cyt]}\right) \quad (5)$$

Consequently, at fixed [cyt] eqn. (3) predicts a family of straight lines with the same slopes (given by eqn. 4). The straight lines are consequently parallel to one another, but they are vertically displaced from each other by a constant amount (given by eqn. 5) when the $[Fe^{3+}$–ADM] is incremented by a constant amount. The plots of Fig. 5 are parallel, as the least-squares-determined values of $slope_1$ are constant well within their standard deviations (Table 2). A secondary plot (plot not shown) of the values of $intercept_1$ versus $[Fe^{3+}$–ADM] is linear, as predicted by eqn. (5), giving a slope of $slope_2$. The ratio of $slope_2$ to $slope_1$, from eqns. 4 and 5 respectively ($slope_1$ is an average for three determinations) from Figs. 4 and 5, yields

$$\frac{Slope_2}{Slope_1} = \frac{K_i^{pentanol}}{K_i^{Fe^{3+}\text{–ADM}}} = \frac{1.8 \times 10^6 \pm 0.2 \times 10^6\ \text{mM}^{-2}\cdot\text{s}}{2.2 \times 10^3 \pm 0.1 \times 10^3\ \text{mM}^{-2}\cdot\text{s}} = 860 \pm 110 \quad (6)$$

This value is in good agreement with the ratio of the independently determined values of $K_i^{pentanol}/K_i^{Fe^{3+}\text{–ADM}} = (13 \pm 3\ \text{mM})/(12 \times 10^{-3} \pm 4 \times 10^{-3}\ \text{mM}) = 1060 \pm 440$. This agreement is good evidence that pentan-1-ol and Fe^{3+}–ADM act as mutually exclusive competitive inhibitors that bind to the same site on cytochrome *c* oxidase. Other types of inhibition such as ordered binding or mixed-type inhibition (Segel, 1975) would not yield the characteristic parallel plots seen in Fig. 5.

Table 3. Protection against the Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase by pentan-1-ol

The reactions were studied in 0.10 *I* phosphate buffer, pH 7.0. The ferrocytochrome *c* concentration was 2.5 μM. The enzyme was incubated for 15 min in the presence or absence of the inhibitors as indicated. The estimated error is in the order of $\pm 15\%$ of the value quoted.

$[Fe^{3+}]$ (μM)	[Adriamycin] (μM)	[Pentan-1-ol] (mM)	Enzyme activity (%)
0	0	0	100
0	0	10	68
0	0	20	74
0	0	20	59*
20	60	0	52
20	60	10	50
20	60	20	52

* No incubation was carried out in this experiment. This value compares with a calculated value of 59% from the expression for competitive inhibition by using the experimentally determined values of K_m and $K_i^{pentanol}$.

Protection against the Fe^{3+}–adriamycin-induced inactivation of cytochrome *c* oxidase by pentan-1-ol

Since the inhibition experiments have shown that the Fe^{3+}–adriamycin complex and pentan-1-ol are mutually exclusive inhibitors, experiments were carried out to test whether pentan-1-ol could offer some protection against the slow inactivation of cytochrome *c* oxidase by blocking binding of Fe^{3+}–adriamycin. The results are shown in Table 3. In these experiments phosphate buffer, pentan-1-ol, cytochrome *c* oxidase and Fe^{3+}–adriamycin were successively added to the reaction cell and incubated together for 15 min, after which ferrocytochrome *c* was added and the initial velocity was measured. The activities reported (Table 3) are in reference to controls run under identical conditions in the absence of either pentan-1-ol or Fe^{3+}–adriamycin. Cytochrome *c* oxidase undergoes a progressive 15% loss in activity over 15 min when diluted from the stock solution, which was compensated for by running a control. The presence of pentan-1-ol, however, partially stabilized the enzyme against some of this loss in activity (Table 3). As seen from Table 3, the activity of cytochrome *c* oxidase, measured when the incubation was carried out in the presence of both Fe^{3+}–adriamycin and pentan-1-ol, is close to that observed (52%) when incubation was with Fe^{3+}–adriamycin alone. Since both are inhibitors of cytochrome *c* oxidase, pentan-1-ol is protecting cytochrome *c* oxidase from the slow inactivation process. Otherwise, the activity of both incubated together would have been significantly less than when each was incubated separately. These results suggest that Fe^{3+}–adriamycin forms a complex with cytochrome *c* oxidase that then subsequently inactivates the enzyme. Since alcohols are known free-radical scavengers (Halliwell & Gutteridge, 1985), pentan-1-ol could also be acting as a scavenger in these experiments.

DISCUSSION

The results of this study have shown that Fe^{3+}–adriamycin is a good competitive inhibitor of cyto-

chrome *c* oxidase and yet free adriamycin is not. Apart from the alcohols investigated in this study and a previous study (Hasinoff & Davey, 1987), several other large organic compounds have also been observed to inhibit or bind to cytochrome *c* oxidase. A variety of hydrophobic amine local anaesthetics (11 in all) were observed to inhibit (K_i from 0.6 to 19 mM) cytochrome *c* oxidase (Singer, 1980; Casanovas *et al.*, 1983). Quinacrine (Casanovas *et al.*, 1985), six fully reduced flavin analogues ($K_{diss.}$ 5–8 μM) (Ahmad *et al.*, 1982) and galactosylsphingosine, which has a long hydrophobic tail (K_i approx. 5 μM) (Igisu & Nakamura, 1986), also bind to cytochrome *c* oxidase. Orii & Yoshikawa (1973) have also shown that hydroxylamine, hydrazine, semicarbazide, salicylaldoxime and ethylxanthate inhibit cytochrome *c* oxidase with inhibition constants as low as 0.5 μM. The diverse range of compounds that have been reported to inhibit or bind to cytochrome *c* oxidase indicates that the enzyme has a relatively non-specific hydrophobic inhibitor-binding site.

The structure or even the stoichiometry of the Fe^{3+}–adriamycin complex that is bound to cytochrome *c* oxidase is unknown. On the basis of the formation constants for the $Fe^{3+}(ADM)_3$ species (May *et al.*, 1980), a mixture of $Fe^{3+}(ADM)_2$ and $Fe^{3+}(ADM)_3$ species may be present in solution under the conditions of the present study. However, the proportion of each cannot be estimated accurately owing to the error limits on the final formation constant of $Fe^{3+}(ADM)_3$ of $10^{4.4\pm0.6}$ M^{-1}. It should be noted that even if the major Fe^{3+}–adriamycin complex in solution can be identified it does not necessarily follow that this is the complex that is bound to cytochrome *c* oxidase. The linearity of the inhibition plot of Fig. 3 could be a result of the experimental concentration range being far enough away from the equilibrium position that the concentration of the complex that binds to the enzyme does not vary by a significant amount. Alternatively, if $Fe^{3+}(ADM)_2$ and $Fe^{3+}(ADM)_3$ were to have similar inhibition constants then the conversion of the one complex into the other would not affect the inhibition. Thus the apparent inhibition constant $K_i^{Fe^{3+}\text{-}ADM}$ represents the maximum value of the inhibition constant for the specific Fe^{3+}–adriamycin complex that does bind to the enzyme. The inhibition constant for this Fe^{3+}–adriamycin complex, whether $Fe^{3+}(ADM)_2$ or $Fe^{3+}(ADM)_3$, therefore has a value that is smaller than 12 μM. This maximum value can be compared with a K_i for ferricytochrome *c* of 8 μM (Yonetani & Ray, 1965) under the same conditions as in the present study. Ferricytochrome *c* is a relatively small protein (radius 1.7 nm) and under the conditions of this study has an overall charge of +7 (Hasinoff *et al.*, 1984). Polycations, including both natural salmine and synthetic polylysine, are well known to inhibit cytochrome *c* oxidase strongly (Mochan *et al.*, 1973) with inhibition constants in the range 0.07–50 μM. The electrostatic component of substrate, product and polycationic inhibitor binding to cytochrome *c* oxidase is thus well established and has been the subject of many studies (Margoliash, 1982). Because adriamycin is positively charged owing to the presence of an amino sugar, adriamycin (Gianni *et al.*, 1983) (Fig. 1) complexed to the positively charged Fe^{3+} would result in a +3 polycationic species in the case of $Fe^{3+}(ADM)_3$ and possibly a +3 species in the case of $Fe^{3+}(ADM)_2$ as well. It thus seems reasonable to suggest that the polycationic Fe^{3+}–adriamycin species that inhibits the enzyme also has a significant electrostatic component to its binding to the enzyme analogous to that for ferrocytochrome *c* and ferricytochrome *c* binding. It is tempting to speculate that it is an $Fe^{3+}(ADM)_2$ species that is bound to cytochrome *c* oxidase. As adriamycin probably acts as a bidentate ligand, an $Fe^{3+}(ADM)_2$ species would leave two free co-ordination sites on the normally six-co-ordinate Fe^{3+}. These two co-ordination sites might be occupied by either water or hydroxide ligands and would be available for further ligand substitution, which could facilitate the catalytic reduction of oxygen to superoxide or hydrogen peroxide. The co-ordination of adriamycin to Fe^{3+} results in the loss of a phenolic proton (Beraldo *et al.*, 1985). Thus the overall charge on $Fe^{3+}(ADM)_2$ could be lowered to +2 or +1 by the loss of either one or two protons from the two remaining bound water molecules. At pH 7.0 at least one proton is likely to dissociate, as the water bound to the analogous $Fe(H_2O)_6^{3+}$ is quite acidic (pK_a 2.7) (Koren & Perlmutter-Hayman, 1972).

The inactivation of cytochrome *c* oxidase in submitochondrial particles by Fe^{3+}–adriamycin can be largely reversed by cholate solubilization (Demant, 1983), suggesting that membrane cardiolipin is damaged or removed by Fe^{3+}–adriamycin. If the Fe^{3+}–adriamycin complex binds adjacent to the cardiolipin bound to cytochrome *c* oxidase, reactive species produced by Fe^{3+}–adriamycin would not have far to diffuse to react with bound cardiolipin. The Fe^{3+}–adriamycin complex has a strong affinity for cardiolipin, 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+} per mol of cardiolipin (Demant, 1984). The loss of unsaturated cardiolipin from pig heart submitochondrial particles caused by Fe^{3+}–adriamycin closely parallels their loss of cytochrome *c* oxidase activity (Demant, 1983; Demant & Jensen, 1983). The loss of haem *a* from cytochrome *c* oxidase (Demant, 1983) suggests also that Fe^{3+}–adriamycin binds close to haem *a* in the cytochrome *c*-binding site. It is possible to locate the lipid bound to cytochrome *c* oxidase as being close to the cytochrome *c*-binding site as an arylazido-cytochrome *c* derivative modified at lysine-22 cross-links only with lipid bound to cytochrome *c* oxidase (Bisson *et al.*, 1980). The lysine-13 derivative cross-linked both to the enzyme and bound lipid, and thus this group is slightly further away from the lipid. Thus if the cytochrome *c* binds adjacent to cardiolipin and Fe^{3+}–adriamycin binds to the same site then it too must bind adjacent to the cardiolipin.

The pentan-1-ol exclusive inhibition experiments confirm that Fe^{3+}–adriamycin is a pure competitive inhibitor of cytochrome *c* oxidase. These results also suggest that if the Fe^{3+}–adriamycin-induced inactivation results from the formation of an initial Fe^{3+}–adriamycin–cytochrome *c* oxidase enzyme–inhibitor complex then pentan-1-ol should act as a protective agent against inactivation by blocking binding of Fe^{3+}–adriamycin. The pentan-1-ol protection experiments demonstrate that this is indeed the case.

It has previously been demonstrated (Demant & Norskov-Lauritsen, 1986) that the strongly bound iron of the iron-transport protein transferrin can be transferred to adriamycin. Thus the Fe^{3+}–adriamycin species

may be formed *in vivo*. It is less problematic to postulate Cu^{2+}–adriamycin species, as Cu^{2+} is present intracellularly and in plasma as more weakly bound species (Gianni *et al.*, 1983).

The reactive oxygen species that have been implicated in Fe^{3+}–adriamycin-induced oxidative damage include hydroxyl radical ($OH^{\cdot}$), superoxide ($O_2^{\cdot -}$) and H_2O_2 (Myers *et al.*, 1982; Gianni *et al.*, 1983; Nakano *et al.*, 1984; Gutteridge, 1984; Mimnaugh *et al.*, 1985). The heart muscle contains all of the necessary metabolic reducing machinery to produce the adriamycin semiquinone free radical ($ADM^{\cdot -}$). Thus a postulated mechanism involving production of the strongly oxidizing $OH^{\cdot}$ might involve these steps:

$$S_{red.} + ADM \rightarrow ADM^{\cdot -} + S_{ox.} \quad \text{semiquinone radical formation} \quad (7)$$

$$ADM^{\cdot -} + O_2 \rightleftharpoons ADM + O_2^{\cdot -} \quad \text{superoxide formation} \quad (8)$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{hydrogen peroxide formation} \quad (9)$$

$$ADM^{\cdot -} + Fe^{3+}\text{–}ADM \rightarrow Fe^{2+}\text{–}ADM + ADM \quad (10)$$

$$O_2^{\cdot -} + Fe^{3+}\text{–}ADM \rightarrow Fe^{2+}\text{–}ADM + O_2 \quad (11)$$

Fe^{3+} reduction

$$Fe^{2+}\text{–}ADM + H_2O_2 \rightarrow Fe^{3+}\text{–}ADM + OH^{\cdot} + OH^- \quad \text{hydroxyl radical formation} \quad (12)$$

In reaction (7) semiquinone free radical production may occur enzymically (Gianni *et al.*, 1983) or it may occur by adriamycin self-reduction (Gianni *et al.*, 1985; Gutteridge, 1984). Reaction (8) has been characterized by pulse radiolysis and is very fast in both directions, with second-order rate constants $k_{forward} = 2.7 \times 10^8\ M^{-1} \cdot s^{-1}$ and $k_{reverse} = 0.44 \times 10^8\ M^{-1} \cdot s^{-1}$ (Land *et al.*, 1983). At pH 7 reaction (9) is fast also, with a rate constant of $5 \times 10^5\ M^{-1} \cdot s^{-1}$ (Halliwell & Gutteridge, 1985). An upper limit of $10^5\ M^{-1} \cdot s^{-1}$ has been placed on reaction (10) and measured directly for several other Fe^{3+} complexes (Butler *et al.*, 1985). The reaction of Fe^{2+} with H_2O_2 (Fenton reaction) to produce $OH^{\cdot}$ is slow, with $k = 76\ M^{-1} \cdot s^{-1}$ (Halliwell & Gutteridge, 1985). However, it is possible that the production of $OH^{\cdot}$ by reaction (12) may occur in two steps, as Rush & Koppenol (1986) have shown that H_2O_2 reacts quickly with Fe^{2+}–EDTA ($k = 1.8 \times 10^4\ M^{-1} \cdot s^{-1}$) to produce what may be a ferryl oxidizing intermediate (FeO^{2+}–EDTA), which then reacts with H_2O_2 again ($k = 3 \times 10^3\ M^{-1} \cdot s^{-1}$) to produce $OH^{\cdot}$. If reactions (10)–(12) can occur while Fe^{3+}–adriamycin is bound to cytochrome *c* oxidase the very reactive strongly oxidizing $OH^{\cdot}$ would be produced *in situ*, where it could damage the adjacent cardiolipin or haem *a*. The $OH^{\cdot}$ is so reactive (Halliwell & Gutteridge, 1985) that if it were produced free in solution it would very quickly be scavenged before it could damage the cytochrome *c* oxidase. An alternative mechanism might involve reactions (7), (8), (10) and (11) followed by formation of the strongly oxidizing perferryl ion (Aust & Svingen, 1982):

$$Fe^{2+}\text{–}ADM + O_2 \rightarrow Fe^{2+}O_2\text{–}ADM \rightleftharpoons Fe^{3+}O_2^-\text{–}ADM$$

which could initiate cardiolipin peroxidation directly without requiring H_2O_2 produced by reaction (9). Further work is required to distinguish among these and other mechanistic possibilities.

This work was supported in part by grants from the Natural Sciences and Engineering Research Council (Canada). A gift of adriamycin from Adria Laboratories is gratefully acknowledged.

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Received 19 May 1987/18 September 1987; accepted 16 November 1987