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Oxidative Destruction of Erythrocyte Ghost Membranes Catalyzed by the Doxorubicin–Iron Complex†

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ABSTRACT: Doxorubicin was shown to form metal complexes with both ferrous and ferric ions. Formation of the ferrous ion complex was characterized by a shift in the absorption maximum of doxorubicin from 479 to 508 nm. In the presence of air, this ferrous ion complex rapidly oxidized, resulting in a product with lowered absorption in the 470–510-nm range and development of an intense absorption band in the 580–610-nm range. A complex with spectral properties identical with those of this oxidized complex formed directly upon addition of ferric ion. The ferric ion was shown to accommodate up to three doxorubicins and to be able to compete with iron bound to acetohydroxamic acid. The 3:1 doxorubicin–iron complex was shown to have a molar extinction coefficient of 11 400 at 600 nm, pH 7.4. The doxorubicin–ferric ion complex catalyzed the reduction of oxygen by both cysteine and glutathione, producing superoxide and hydrogen peroxide. This catalysis was shown to be consistent with Michaelis–Menten kinetics. The apparent K_m for oxygen under these conditions with glutathione as a thiol was 280 μM as compared with 191 μM with cysteine as a thiol. Conversely, the apparent K_m for glutathione was 20 mM as compared to 1 mM for cysteine. Analysis by high-pressure liquid chromatography showed no alteration in the doxorubicin where the reaction was allowed to proceed to completion. The doxorubicin–ferric ion complex was shown to bind to human erythrocyte ghost membranes and cause efficient destruction of these ghosts in the presence of glutathione. This damage was shown to depend upon the production of both superoxide and hydrogen peroxide by the complex. Mannitol partially (50%) inhibited complex-mediated ghost destruction, suggesting a role for the hydroxyl radical. On the basis of these observations, a model is proposed in which the destruction of erythrocyte ghosts depends on the ability of the complex to first bind to the ghost membrane and then generate high local concentrations of reactive oxygen species.

Several recent observations have focused attention on the interaction of doxorubicin and iron. Thus, May et al. (1980) have shown that doxorubicin can bind iron in drug–iron ratios of 1:1, 2:1, and 3:1. In that study, done at pH 3, the affinity of doxorubicin for iron appeared impressive, with step association constants of 10^{18}, 10^{11}, and $10^{4.4}$, respectively, for the association of the first, second, and third doxorubicin with ferric ion. This results in an overall association or β constant of $10^{33.4}$. Sugioka et al. (1981) have reported that iron–ADP–doxorubicin complexes can form and could be reduced by P450 reductase and in the process cause marked decomposition of unsaturated fatty acids. This decomposition was dependent upon oxygen and was partially blocked by catalase and tocopherol, suggesting the mediation by oxygen radical species. In this study, doxorubicin alone did not stimulate destruction of the unsaturated fatty acids. In a parallel in vivo study, Kappus et al. (1980) also showed marked lipid peroxidation with doxorubicin plus iron but not with either doxorubicin or iron alone. Finally, while Gosalvez et al. (1978) reported that the doxorubicin–iron chelate lacks cardiac toxicity, other workers have found that it yields increased cardiac toxicity as compared to doxorubicin alone (Young et al., 1978).

The results outlined above have led us to examine the behavior of doxorubicin–iron chelates in more detail. In the process, we have observed that these chelates do occur at physiologic pH and can act as redox catalysts without the need for enzymic activation as reported by Sugioka et al. (1981). Furthermore, the properties of the doxorubicin–iron complex are such as to offer an explanation for some of the observations discussed above.

Materials and Methods

Doxorubicin hydrochloride was provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Ferrous ammonium sulfate hexahydrate (99.999%) was obtained from Aldrich Chemical Co. All other chemicals were reagent grade or better and used as received. Glass-distilled water was used throughout the experiments. Absorption spectra were measured in aqueous solution (pH 7.4) at room temperature in quartz cuvettes with a Perkin-Elmer recording spectrophotometer (Model 556). Anaerobic conditions were obtained by using previously published techniques (Burleigh et al., 1969) except that the solutions were purged alternating with argon and vaccum rather than with nitrogen and vacuum.

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Unless otherwise stated, doxorubicin–ferric complex was prepared from a 2 mM solution of doxorubicin by combining equal volumes of 1 mM ferric ion and doxorubicin at pH 3. The pH of this solution was then slowly adjusted to pH 7.4 with 0.05 N NaOH. The spectrum of this complex was obtained with a 1:10 dilution of the solution with 0.1 M cacodylate buffer, yielding a final total doxorubicin concentration of 0.1 mM and 0.05 mM iron.

Oxygen consumption studies were performed with a Clark type electrode (Yellow Spring Instruments No. 5331) on a Gilson Model 5/6 oxygraph. Doxorubicin–ferric ion complex was prepared as specified previously or with the ferric ion–acetohydroxamic complex as an iron donor. For reasons discussed under Results, most of the experiments were performed by using complexes made with the acetohydroxamic acid acting as an iron source. The native pH of the complex prepared with the acetohydroxamic acid complex was not adjusted before addition to the cell of the oxygraph. In the final range of concentrations used (1.5–15 mM), glutathione dissolved in distilled water and adjusted to pH 7.4 with 1 mM NaOH had enough buffering capacity to maintain the pH of the solution in a range between 7.2 and 7.3 throughout the reaction. This allowed us to avoid any effects from buffer anions, many of which can react with iron chelates. For the above-mentioned reasons, all of the experiments with glutathione were performed in glass-distilled water without added buffer. The distilled water was aerated by equilibration with air for 2 h. In experiments with other reducing agents of weaker or absent buffering capacity, 20 mM acetate–barbital buffer at pH 7.3 was used.

The experiments were performed according to the following procedure: 1.7 mL of glass-distilled water or buffer was put into the 2.0-mL cell of the oxygraph, and the cell was sealed with a capillary bore stopper. After 3 min, glutathione, cysteine, NADPH, or NADH as electron donors was added through the capillary in a 0.1-mL volume. After an equilibrium delay of 5 min, the reaction was started with either the drug–metal ion complex or the control reagents added in an 0.2-mL volume. In the experiments with superoxide dismutase or catalase, the enzymes were added in an 0.025-mL volume to minimize the dilution error in the reaction cell. The final reaction mixture concentrations were 0.1 mM doxorubicin and 0.05 mM ferric ion from the acetohydroxamic acid complex.

For the purposes of calculation, the 2-mL reaction mixture was assumed to contain the same amount of dissolved oxygen as plain water (251 μM at 25 °C). The actual concentration of reduced thiols in the stock solutions was measured by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reaction. The same method was applied to measure the reduced thiols in the reaction cell at various times during the reaction.

Preparation of resealed ghosts was done as previously described (Simone & Henkart, 1980). Fresh washed human red cells were trinitrophenyl (TNP) modified by reaction with 1.0 mM trinitrobenzenesulfonic acid in phosphate-buffered saline (PBS) at pH 7.4 for 15 min at 37 °C. After being washed, the packed cells were resuspended with an equal volume of 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffered saline, pH 7.4 (BSS–Hepes). One volume of this cell suspension was added to 10 volumes (20 mL total) of ice-cold $MgSO_4$ buffer (4.0 mM $MgSO_4$–3.5 mM acetic acid adjusted with NaOH to pH 6.2) maintained at 0 °C and pH 6.2. After 5 min, 1.2 volumes of a 10× stock of BSS divalent ions and 1.2 volumes of a 10× stock of PBS monovalent ions plus 0.1 M Hepes were added, thus raising the pH to 7.4. The hemolysate was incubated at 37 °C for 1 h to reseal the ghosts. The ghosts were then washed 3 times with BSS–Hepes by using a Sorval centrifuge SS34 rotor at 15000 rpm for 10 min at 0 °C.

A monolayer of ghosts was made in flat-bottom microliter wells which were first treated with 25 μL of sheep anti-TNP $F(ab')_2$ (1 mg/mL in PBS) at room temperature and washed with PBS. The ghost monolayer in each well contained about 7×10^5 ghosts. This adherent ghost monolayer formed the target for subsequent testing of the doxorubicin–iron complex membrane action. The wells were all refilled with 180 μL of BSS–Hepes and the desired reaction components added. The plate was incubated at 37 °C for 30 min and agitated on a Micro mixer (Cooke Laboratory Products, Alexandria, VA). Destruction of erythrocyte ghosts was quantitated by counting the number of ghosts per well before and after a 30-min incubation of the ghosts with the reaction components.

Binding of Doxorubicin–Iron to Red Cell Ghost Membranes. A pellet of ghosts prepared as previously described was resuspended and then hypotonically lysed again as described above. This preparation was then washed several times with phosphate buffer at pH 9.0 to remove as much additional hemoglobin as possible. The final hemoglobin concentration was less than 1% after four washings. From the final suspension of 2×10^6 ghosts/mL in phosphate-buffered saline, pH 7.2, 1.8-mL aliquots were pipetted in ultracentrifuge tubes. A 0.2-mL sample of doxorubicin–iron complex was then added to achieve a final concentration of 0.1, 0.5, and 1 mM. In a separate tube, 0.2 mL of 1 mM doxorubicin was added to measure binding due to doxorubicin alone. A control was run in which 0.2 mL of water was added to the ghost suspension in place of either complex or doxorubicin. After incubation for 60 min at 37 °C, the tubes were centrifuged at 15000*g* for 10 min, and the resultant pellets were washed twice with 5 mL of distilled water, centrifuged at 15000*g* for 10 min, and resuspended in 1 mL of 0.1% Triton X-100. Distilled water (4 mL) was added to each tube, and the ghosts were then sonicated for 3 min at setting 5 on a Branson sonicator. The absorption spectra of the final homogeneous suspensions were measured in quartz cuvettes of 1-cm path by using a Perkin-Elmer Model 556 double-wavelength double-beam spectrophotometer.

Results

Chelation of Iron by Doxorubicin. Previous papers dealing with iron chelation by doxorubicin have lacked experimental details on how to form the complex (Gosalvez et al., 1978) or reported difficulties in obtaining a chelate with reproducible properties (May et al., 1980). In order to clarify these problems, we have initially examined the following problems: (1) the spectra of the ferrous and ferric chelates; (2) the conditions for formation at physiologic pH of iron chelates with consistent properties; and (3) the stoichiometry of the complex which forms at physiologic pH. As a preliminary experiment, we added ferrous ammonium sulfate anaerobically to a 0.1 mM solution of doxorubicin (Figure 1). As is apparent, the addition of ferrous ion resulted in a shift of the absorption maximum from 479 to 508 nm. The cuvette was then opened to air and inverted to ensure mixing and the spectrum remeasured. Introduction of oxygen resulted in a marked loss in the absorption at 479 nm and the appearance of a band between 580 and 610 nm. These results suggested that a doxorubicin–ferrous ion complex can form and that it can react upon exposure to air. In order to study these spectral changes in more detail, a 0.1 mM aerated solution of doxorubicin was titrated with ferrous ion. The results, presented in Figure 2, show that formation of the oxidized complex is characterized

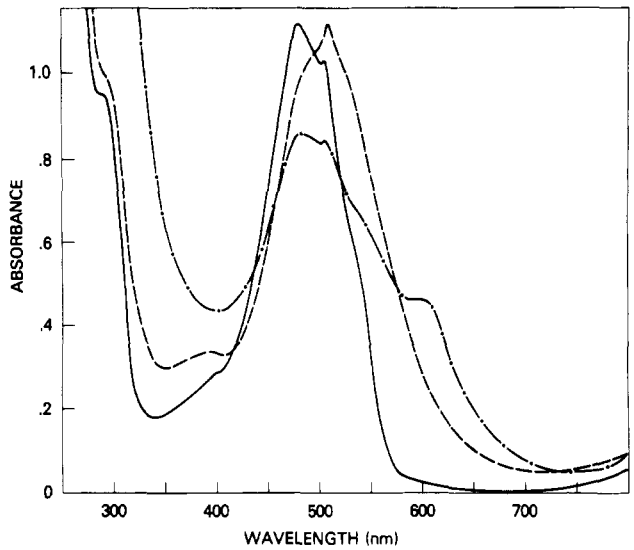

FIGURE 1: Interaction of ferrous ion with doxorubicin. The spectrum of doxorubicin (—) is compared with those of doxorubicin plus ferrous ion in the absence (- - -) and presence (-·-) of air. Doxorubicin concentration was 0.12 mM in 0.1 M sodium cacodylate buffer (pH 7.4). The ferrous ion was added as ferrous ammonium sulfate crystals to attain a final ferrous ion concentration of 0.12 mM.

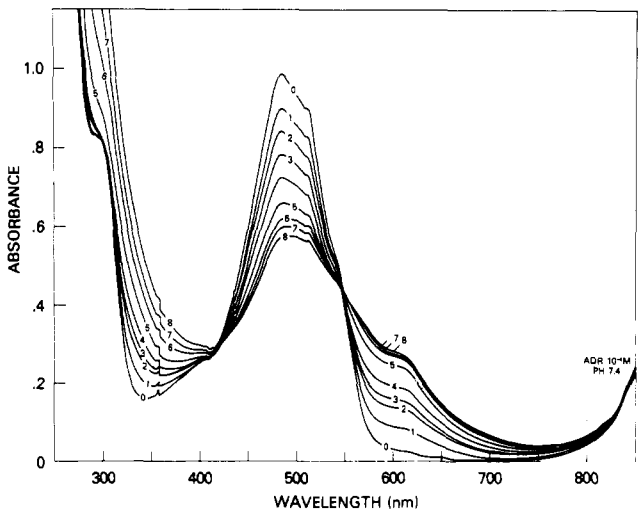

FIGURE 2: Titration of doxorubicin with ferrous ion. The doxorubicin concentration was 0.1 mM in 0.1 M sodium cacodylate buffer, pH 7.4. Ferrous ammonium sulfate solutions were prepared at 0.1 M in water and used within 15 min. This stock solution was added to the doxorubicin to give drug/metal ion ratios which ranged from 0.05 to 4: doxorubicin alone is represented by curve 0, 10:1 by 1, 5:1 by 2, 3:1 by 3, 2:1 by 4, 1:1 by 5, 1:2 by 6, 1:3 by 7, and 1:4 by 8. Ferrous ion can be expected to rapidly oxidize to ferric ion at the final cuvette concentrations used.

by the same loss in absorption at 479 nm and development of a pronounced shoulder between 580 and 610 nm similar to that seen in Figure 1 after the addition of air. Figure 2 also exhibits two clearly defined isosbestic points at 430 and 535 nm, suggesting the existence of only two absorbing species in solution, doxorubicin and the doxorubicin–iron complex. Identical spectral changes were obtained if doxorubicin and ferric ion were mixed at pH 3 and then brought to pH 7.4 with 0.05 N NaOH.

Ferric ion is unstable in aqueous solution at neutral pH and rapidly forms insoluble ferric hydroxide polymers (Spiro et al., 1966). We have found that the iron in such polymers is not readily available for chelation by doxorubicin and that the rapid formation of the ferric hydroxide polymers at physiologic pH made reproducible direct formation of a doxorubicin–ferric ion complex difficult. Also, the complex, once formed, was not stable for more than a few hours. We reasoned that these problems might be lessened if iron could be bound in a chelate which, while holding free iron to low levels, might also be able to donate iron to doxorubicin. In order to accomplish this, we evaluated the ferric ion–acetohydroxamic acid complex. The ferric ion–acetohydroxamic acid complex has itself been well characterized and has step constants for association of $10^{11.4}$, $10^{9.68}$, and $10^{7.23}$ (Brown et al., 1978). We have found no qualitative or quantitative difference in the spectral changes obtained by using freshly made ferrous ammonium sulfate or the acetohydroxamic acid complex. It is also important to note that the spectra obtained revealed the same two isosbestic points as were noted in Figure 2. This suggests that the complex formed from air oxidation of the doxorubicin–ferrous ion complex is the ferric ion–doxorubicin complex. The ability of doxorubicin to compete effectively with acetohydroxamic acid for ferric ion is consistent with their relative published association constants. In addition, the occurrence of two well-defined isosbestic points and the titration data presented show mixed complexes containing doxorubicin and acetohydroxamic acid do not occur to a significant degree under the conditions employed. The acetohydroxamic acid complex did make a much more stable iron source, yielding essentially identical results in experiments a month apart with the acetohydroxamic acid as the iron donor. We have found that both the ratio and slope methods (Harvey & Manning, 1950) (Figure 3A,B) give results consistent with an empirical formula of three doxorubicins to each ferric ion. Parallel titrations with ferric ion done according to the ratio method yielded plots which, although consistent with a 3:1 ratio, did exhibit considerable variability. Based upon this 3:1 stoichiometry and the data in Figure 3, it is possible to calculate a molar extinction coefficient for the complex at 600 nm of 11 400 for the 3:1 complex.

These results are consistent with the results of May et al. (1980), who found the maximum number of doxorubicins which can be accommodated by ferric ion under acidic conditions to be 3. Thus, the stoichiometry and spectral properties of the doxorubicin complex are identical whether ferric ion or the acetohydroxamic acid–iron chelate is used as an iron source.

Redox Catalysis by Doxorubicin–Iron Complex. The initial studies presented in Figure 1 suggested that the doxorubicin–ferrous ion complex was capable of reacting with oxygen. This chelate might potentially catalyze the reduction of oxygen to superoxide, hydrogen peroxide, or water in the presence of an appropriate electron donor able to reduce the oxidized complex. In order to evaluate this possibility, the effect of a variety of reducing agents on oxygen consumption by the ferric complex was evaluated. In contrast to the system described by Sugioka et al. (1981), NADPH and NADH did not stimulate oxygen consumption. Significant activity was observed for two naturally occurring thiols, cysteine and glutathione. As can be seen in Table I, glutathione plus ferric complex exhibited greater stimulation of oxygen consumption than was seen with any of the various controls. However, it is important to note that appreciable thiol-dependent oxygen consumption was seen with the ferric ion–acetohydroxamic acid complex. As with the spectral studies, ferric ion yielded results with considerable variability as compared with the doxorubicin–iron complex or the ferric ion–acetohydroxamic acid complex. The oxygen consumption by the doxorubicin–iron complex was lessened measurably by superoxide dismutase and by catalase. Again, qualitatively similar results were obtained for the ferric ion–acetohydroxamic acid complex.

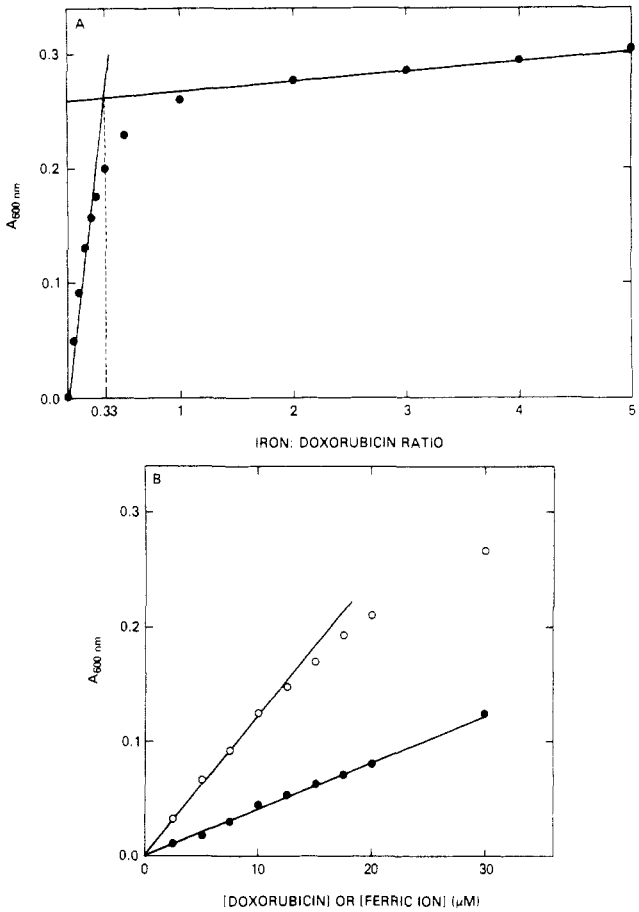

FIGURE 3: Stoichiometry of the doxorubicin–iron complex. Panel A shows the results of the ratio method. A titration of 0.1 mM doxorubicin was carried out as specified in Figure 2. The lines represent a least-squares fit to the first five and last four points. The *x*-axis value at the intersection of the two lines is taken to represent the ratio of iron to doxorubicin. The value obtained here is 0.33 or one iron to three doxorubicins. Panel B shows the results of the slope method. Here first a constant iron concentration (0.1 mM iron–acetohydroxamic acid complex) was titrated with doxorubicin (●). Second, a constant doxorubicin concentration (0.1 mM) was titrated with iron–acetohydroxamic acid complex (○). The slope in the first case was 0.039, and the slope in the second, 0.118. The ratio of the slopes, 3.02, gives an estimate of the ratio of doxorubicin to iron in the complex.

Table I: *Catalysis of Oxygen Consumption by the Complex*[a]

agent	oxygen consumption (nmol mL^{-1} min^{-1})
Dox	*b*
Dox-Fe	*b*
Dox-Fe + glutathione	21.0 ± 2.59
Dox-Fe ($FeCl_3$ as iron source) + glutathione	24.0
Dox-Fe + glutathione, SOD	16.48 ± 3.6
Dox-Fe + glutathione + catalase	10.67 ± 3.6
Dox-Fe + glutathione + EDTA	*b*
AHA-Fe	*b*
AHA-Fe + glutathione	13.3 ± 3.7
AHA-Fe + glutathione + SOD	9.98 ± 3.3
AHA-Fe + glutathione + catalase	8.69 ± 2.7

[a] The concentrations used were doxorubicin (Dox), 0.1 mM; glutathione, 15 mM; superoxide dismutase (SOD), 120 units/mL; catalase, 400 units/mL; doxorubicin-iron (Dox-Fe), as described under Materials and Methods. AHA is acetohydroxamic acid. [b] Value not significant.

These results suggest that the products of oxygen reduction in both cases include both superoxide and hydrogen peroxide.

We next examined the possibility that this catalysis might occur via a mechanism consistent with Michaelis–Menten kinetics. The results yielded a linear Lineweaver–Burk plot with either oxygen or the thiols cysteine and glutathione as the variable substrate. The calculated constants with glutathione as the thiol were the following: (1) for oxygen, K_m = 280 μM and V_{max} = 39 nmol mL^{-1} min^{-1}; (2) for glutathione, K_m = 21 mM and V_{max} = 11 nmol mL^{-1} min^{-1} where the fixed substrates had concentrations of 251 μM for oxygen and 15 mM for glutathione. The calculated constants with cysteine as a thiol were the following: (1) for oxygen, K_m = 191 μM and V_{max} = 38 nmol mL^{-1} min^{-1}; (2) for cysteine, K_m = 1.2 mM and V_{max} = 42.4 nmol mL^{-1} min^{-1} where the fixed substrates had concentrations of 251 μM for oxygen and 1.3 mM for cysteine. The concentration of complex in these experiments was 50 μM.

In the process of performing these experiments, we have noted that the actual values obtained for K_m and V_{max} are affected by alterations in the concentration of thiol and oxygen, as might be expected for a double-substrate reaction. Also, the doxorubicin/iron ratio also affected the K_m and V_{max}. Finally, it must be pointed out that these complexes are not enzymes but catalysts whose behavior approximates Michaelis–Menten kinetics. Thus, the actual K_m and V_{max} values reported are unique to the specific experimental conditions used and cannot be interpreted as having the same physical meaning they do in enzyme kinetics.

The acetohydroxamic acid–iron complex also yielded a linear Lineweaver–Burk plot with oxygen as the variable substrate and glutathione concentration set at 15 mM. The V_{max}, at 60.9 μmol L^{-1} min^{-1}, compared favorably with the values for the doxorubicin–iron complex; the K_m, however, was 831 μM which is considerably in excess of both ambient oxygen concentrations (approximately 251 μM) and the K_m for oxygen exhibited by the doxorubicin–iron complex (280 μM). Nevertheless, we have shown that, like many iron chelates, the doxorubicin–iron complex will catalyze the reaction between thiols and oxygen.

During the cyclic oxidation and reduction of doxorubicin catalyzed by P450 reductase, cleavage of doxorubicin to its aglycon occurs as oxygen is depleted. The reaction between glutathione and the complex was allowed to proceed until no detectable oxygen remained in order to test whether a similar phenomenon occurred in the doxorubicin–iron system. The reaction mixture was then extracted, and the products were examined via high-pressure liquid chromatography (HPLC) according to previously published methods (Israel et al., 1978). Only intact doxorubicin was detected. Thus, in the process of this reaction, irreversible alteration of the doxorubicin does not occur. In addition, if the reaction is allowed to proceed until the oxygen is consumed, it may be reinitiated by reintroduction of fresh oxygen and glutathione, and the resultant reaction velocity is then comparable to the original initial velocity.

Erythrocyte Ghost Destruction by Doxorubicin–Iron Complexes. Because the doxorubicin–iron complex and the acetohydroxamic acid–iron complex catalyze oxygen radical formation, we next tested to see if they could mediate oxidative damage to erythrocyte ghost membranes. In preliminary experiments, we found that the doxorubicin complex would agglutinate erythrocyte ghosts from a suspension. Since this made measurement of ghost destruction difficult, we utilized a technique in which the ghosts were attached to a microtiter well. This prevented agglutination and allowed easy, direct assessment of ghost destruction. As is apparent in Table II,

Table II: Damage to Erythrocyte Ghosts after 30-min Contact Time with Doxorubicin-Iron Complex[a]

agent	% intact ghosts
Dox	100
ferric ion	100
ferric ion + glutathione	100
acetohydroxamic acid complex	100
acetohydroxamic acid complex + glutathione	100
Dox + glutathione	100
Dox-Fe	75
Dox-Fe + glutathione	5
Dox-Fe ($FeCl_3$ as iron source) + glutathione	9
Dox-Fe + glutathione + SOD	65
Dox-Fe + glutathione + catalase	80
Dox-Fe + glutathione + SOD + catalase	100
Dox-Fe + glutathione + boiled SOD + catalase	5
Dox-Fe + glutathione + mannitol (10 mM)	50
Dox-Fe + glutathione + mannitol (50 mM)	50
Dox-Fe + glutathione + EDTA	100

[a] The concentrations used were doxorubicin (Dox), 0.1 mM; iron as the acetohydroxamic acid complex, 50 μM; glutathione, 15 mM; superoxide dismutase (SOD), 120 units/mL; catalase, 400 units/mL; doxorubicin-iron complex (Dox-Fe), formed as described under Materials and Methods; EDTA, 1 mM.

the doxorubicin–iron complex plus glutathione leads to rapid destruction of the ghosts which is more complete than that caused by this complex alone or by iron plus glutathione or doxorubicin plus glutathione. Again, it did not matter whether the iron came from ferric chloride or the acetohydroxamic acid complex. In addition, this destruction of the ghosts is lessened by superoxide dismutase and by catalase and completely blocked by both enzymes used in combination. Because superoxide and hydrogen peroxide are known to react in the presence of iron to yield the hydroxyl radical, we also tested the effect of mannitol, a known hydroxyl radical scavenger. The protective effects appeared to be limited to 50%, suggesting that while the hydroxyl radical was involved superoxide or hydrogen peroxide was causing damage by other mechanisms as well. Finally, ethylenediaminetetraacetic acid (EDTA) blocked the erythrocyte ghost destruction and resulted in reversion of the spectrum from that of the complex to that of the parent doxorubicin. These results suggest that EDTA operates by removing iron from the doxorubicin complex.

The erythrocyte ghost data in Table II show the doxorubicin–iron complex possesses much more activity relative to the acetohydroxamic acid complex than might be expected from the relative activity of these agents as redox catalysts. When these experiments were performed, the ghost membranes became stained purple, suggesting direct binding of the doxorubicin–iron complex to the membranes. Since doxorubicin is known to bind to cell membranes, we measured the ability of the doxorubicin–iron complex to bind red cell ghost membranes by measuring the absorbance at 535 nm and the isosbestic point for doxorubicin and doxorubicin–iron complex. Ghost membranes exposed to 1 mM doxorubicin exhibited an absorption of 0.06 as compared to 0.02, 0.17, and 0.19 for 0.1, 0.5, and 1.0 mM doxorubicin–iron complex. The results confirm that the doxorubicin–iron complex can also bind erythrocyte ghost membranes and that this cannot be removed by repeated washing.

Discussion

This paper has described four properties of the doxorubicin–iron complex at physiologic pH. First, we have confirmed that the stoichiometry of the binding under conditions of doxorubicin excess is three doxorubicins to one ferric ion. Second, the resulting complex can act as a catalyst for the reduction of oxygen by thiols. Third, the complex will bind to erythrocyte membranes. Fourth, in the presence of glutathione, the complex causes rapid destruction of the erythrocyte ghost via a mechanism mediated by superoxide radicals and hydrogen peroxide.

The chelation of iron by doxorubicin has several interesting properties. The intense apparent charge transfer band formed when doxorubicin binds ferric ion is characteristic of ligands with strong electron-donating properties, and the absence of the same apparent charge transfer band in the ferrous complex is consistent with this (Williams, 1959). The stoichiometry of three doxorubicins to one ferric ion permits some speculation as to the structure of the complex. Ferric ion has six preferred coordinate positions in an octahedral geometry. This would allow the binding of three doxorubicins as bidentate ligands. Doxorubicin has several possible iron binding functionalities including the sugar amino groups, the quinone–hydroquinone functionalities, and the side-chain carbonyls (Gosalvez et al., 1978). Of these, the quinone–hydroquinone functionalities are most attractive as bidentate ligands in that they would bind iron into a stable six-membered ring, would provide for the strong electron donation inferred above, and would be consistent with the marked changes observed in the absorption at 479 nm. Further studies are, however, obviously needed to determine the structure of the complex.

The doxorubicin–iron complex has also proved an efficient catalyst for the reduction of oxygen by thiols such as glutathione and cysteine. The doxorubicin–iron complex exhibits several properties common to iron chelates capable of catalyzing the reduction of oxygen by thiols. It is, for example, common for such chelates to exhibit a charge transfer band with ferric but not ferrous ion. In addition, such complexes usually have a readily exchangeable ligand: the doxorubicin–iron complex, with a step constant of $10^{4.4}$ for the third ligand, seems to fit this general pattern. Furthermore, the catalysis by the doxorubicin–iron complex appears to exhibit saturation kinetics with regard to both oxygen and thiol. These results suggest the possible existence of rate-limiting transition-state complexes with oxygen, thiol, or both. While these results are consistent with the ordered sequential oxidation and reduction mechanism usually proposed for reactions of this type, a bi-bi mechanism in which both thiol and oxygen are bound simultaneously cannot be ruled out. It is of interest that the acetohydroxamic acid–iron chelate also exhibits a similar pattern of behavior.

The redox reaction catalyzed by the doxorubicin–iron complex differs markedly from the previously described P450 reductase mediated reaction by which doxorubicin can give rise to oxygen radicals. First, the reductant for the doxorubicin–iron chelate is glutathione, not NADPH as is the case for the P450 reductase system. Second, under anaerobic conditions, the P450 reductase dependent reaction leads to conversion of doxorubicin to its 7-deoxyaglycon (Bachur et al., 1978). This is thought to result from rearrangement of the semiquinone radical, which is the first radical produced, in which the bond between the C-7 carbon on the chromophore and the amino sugar cleaves, resulting in an aglycon C-7 radical. The doxorubicin–iron complex does not undergo such a rearrangement following reduction under anaerobic conditions. We suspect this results from alterations in the π cloud distribution of the chromophore as a consequence of the electron-withdrawing effect of the iron.

Because the results presented in Table I suggested that superoxide and hydrogen peroxide were products of the re-

action catalyzed by both the acetohydroxamic acid–iron and the doxorubicin–iron complexes, we tested both for their ability to damage erythrocyte ghosts. Although, under the conditions of the test, oxygen consumption was nearly equivalent with the two complexes, the difference between the two chelates in terms of ghost destruction was marked: 95% lysis for the doxorubicin–iron complex as compared to 0% lysis for the acetohydroxamic acid–iron chelate. These results suggest that factors other than the rate of oxygen consumption are major determinants of ghost destruction in this system. One factor which we have identified appears to be the ability of the doxorubicin–iron chelate to bind to erythrocyte membranes. The result of this binding is to position the doxorubicin–iron chelate close to the surface of the membrane. Such membrane binding may enhance membrane damage by resulting in high local concentrations of superoxide and hydrogen peroxide at the membrane surface. This might have several consequences. First, it might facilitate hydroxyl radical generation via a Haber-Weiss-like reaction. While the Haber-Weiss reaction is very slow without catalysis, it is possible that the doxorubicin–iron complex might directly catalyze the Haber-Weiss reaction. There is precedence for such catalysis in that other iron chelates have been described as catalyzing the formation of hydroxyl radical from hydrogen peroxide and superoxide (McCord & Day, 1978).

Regardless of the mechanism by which the hydroxyl radical is generated, its involvement in erythrocyte ghost damage has certain implications. The very reactivity of the hydroxyl radical means that it must have a short lifetime in aqueous solution. As a result, it is unreasonable to expect the hydroxyl radical to diffuse any great distance from the site of its generation. In such a situation, binding of the doxorubicin complex to the erythrocyte membrane can be seen as an effective way of reducing the diffusion distance from the site of oxygen radical generation to the targets in the membrane.

There is a portion of the ghost destruction which is not inhibitable by mannitol and which is therefore probably not mediated by the hydroxyl radical yet is inhibited by catalase plus superoxide dismutase, indicating that superoxide and/or hydrogen peroxide are involved. While superoxide alone has not been implicated in erythrocyte ghost destruction, hydrogen peroxide does appear to be able to cause membrane damage by a pathway not yielding hydroxyl radical (Kong & Davison, 1980). A similar mechanism may be operating here.

To some degree, the model proposed here for the doxorubicin–iron chelate parallels that proposed for the damage of DNA by the bleomycin–iron complex (Sausville & Horwitz, 1979). In both cases, the drug combines structural features capable of binding iron to create a redox catalyst with structural features which attach the redox catalyst to a macromolecular target. The iron chelate then catalyzes the reduction of oxygen by the thiols to oxygen radical species. In the case of bleomycin, the only known target seems to be DNA; one target of the doxorubicin–iron complex is the cell membrane. However, in the present study, we have made no attempt to study the interaction of the doxorubicin–iron complex with DNA, and this macromolecule might also be a potential target in view of the ability of the parent drug, doxorubicin, to bind DNA.

In previous publications on iron binding by doxorubicin, there have been references to varability in the behavior of the complex (May et al., 1980). We have confirmed these difficulties. It seemed to us that the problems were similar to that previously reported for transferrin (Bates & Schlabach, 1973). With transferrin, ferric salts have proved to be an unreliable source of iron, primarily because of the complex chemistry of free ferric ion in neutral aqueous solution. For transferrin, this problem may be overcome by use of appropriate ferric chelates as the iron source. For this reason, we examined the acetohydroxamic acid–ferric ion chelate as an iron source and have found that its use was associated with less variable formation of doxorubicin–ferric ion complex than was obtained with ferric ion. We have also not detected any spectral evidence of mixed doxorubicin–acetohydroxamic acid–iron chelates. It should also be pointed out that high concentrations of free ferric ion do not exist in vivo, and for this reason, formation of the doxorubicin–iron complex from chelate-bound ferric ion is probably more relevant than the use of ferric salts.

At present, only circumstantial evidence is available to suggest activity of the doxorubicin–iron complex in vivo. The complex does provide an explanation for the observations of Kappus et al. (1980) that lipid peroxidation was much more marked after doxorubicin plus iron than after iron or doxorubicin alone. In terms of specific organ toxicity, lipid peroxidation has been invoked as the etiology of the cardiac toxicity induced by doxorubicin (Myers et al., 1977). Thus, free-radical scavengers have been reported to lessen the cardiac toxicity of doxorubicin in mice (Myers et al., 1977; Doroshow et al., 1981), and cardiac tissue has been found to have defects in its oxygen radical defense mechanisms (Doroshow et al., 1980). However, the biochemical mechanism by which doxorubicin might induce lipid peroxidation in the heart has never been adequately defined. The recent observation that chelating agents such as ICRF 187 lessen the cardiac toxicity of doxorubicin suggests involvement of metal ions and would be consistent with the properties now established for the doxorubicin–iron chelate (Herman et al., 1979). Unfortunately, these observations might also be explained by competition between ICRF-187 and doxorubicin for other metal ions such as calcium, known to be important in myocardial function. Also, ICRF-187 may exert its protection by mechanisms other than chelation. Herman et al. (1974) showed that a related compound, ICRF-159, decreased doxorubicin aglycon formation. Thus, ICRF-187 may operate by preventing in vivo bioactivation of doxorubicin to the semiquinone.

In spite of the above difficulties, it is now apparent that the doxorubicin–iron complex exhibits interesting behavior in terms of its stability (association constant of $10^{33.4}$), its redox catalysis, and its membrane binding and destruction. While the present report does not address the in vivo role of the doxorubicin–iron complex, it has documented that the complex does possess characteristics which warrant further study.

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Association of Membrane and Cytoplasmic Proteins with the Cytoskeleton in Blood Platelets†

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ABSTRACT: The association of membrane and cytoplasmic proteins with the cytoskeleton of resting and activated platelets was studied. Glycoproteins were identified by labeling with ^{125}I-labeled lectins (concanavalin A, wheat germ agglutinin, and Lens culinaris). Polypeptides, which are embedded in the lipid bilayer, have been identified by their photolabeling with the lipid-soluble reagent 5-[^{125}I]iodonaphthyl 1-azide (^{125}INA). Cytoplasmic proteins were identified by their photolabeling with the intracellular probe azidofluorescein diacetate. Results indicate that the Triton X-100 residue contains the membrane-associated glycoprotein Ia, a 95 000-dalton protein, and two other acidic proteins of molecular weights of 35 000–40 000, one labeled with ^{125}INA and the other with azidofluorescein diacetate. The presence of part of these proteins in the Triton residue is dependent upon the mode of platelet activation. Glycoproteins IIb and III are embedded in the membrane lipid bilayer but sedimented with the Triton residue only after thrombin activation. Another protein with M_r 70 000, which is highly labeled by ^{125}INA in resting platelets, is found only in the Triton-soluble fraction.

The association of platelet membrane proteins with the cytoskeleton and the changes in the membrane proteins as a result of activation were the focus of a few studies in recent years (Phillips et al., 1980; George et al., 1980). Thus, Phillips et al. have recently shown that the amount of actin present in the cytoskeleton of thrombin-aggregated platelets is almost double that of actin in the cytoskeleton of unstimulated platelets. This is in accordance with observations made by Pribluda et al. (1981) and by Carlsson et al. (1979) that there is a significant mobilization of actin (decrease in DNase-available actin) as a result of platelet aggregation. A few membrane proteins were identified by Phillips et al. (1980) as being associated with the cytoskeleton, among them glycoprotein IIb and glycoprotein III. The association of actin with platelet membrane was shown morphologically by Zucker-Franklin (1970), biochemically by Taylor et al. (1976), and by immunofluorescent technique (Bouvier et al., 1977; Diggle et al., 1979). Some evidence was presented recently that platelet membrane glycoprotein III may be α-actinin (Gerard et al., 1979) and that this protein spans the plasma membrane (Phillips & Agin, 1974) and can function to anchor actin to the membrane. Thus, data accumulated so far indicated that glycoprotein III and glycoprotein IIb are associated with the cytoskeleton of aggregated platelets (Phillips et al., 1980) and that they may participate in the mediation of platelet–platelet interaction.

In this contribution we report the identification of those membrane proteins embedded in the lipid bilayer and their association with the cytoskeleton, using [^{125}I]iodonaphthyl azide (^{125}INA). We used the lectins wheat germ agglutinin (WGA), Lens culinaris lectin, and concanavalin A (Con A) to determine the association of glycoprotein Ia, glycoprotein Ib, glycoprotein IIb, and glycoprotein III with the cytoskeleton. We also identified those cytoplasmic proteins associated with the cytoskeleton by the use of the fluorescent intracellular probe azidofluorescein diacetate (Rotman & Heldman, 1980).

Materials and Methods

Platelets were prepared according to the method of Peerschke et al. (1980), with the modification that albumin was omitted from the medium. [^{125}I]Iodonaphthyl azide was

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