Characterization of the Cycle of Iron-mediated Electron Transfer from Adriamycin to Molecular Oxygen

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The anticancer drug adriamycin binds iron and these complexes cycle to reduce molecular oxygen (Zweier, J. L. (1984) J. Biol. Chem. 259, 6056–6058). Optical absorption, EPR, and Mössbauer spectroscopic data are correlated with polarographic O₂ consumption and chemical Fe²⁺ extraction measurements in order to characterize each step in this cycle. Fe³⁺ binds to adriamycin at physiologic pH forming an optical absorbance maximum at 600 nm. EPR signals at g = 4.2 and g = 2.01, and a doublet Mössbauer spectrum with isomer shift δ = 0.57 mm/s and quadrupole splitting ΔE₀ = 0.74 mm/s are observed indicating that the Fe³⁺ bound to adriamycin is high spin S = 3/2. Under anaerobic conditions the absorbance maximum at 600 nm decreases with an exponential decay constant = 0.77 h⁻¹ and the EPR and Mössbauer spectra of Fe³⁺-adriamycin similarly decrease as the Fe³⁺ is reduced to EPR silent Fe⁴⁺. The Fe⁴⁺-adriamycin complex which is formed exhibits a Mössbauer spectrum with δ = 1.18 mm/s and ΔE₀ = 1.82 mm/s indicative of high spin Fe⁴⁺. As the EPR spectra of Fe⁴⁺-adriamycin decrease on reduction of the Fe⁴⁺ to Fe⁵⁺ a signal of the oxidized adriamycin free radical appears at g = 2.004 with line width of 8 G. On exposure to O₂ the absorption maximum at 600 nm, the Fe⁵⁺ EPR, and the Fe⁵⁺ Mössbauer spectra all return. Polarographic measurements demonstrate that O₂ is consumed and that H₂O₂ is formed. Additon of high affinity Fe⁵⁺ chelators block O₂ consumption indicating that Fe⁵⁺ formation is essential for O₂ reduction. This cycle of iron-mediated O₂ reduction can explain the formation of the reactive reduced oxygen and adriamycin radicals which are thought to mediate the biological activity of adriamycin.

Adriamycin is known to chelate iron. Step association constants of 10⁻¹⁸, 10⁻¹⁴, and 10⁻¹⁰ have been estimated for the association of the first, second, and third adriamycin, respectively, with Fe³⁺ yielding an overall association constant of 10⁻³¹ (10).

In the past we have shown that adriamycin, through chelation of Fe³⁺, can function as a catalyst for O₂ reduction by the physiologic reducing agents glutathione and cysteine (6, 7). Activation of molecular O₂ to radical species is a feature common to several iron-chelates, but the reaction catalyzed by Fe³⁺-adriamycin appears unusual because the complex binds tightly to erythrocyte-glucose membranes and catalyzes their destruction in the presence of glutathione (6). More recently, we have shown that Fe⁵⁺-adriamycin is also able to bind to and cleave double-stranded DNA (7). In both sets of experiments, we observed that the chelate formed by adriamycin and Fe⁵⁺ catalyzes destruction of the erythrocyte ghosts or DNA in the absence of reducing agents. In the case of DNA, this activity is completely blocked by addition of catalase, suggesting that H₂O₂ had been generated and is a critical intermediate in DNA cleavage. The generation of H₂O₂ under these circumstances suggests that iron-adriamycin is able to reduce molecular oxygen in the absence of added electron donors such as the thiols. In support of this hypothesis, it has been reported that a complex of Fe⁵⁺-ADP and adriamycin can reduce ferricytochrome c in the absence of O₂ (8). Finally, EPR studies of the iron-adriamycin complexes suggest that adriamycin reduces its bound Fe⁵⁺ to Fe⁴⁺ with subsequent electron transfer to molecular oxygen (5).

In the present study we definitively demonstrate that the iron-adriamycin complexes cycle to reduce O₂. By correlating optical absorbance, electron paramagnetic resonance, and Mössbauer spectroscopic data with polarographic O₂ consumption and chemical Fe⁵⁺ extraction measurements each step in the cycle of O₂ reduction is characterized.

EXPERIMENTAL PROCEDURES

Materials

Adriamycin hydrochloride (>98% pure by HPLC) was supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute or purchased from Aldrich. Bathophenanthroline disulfonic acid disodium salt hydrate, ultrapure NH₄FePO₄, acetohydroxamic acid, and ferrous ammonium sulfate betahydrate (99.999%) were purchased from Aldrich. Ferric-acetohydroxamic acid was prepared daily by dissolving 3 molar eq of the chelating agent in doubly distilled water and adding 1 molar eq of solid FeCl₃·6H₂O (ACS grade, Allied Chemicals). For EPR experiments a ferric chloride standard solution was prepared by the methods of Aisen et al. (11). For the Mössbauer

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† The abbreviations used are: HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BPS, bathophenanthroline; AHA, acetohydroxamic acid.
experiments a similar $^{57}$FeCl₃ solution was prepared from 97% isotopically pure $^{57}$Fe metal purchased from New England Nuclear. All other reagents were of the highest quality commercially available. Doubly distilled water was used in all experiments.

Methods

Optical Studies—Optical absorption spectra were obtained with a Hewlett-Packard UV/Vis spectrophotometer model 8450A, which is equipped with microprocessor and storage memory that allows the recording and storage of spectra from 200 to 800 nm each second. The anaerobic time course of the reaction of adriamycin with Fe$^{3+}$ was performed in a quartz anaerobic cuvette equipped with a side arm stoppered with a silicon rubber gas chromatography septum. Four ml of buffered adriamycin were degassed according to previously published techniques (6). Stock ferric acetohydroxamate was added through a gas-tight 10-μl syringe to reach the final concentration desired.

EPR Experiments—Fe$^{2+}$-adriamycin was prepared by dissolving the desired amount of the drug in doubly distilled water, mixing the desired amount of FeCl₃, and then titrating the pH from the initial value of approximately 2.5 to the desired pH with 0.05 M NaOH. Titration of the pH by addition of 50 mM Hepes, 0.1 M KCl buffer at pH 7.4 yielded spectral results identical to those obtained with NaOH. Anaerobic Fe$^{2+}$- or Fe$^{3+}$-adriamycin complexes were prepared either in vacuum or in 100% nitrogen atmosphere as described previously (6). EPR spectra were recorded with a Varian E-9 spectrometer operating at X band using 100 kHz modulation frequency and a TE 102 cavity. EPR spectra were obtained over the temperature range 10-100 K using an Air Products variable-temperature apparatus with EPR dewar insert. Spectra were also obtained at 77 K using a liquid nitrogen EPR dewar. The microwave frequency and magnetic field were calibrated using techniques similar to those described previously (12).

Mössbauer—Anaerobic complexes were prepared by purging with nitrogen gas in a specially constructed glass apparatus fused to a transfer tube which connects to a lucite Mössbauer cell. After the desired time the anaerobic sample was poured through the transfer tube into the Mössbauer cell which was then immediately frozen in liquid nitrogen. After freezing the seal between the glass transfer tube and the Mössbauer cell was opened allowing insertion of the cell into the Mössbauer dewar.

Transmission Mössbauer spectra were obtained using a 512-channel spectrometer operated in a constant acceleration mode. A 75-reCi source of $^{57}$Co diffused into a Rh matrix in conjunction with a Jannis flow cryostat were employed to carry out the measurements at the various temperatures. The theoretical fit of the data was performed assuming a lorentzian shape for the absorption lines, and the isomer shifts (δ) are given relative to metallic iron at room temperature.

Liquid Chromatographic Experiments—Solutions of Fe$^{3+}$ and adriamycin were prepared under anaerobic conditions as described for the optical spectroscopy studies. The reaction was monitored spectroscopically for loss of absorbance at 600 nm. After 1.5-h incubation, iron in Fe$^{3+}$ was then extracted according to a modified method (13). This technique depends upon the fact that the Fe$^{2+}$-phenanthroline complex is stable in the presence of oxygen. Four hundred μl of 0.01 M 4,7-diphenyl-1,10-phenanthroline (Sigma) in 95% ethanol was added quickly with rapid mixing. The solution had a final pH of 2.5 and was extracted by mixing with 4.4 ml of chloroform/methanol (4:1, v/v). At this pH, only the aglycon forms of adriamycin and the Fe(II)-phenanthroline chromogen can be extracted. The organic phase was collected and dried under a stream of N₂, resuspended in 2 ml of methanol, and 10-μl aliquots were analyzed by HPLC. The liquid chromatographic analysis of the samples was performed by automatic injection into an HPLC system fitted with a Shoefield UV/Via detector model GM 770 and a Shoefield fluorimetric detector model 970. Simultaneous analysis by detection of absorption at 535 nm through the side arm of the anaerobic cuvette. The cuvette was opened, and 3.1 ml of a degassed solution containing 10% NH₄H₂PO₄, 4,7-phenyl-1,10-phenanthroline, and 1 M HCl (20:8:0.3, v/v/v) was added quickly with rapid mixing. The solution had a final pH of 2.5 and was extracted by mixing with 4.4 ml of chloroform/methanol (4:1, v/v). At this pH, only the aglycon forms of adriamycin and the Fe(II)-phenanthroline chromogen can be extracted. The organic phase was collected and dried under a stream of N₂, resuspended in 2 ml of methanol, and 10-μl aliquots were analyzed by HPLC. The liquid chromatographic analysis of the samples was performed by automatic injection into an HPLC system fitted with a Shoefield UV/Via detector model GM 770 and a Shoefield fluorimetric detector model 970. Simultaneous analysis by detection of absorption at 535 nm and by fluorescence (excitation 229 nm; emission 580 nm) was performed. Samples were run isocratically at 2 ml/min using a Waters RCM-100 siliconized column and a mobile phase containing 85% CH₃CN and 15% of a 0.2% solution of ammonium hydroxide adjusted to pH 4 with formic acid. The 4,7-phenyl-1,10-phenanthroline was eluted as a single peak at 8 min under these conditions. A standard curve for quantitation was constructed from a stock of Fe(Ⅲ)H₂SO₄ extracted as above and serially diluted in methanol in the range of concentrations of 1 to 100 μM. The correlation between the area under the peak eluted at 8 min, and concentration was linear with r > 0.99. The same samples described above were analyzed by HPLC according to a previously described method for adriamycin and its metabolites. The analysis showed that less than 5% of the total drug present was extracted as the aglycon plus a poorly resolved peak with the same retention time as adriamycin.

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Optical spectra recorded during the course of the anaerobic reaction of Fe$^{3+}$ with adriamycin and changes after addition of oxygen. Four ml of the 0.01 μM adriamycin in 50 mM Tris-HCl (pH 7) was degassed as described (see "Methods"). Panel A, after Fe$^{3+}$ addition the 600-nm absorption band appears. At time 0, 10 μl of 10 mM Fe(AHA)$_2$ was added to the anaerobic cuvette and spectra recorded every 20 s for the first 3 min. Dashed line, t = 0; lower solid line, at 600 nm t = 20 s; solid upper line, t = 40 s-3 min. Panel B, the absorption at 600 nm gradually decreases. Dashed line, t = 3 min; solid lines from highest to lowest, at 600 nm t = 18, 33, 48, 55, 68, and 90 min, respectively. Panel C, after O₂ addition the 600-nm absorption band returns. O₂ was introduced as 200 μl of air equilibrated buffer (25°C) at t = 0. Spectra from lowest to highest: dashed line, t = 0; solid lines, t = 30, 60, 90, 120, and 150 s and 18 min, respectively.
Polarographic Experiments—O₂ consumption studies were performed with a Clark-type electrode (Yellow Spring Instruments) on a Gilson model 5/6 Oxygraph equipped with a water-jacketed cell for temperature control. Superoxide dismutase (Sigma, type I from bovine blood) was used without further purification. Catalase (Sigma, from bovine liver, 2 × crystallized) was pretreated by mixing with a slurry of Chelex 100 resin in water at 4 °C for 30 min. This preparative step resulted in higher activity of the enzyme and more reproducible results. Control experiments were performed with enzymes that had been boiled for 10 min. The different buffer solutions were equilibrated with air at 37 °C and assumed to contain the same concentration of dissolved O₂ as water (207 μM at 37 °C). The oxygraph was calibrated daily with a solution of water equilibrated with air for 2 h at 37 °C. All experiments were performed in the dark.

Polarographic studies were performed with ferric-acetohydroxamate as the source of Fe⁶⁺ for adriamycin. Control experiments were performed with FeCl₃ to rule out possible artifacts due to hydroxamic acid. No significant differences were observed, and ferric-acetohydroxamate was routinely used because it results in a lesser degree of variability at neutral pH.

Calculations and Computer Fitting—Equations were fitted to the data using the MLAB fitting routine program. The rate constant for the 600-nm absorption decay of the anaerobic Fe³⁺-adriamycin complex was calculated by least squares fit to the equation,

\[ a = a_0 e^{-Kt} \]

where \( a \) is the measured absorbance at time \( t \); \( a_0 \) the extrapolated absorbance value at time 0; and \( K \) the calculated rate constant. The pH titration curves of O₂ consumption were similarly fitted to the Henderson-Hasselbalch equation.

RESULTS AND DISCUSSION

The anticancer drug adriamycin forms complexes with Fe³⁺ over the physiologic pH range, pH 6.5–8.5, which give rise to distinctive optical absorption, EPR, and Mössbauer spectra. On addition of Fe³⁺ to adriamycin a new absorption band centered at 600 nm appears along with hypochromicity of the main peak at 480 nm (Figs. 1A and 2). These complexes give rise to EPR spectra at 77 K with signals at \( g = 4.2 \) and \( g = 2.01 \) (Fig. 3). Over the temperature range from 10–100 K the EPR spectra are similar to those observed at 77 K. No new signals are observed on lowering the temperature to 10 K. Mössbauer spectra of these complexes at 80 K exhibit a single absorption doublet with an isomer shift of \( \delta = 0.57 \pm 0.02 \) mm/s and a quadrupole splitting of \( \Delta E_Q = 0.74 \pm 0.02 \) mm/s (Fig. 4A).

Both the temperature behavior of the EPR spectrum and the isomer shift values of the Mössbauer spectra indicate that the Fe³⁺ added to adriamycin is bound as high spin Fe⁶⁺, \( S = 5/2 \) (14).

It has previously been determined from optical absorbance titrations of Fe³⁺ versus adriamycin that the Fe⁶⁺-adriamycin complex has a stoichiometry of 1:3 (6). In the presence of an excess of adriamycin the spectra of aerobic Fe⁶⁺-adriamycin complexes remain unchanged. The optical, EPR, and Mössbauer spectra of complexes with an Fe⁶⁺-adriamycin ratio of 1:10 are unchanged after 6 h under aerobic conditions at room temperature. Under anaerobic conditions, however, the spectra of the Fe⁶⁺-adriamycin complexes dramatically change as a function of time. As shown in Figs. 1A and 2 the addition of Fe⁶⁺ to an anaerobic solution of adriamycin at pH 7.7 results in the appearance of an absorbance at 600 nm that reaches a maximum in 30 s. After the first 3 min, however, the intensity of the absorbance at 600 nm progressively declines over the next 1.5 h, following a monoexponential decay with an apparent rate constant of 0.77 h⁻¹ (Figs. 1B and 2). After 2 h the optical absorption spectra appear identical to that of anaerobic preparations of Fe⁵⁺-adriamycin (Fig. 5). Introduction of O₂ rapidly restores the 600-nm absorbance to its initial value (Figs. 1C and 2). The EPR spectra of anaerobic complexes of Fe⁶⁺-adriamycin also decrease as a function of time (Fig. 3). As reported previously Fe⁵⁺-adriamycin is EPR silent, so the decrease in the Fe⁵⁺-adriamycin spectra suggest the reduction of the bound Fe⁶⁺ to Fe⁵⁺ (5). A sharp signal appears at \( g = 2.004 \) and increases as the Fe⁵⁺ signals decrease (Fig. 3). The signal at \( g = 2.004 \) has a line width of only 8 G, and it is superimposed on the much broader 225-G line width Fe⁵⁺ signal at \( g = 2.01 \). The line width and \( g \) value of this signal suggest that it is due to a free radical. This signal
appears to correspond to the oxidized adriamycin free radical which is formed as adriamycin reduces its bound Fe^{3+} to Fe^{2+}. On re-exposure to O_2, the Fe^{2+} EPR spectra reappear, and the free radical signal disappears with both of these spectral changes completed within 5 min. Under anaerobic conditions the Mössbauer spectrum of Fe^{3+}-adriamycin also decreases as a function of time. After incubating the sample under anaerobic conditions for 3 h one can notice the emergence of an absorption peak in the high velocity range accompanied by a decrease in the intensity of the higher velocity peak of the original doublet (Fig. 4B). Analysis and simulation of this spectrum indicates that the observed change is due to a decrease in the intensity of the original doublet with the formation of a new component which contributes a doublet with an isomer shift $\delta = 1.18 \pm 0.02$ mm/s and quadrupole splitting $\Delta E_q = 1.82 \pm 0.02$ mm/s. These parameters are characteristic of Fe^{2+}, $S = 2$ (14), which indicates that the Fe^{3+} with $S = 5/2$ is reduced to high spin Fe^{2+}, $S = 2$. On exposure to O_2, the Fe^{2+} doublet decreases with a corresponding reappearance of the original Fe^{3+} doublet (Fig. 4C). After 5 min the reaction is completed and only the Fe^{3+} doublet remains.

In order to chemically confirm the reduction of Fe^{3+} by the drug, we tested the ability of the specific Fe^{2+} chelator 4,7-diphenyl-1,10-phenanthroline to compete with and displace iron from adriamycin after an anaerobic incubation of the ferric chelate of the drug. Because the drug and Fe^{2+}-phenanthroline absorb in the same region, we extracted the Fe^{2+} complex of phenanthroline with CHCl_3-CH_3OH (4:1, v/v). Fig. 6 shows the results of the HPLC analysis of the organic extract obtained after a 1.5-h anaerobic incubation of adriamycin with Fe^{3+}. Panel A of the figure shows that a Fe^{2+}-phenanthroline standard eluted as a single symmetric peak absorbing at 535 nm. The sample in which adriamycin and Fe^{3+} had been incubated anaerobically yielded a peak with the same retention time (panel B). Identical results were obtained on aerobic incubation. Simultaneous analysis by fluorimetric detection showed that less than 5% of the drug had been extracted in the organic phase and eluted from the column with the solvent front. Quantitation through the use of an external standard curve indicated that all the iron initially added as Fe^{3+} had been reduced to Fe^{2+}. Panel C shows that a solution of ferric acetylhydroxamate, when assayed for the presence of Fe^{2+}, yielded minimal amounts of phenanthroline-iron. Similar amounts of Fe^{2+} (1 $\mu$M) were found in buffer alone (Fig. 6D) or adriamycin alone (data not shown).

Because of the reaction with O_2 elucidated by the spectroscopic studies, one would expect the reaction of Fe^{3+} with adriamycin to be associated with O_2 consumption. Measurement of O_2 concentration by polarography did in fact show that addition of Fe^{3+} to adriamycin triggered the consumption of O_2. At pH 7.4 and 0.2 mM adriamycin, the addition of 0.5 eq of Fe^{3+} resulted in a rate of 2.4 $\mu$mol/liter/min. In addition, the rate of O_2 consumption was a function of adriamycin concentration yielding a linear double reciprocal plot (Fig. 7). As in the case of ferricytochrome c reduction by adriamycin-Fe^{3+}-ADP (8), the rate also progressively increased at more basic pH. The effect of pH on the rate of oxygen consumption
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**Fig. 6.** Evidence for Fe$^{2+}$ presence in anaerobic solutions of Fe$^{3+}$-adriamycin. Panel A represents the peak of standard Fe$^{3+}$-4,7-diphenyl-1,10-phenanthroline in 10 µl of 25 µM solution. Panel B is the peak present in the sample extracted from an anaerobic solution of adriamycin (100 µM) and ferric acetohydroxamate (25 µM) after 1.5 h of reaction at pH 7.4. The apparent rate constant in this experiment was 0.94 h$^{-1}$. The peak corresponded to a concentration of 27 µM Fe$^{3+}$. Panels C and D are the peaks present in samples of ferric acetohydroxamate (25 µM) and buffer alone, with peak height which corresponds to 1 and 1.22 µM, respectively, of Fe$^{3+}$.

**Fig. 7.** Oxygen uptake as a function of adriamycin concentration. The triangles represent the titration of 200 µM Fe(AHA)$_3$ with adriamycin. The points represent the mean of two consecutive experiments. Each experiment was performed by adding stock adriamycin to the desired final concentration in barbital buffer (pH 8.5, 0.1 M) equilibrated with air at 37 °C. The rate after addition of the desired amount of Fe(AHA)$_3$ was recorded. The final volume in the oxygraph cell was 2 ml. The inset shows the same data values grouped as the double reciprocal.

by both adriamycin and its iron complex is shown in Fig. 8. The presence of iron shifted the apparent pK from 9.65 to 7.95. Thus, in the physiologic pH range the Fe$^{3+}$-adriamycin complex reduces O$_2$ while adriamycin alone does not. The central role of Fe$^{3+}$ reduction in the reaction leading to O$_2$ consumption is clearly indicated by the experiment shown in

**Fig. 8.** Effect of pH on the rate of oxygen consumption by adriamycin and the iron-adriamycin complex. The solid triangles represent the results obtained with addition of 200 µM adriamycin. The results shown in the open circles represent the rate obtained after the addition of 200 µM adriamycin and 100 µM Fe(AHA)$_3$ minus the rate for adriamycin alone at each pH. From pH 7 to 9, the buffer used was barbital-HCl, 0.1 M. From pH 9–12, the buffer used was glycine-NaOH, 0.1 M. The solid lines represent a least squares fit of the data to the Henderson-Hasselbach equation. The apparent pK was 7.95 for the drug-iron complex and 9.65 for adriamycin.

**Fig. 9.** Effect of BPS on oxygen uptake by Fe$^{3+}$-adriamycin (Dox-Fe(III)). The oxygraph tracings were obtained in Tris-HCl buffer (pH 8.5, 50 µM). The concentrations used in the experiment were 200 µM adriamycin, 100 µM Fe(AHA)$_3$, and 300 µM BPS.
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Experiments were performed in duplicate in Tris buffer at pH 8.5. Adriamycin-Fe(III) was obtained by adding ferric acetohydroxamate, anthroline sulfonate (bathophenanthroline, BPS) blocked peroxo-like form is susceptible to nucleophilic displacement prompted an analysis of the enzyme effects shown in Table I. 

| Additions          | Rate (µmol O₂/liter/min) |
|--------------------|--------------------------|
| Complex            |                          |
| + Catalase         | 6.67                     |
| + Boiled catalase  | 6.74                     |
| + Superoxide dismutase | 5.58                   |
| + Boiled superoxide dismutase | 5.11            |
| + Superoxide dismutase + catalase | 4.17          |

Fig. 9. Addition of the water-soluble 4,7-diphenyl-1,10-phenanthroline sulfonate (bathophenanthroline, BPS) blocked O₂ consumption either before or after the reaction with the complex was initiated. The known respective affinities of adriamycin and BPS for Fe³⁺ rule out the possibility that, at the concentrations used (see legend to Fig. 9), the effect of BPS could be due to successful competition with adriamycin for ferric iron. 

Fe³⁺ chelates can either react with O₂ to yield superoxide and H₂O₂ or bind O₂ in a superoxo- or peroxo-like form (15). In some systems, O₂ coordinated to iron in a superoxo- or peroxo-like form is susceptible to nucleophilic displacement (16). In the case of adriamycin (200 µM), however, the addition of excess of NaN₃ (10 mM), NaCN (10 mM), or NaCl (100 mM) with respect to iron did not inhibit O₂ consumption by Fe³⁺-adriamycin. To rule out the possibility that O₂ might still be bound to iron but displaceable as either superoxide or H₂O₂ rather than O₂, nucleophile addition was repeated at the same concentrations used above, but now in the presence of superoxide dismutase and catalase. The introduction of the two enzymes (superoxide dismutase, 120 units/ml; catalase, 400 units/ml) into the oxygraph cell resulted in the immediate evolution of O₂ that was, however, unrelated to the presence or absence of the nucleophiles. The latter observation prompted an analysis of the enzyme effects shown in Table I. Catalase caused a 35% drop in O₂ consumption. Since catalase converts each 2 eq of H₂O₂ to 1 eq of O₂, this indicates that H₂O₂ can account for 70% of the O₂ consumed. The initial rates of O₂ uptake were affected to the same extent by addition of catalase alone or catalase and superoxide dismutase. Superoxide dismutase alone did not appear to have any measurable specific effect, but boiled superoxide dismutase did decrease the rate of O₂ consumption. This surprising decrease could be due to the release of copper and zinc ions which interfere with iron-mediated O₂ reduction, or the denatured protein itself may bind the drug-iron complex altering the rate of O₂ consumption. From the above experiments, it appears that the observed O₂ consumption is not due to the formation of a stable adriamycin-iron-O₂ complex between O₂ and the drug-iron chelate, but to a reaction that yields H₂O₂ as the predominant product.

The present investigation demonstrates that iron binds to adriamycin and that these complexes cycle to reduce molecular O₂. Optical absorption, EPR, and Mössbauer spectroscopic studies as well as Fe³⁺ chemical extraction experiments all indicate that the Fe³⁺ bound to adriamycin is reduced to Fe²⁺. The isomer shift values observed in the Mössbauer spectrum indicate that the Fe²⁺ formed is high spin S = 2. The EPR experiments demonstrate that an oxidized adriamycin free radical is formed as Fe²⁺ is reduced to Fe³⁺. An unusual aspect of this reaction sequence is the slow rate of Fe³⁺ reduction, as indicated by the apparent rate constant of the spectral shift seen in Fig. 2. This behavior is confirmed by the EPR and Mössbauer experiments (Figs. 3 and 4) that show a similar slow rate for the disappearance of the Fe³⁺ signals. This observation is unusual because electron transfer reactions are fast processes, as in the case of Fe³⁺-thiol complexes (17). The rapidity of the subsequent reaction (Fig. 2) after O₂ addition suggests that the Fe³⁺ reduction is the rate-limiting step in the reaction sequence. This is further corroborated by the ability of BPS to immediately stop the O₂ uptake by the drug-metal chelate. Perhaps the closest precedent in the chemical literature for these observations is the reaction of O-quinones and catechols (o-hydroquinones) with iron. The recent report of phenanthrenequinone-phenanthrenechelate complexes is of particular interest. These complexes can exhibit either strong apparent charge transfer bands without Fe³⁺ reduction or, in contrast, full reduction of Fe³⁺ with formation of a radical semiquinone ligand depending upon the dielectric strength of the solvent (18). Parallel detailed studies do not appear to have been done on hydroquinones analogous to adriamycin. However, this study indicates that the Fe³⁺-adriamycin complex at neutral pH also exhibits both a strong charge transfer band and slow electron transfer from the ligand to the iron.

The second step in this reaction cycle is the reduction of oxygen accompanied by oxidation of the ferrous iron. Our polarographic experiments, Table I, indicate that 70% of the O₂ consumed is transformed to H₂O₂. Recently we have shown that H₂O₂ reacts with the complex leading to the oxidative cleavage of DNA. The latter suggests the complication that H₂O₂ is also a transient product able to react with the iron-chelate of adriamycin. Thus, while it is clear that H₂O₂ is a product, the formation of other reactive O₂ species, such as hydroxyl radical, may also occur. As a result of electron transfer to molecular oxygen Fe³⁺-adriamycin is regenerated, and in the presence of excess adriamycin this cycle of O₂ reduction will continue.

In recent years, oxidative damage to macromolecular targets has been advocated as a mechanism responsible for the cytotoxicity and/or the cardiac toxicity of adriamycin (2-4). The cycle of iron-mediated electron transfer from adriamycin to molecular oxygen results in the formation of reduced oxygen and oxidized adriamycin radicals which may mediate the therapeutic and toxic effects of the drug.

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