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TIME-DEPENDENT MODIFICATIONS OF FERRIC–ADRIAMYCIN

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Abstract—The biological and chemical properties of the ferric–Adriamycin complex changed with time after its preparation. Our experiments demonstrated that the toxicity of the iron-chelate in mice decreased as a function of its age. The reduced toxicity can be correlated with changes in the difference spectrum of ferric-Adriamycin vs Adriamycin® (ADR), where a peak around 610 nm shifted to the 570 nm region. When ferric-Adriamycin "aged", the affinity of the drug for iron increased. Concurrently, the redox properties of the chelate changed, such that the bound iron was no longer reduced by glutathione or cysteine. The time-dependent changes observed did not involve the formation of polynuclear iron, as shown by electron spin resonance. Thin-layer chromatography showed that ADR undergoes accelerated degradation in the presence of iron. The iron-catalyzed degradation was oxygen independent. The changes evolving in the spectral and chemical properties of the chelate were shown to stem from transfer of the iron from ADR to one of the degradation products.

The anthracycline drug, Adriamycin® (ADR)‖, is widely used in cancer chemotherapy. The drug has serious cardiotoxic side-effects which impose severe limitations on its use. Its mode of action has not yet been elucidated, but evidence suggests that ADR has more than one mechanism of action [1]. ADR is an effective chelator for metal cations, especially iron [2, 3]. The iron–Adriamycin complex is capable of producing highly reactive oxygen-derived free radicals by redox cycling [4, 5], which has been proposed as one of the mechanisms of action for the drug [1]. The iron-complex of ADR, but not ADR alone, has been shown to precipitate in certain organs *in vivo* [6], to undergo chemical modification in artificial and natural membranes [7], and to cause lipid peroxidation *in vivo* [8]. Furthermore, it has been suggested that ADR can bind adventitious iron ions *in vivo* [1, 2, 7, 9–11]. Thus, determination of the physico-chemical properties of ferric-ADR may be important to the understanding of the mode of action of this drug.

Gosalvez *et al.* [12] originally proposed that iron and ADR form a "triferric–Adriamycin complex", quelamycin. While this preparation has been reported to have considerable antitumor activity but reduced toxicity, animal experiments and clinical trials have been ambiguous [13]. No evidence has been found for the existence of the proposed 3:1 iron–ADR complex. Moreover, it has been shown that the Fe(III)-ion can bind three ADR molecules [2]. In the presence of excess ADR, a distinct Fe(III)ADR_3 chelate [2, 14, 15] with defined physico-chemical properties can be formed [16]. The nominal formation constant, β, of this chelate has been reported to be $10^{33.4}$ [2] or $10^{28.4}$ [14], and its effective binding constant, K_{eff}, is $10^{16.2}$ at pH 7.4 [16].

The 3:1 iron:ADR mixture has been shown to form precipitable aggregates which lodge in the pulmonary capillary bed when injected into mice, whereas 1:1 mixtures precipitated only if the drug–iron mixture was allowed to stand for a prolonged period of time between preparation and injection [6]. A detailed study on the formation of Fe(III)ADR_3 has determined that the complex exists in two distinct forms, and that the transition from the transient to the stable form may take as much as a day [14]. Other reports [4, 15] have also mentioned time-dependent changes occurring in Fe(III)-ADR preparations, eventually resulting in the precipitation of the complex. Recently, it has been reported that iron may interact with the C_9-ketol sidechain of ADR to promote a unique type of degradation, leading to 9-carboxy-ADR [17].

Inconsistencies observed in preliminary studies of the properties of the Fe(III)ADR_3 chelate suggested that this complex undergoes time-dependent changes. This notion was supported by the reports mentioned above. In view of the apparent biological importance of the association between ADR and iron, the nature of the time-dependent changes occurring in Fe(III)ADR_3 was investigated. By determining the properties of the chelate at various times after preparation, it was found that Fe(III)ADR_3 degrades spontaneously to form chelates with lower toxicity and altered iron-binding properties.

MATERIALS AND METHODS

Chemicals and solutions. Adriamycin, supplied as

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‖ Abbreviations: ADR, Adriamycin®; ADR-ol, adriamycinol; DFO, desferrioxamine; DTPA, diethylenetriaminepentaacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TDW, triple distilled water; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; and Tl_{50}, time required for 50% mortality.

a 1:5 (w/w) mixture with lactose, was produced by Farmitalia Carlo Erba (Milan, Italy). $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was from the Fisher Scientific Co. (Silver Spring, MD); DTPA, ascorbate, bathophenanthroline-sulfonate and HEPES were from the Sigma Chemical Co. (St Louis, MO), cysteine and glutathione from the Nutritional Biochemicals Corp. (Cleveland, OH) and EDTA, trisodium citrate and $FeCl_3 \cdot 6H_2O$ from BDH Chemicals Ltd (Poole, U.K.). Desferrioxamine (DFO) was a gift from the CIBA Pharmaceutical Co. (Summit, NJ). Triple distilled water (TDW) was used throughout.

Stock solutions of 4 mM ADR were prepared in TDW and kept in the dark at 4°. To prevent photolytic degradation, exposure of the ADR and iron–ADR solutions to light was minimized. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was dissolved in 10 mM H_2SO_4 (pH 1.9) to give a 10 mM solution. Buffers, ADR, and H_2SO_4 were essentially iron-free, though not chelex-treated. The buffer was chosen because of its low affinity for metal ions [18]. The lactose introduced with the ADR has been noted previously not to interfere with iron chemistry [16].

To prevent hydrolysis during preparation, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (in sulfuric acid at pH 1.9) was added to an excess of ADR at acid pH. The concentration was adjusted to 1 mM ADR with TDW and, as the final step, the pH was brought to 7.4 with HEPES buffer (final concentration 40 mM). The iron was presented as Fe(II) which oxidizes rapidly upon neutralization, since this gave the best complex yields [14, 16]. The chelates were used within 3–5 min of their preparation in chemical experiments, or kept on ice and used within 45 min of preparation in animal experiments. Alternatively, the chelates were incubated in the dark at the temperature and for the period stated in each experiment.

Iron concentrations were routinely monitored by the bathophenanthroline–sulfonate method [16].

Animal experiments. Ten-week-old male BALB/C mice were injected i.v. with a single dose (0.01 mL/g body weight) of one of the following: fresh ADR, 48-hr-old ADR, freshly prepared Fe(III)ADR_3, or 48-hr-old Fe(III)ADR_3, to provide either 16 or 20 mg ADR/kg body weight. Fe(III)ADR_3 was prepared as a 1:5 mixture of iron and ADR, and all injectable solutions were adjusted to pH 7.4 with 40 mM HEPES buffer. The mortality was followed for 40 days.

Ligand substitution of Fe(III)*ADR_3.* DFO, EDTA or DTPA at neutral pH was added to Fe(III)ADR_3 (0.1 mM iron and 1 mM ADR) to provide a 20 mM final concentration of the respective chelating agent. The concentration of the chelators was chosen so that $[\text{chelator}] \cdot K_{\text{eff}}^{\text{Fe chelator}} \gg [\text{ADR}]^3 \cdot K_{\text{eff}}^{\text{Fe ADR}_3}$. The substitution of ADR by the chelators was monitored by the decrease of A_{602}, where Fe(III)ADR_3, but not the Fe(III)-chelates of DFO, DTPA and EDTA, absorbs.

Iron reduction. Fe(III)ADR_3 solutions (0.05 mM iron and 1 mM ADR) were made anaerobic by bubbling with N_2. A small volume of an anaerobic concentrated cysteine, glutathione or ascorbate solution, providing 2, 10 or 20 mM final concentration, was added to reduce the iron. The reduction of the iron was followed by monitoring A_{602} and correcting for the absorption of Fe(II)-ADR.

ESR spectroscopy. Fe(III)ADR_3 (1 mM iron and 5 mM ADR) was prepared as above, and either frozen in liquid N_2 within 4 min of preparation or kept at room temperature, in the dark, for 24 hr before freezing. Fe(III)–citrate at ratios of 1:25 and 1:1, containing 1 mM Fe(III), were prepared by adding $FeCl_3$ (dissolved in H_2SO_4 at pH 1.9) to the required amount of citrate and neutralizing with solid $NaHCO_3$. These solutions were subsequently frozen in liquid N_2. ESR spectra were recorded at 77°K with a Varian E-4 spectrometer operating at 8.995 GHz using 20 mW microwave power. 100 KHz modulation frequency and a modulation amplitude of 25 G.

Thin-layer chromatography. ADR and its degradation products were separated by ascending TLC on silica gel-60 plates. Three different developing solution systems were used:
(A) chloroform:methanol:H_2O 80:20:3 (by vol.);
(B) chloroform:methanol:glacial acetic acid:H_2O 80:20:14:6 (by vol.);
(C) ethyl acetate:ethanol:glacial acetic acid:H_2O 80:10:5:5 (by vol.).

After chromatography, the migrating spots were visualized by their fluorescence under UV or visible light. Non-fluorescent products were detected by charring with concentrated H_2SO_4.

Spectroscopic measurements. All spectral measurements were made at room temperature using a dual beam Kontron model Uvikon 860 spectrophotometer.

Data presentation. In the animal experiments three separate repeats were pooled to give a total of 28–30 animals in each treatment group. In all other experiments, typical results of at least three similar repeats are presented.

RESULTS

Toxicity of fresh and "aged" chelates. Preliminary experiments with Fe(III)ADR_3 indicated that the toxicity of the chelate is altered with the elapsed time between its preparation and use. To characterize this effect more clearly, 10-week-old male BALB/C mice were injected i.v. with a single lethal dose of ADR, a freshly prepared Fe(III)ADR_3 chelate, or a 48-hr aged chelate. Since iron binding to ADR is not complete unless ADR is present in excess [16], and unbound Fe(III) precipitates as polynuclear aggregates at physiological pH, the chelates were prepared with a defined excess of ADR ([iron]:[ADR] = 1:5), which prevented the formation of precipitable material during the time–course of the experiment. The survival of the experimental animals was followed for 40 days. At doses providing 16 mg ADR/kg body weight the time required for 50% mortality (Tl_{50}) was 17 days with ADR and 18 days with the fresh Fe(III)ADR_3 chelate, whereas no mortality at all was observed for the entire duration of the experiment in the group receiving the 48-hr aged chelate (Fig. 1A). At 20 mg ADR/kg body weight, Tl_{50} was 7.5 days for ADR, 8.5 days for the fresh

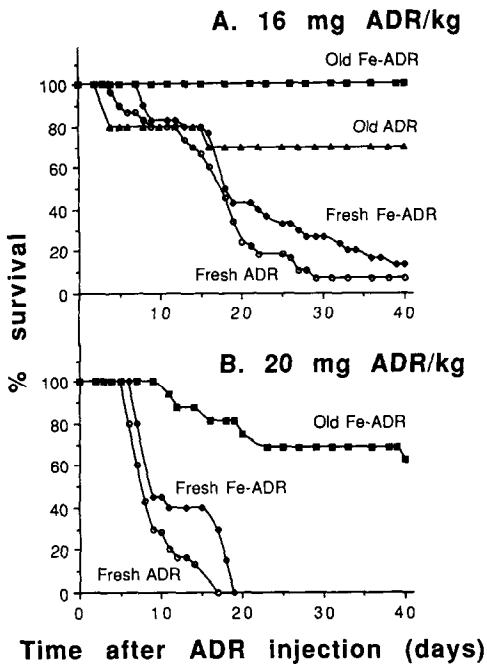

Fig. 1. Toxicity of ADR and Fe(III)ADR_3 to mice. Ten-week-old male BALB/C mice (28–30 animals per group) were given a single i.v. injection with a lethal dose of either fresh ADR, 48-hr-old ADR, a freshly prepared Fe(III)ADR_3 chelate or a 48-hr-old chelate, all at pH 7.4. Fe(III)ADR_3 was prepared as a 1:5 molar ratio mixture of iron and ADR. The survival was followed for 40 days. The dose of ADR provided by each compound was: (A) 16 mg/kg body weight; and (B) 20 mg/kg body weight.

chelate and >40 days for the aged Fe(III)ADR_3 chelate (Fig. 1B).

Time-dependent spectral changes. The time-dependent decrease in the toxicity of the chelate probably resulted from a chemical change. Therefore, the absorption spectra of the fresh and the aged Fe(III)ADR_3 chelates were compared. Fe(III)ADR_3 chelates were prepared at 0.05 mM iron and 1 mM ADR in 40 mM HEPES buffer, pH 7.4, and kept at room temperature. Difference spectra (550–700 nm) were recorded at various times after preparation versus freshly prepared ADR (Fig. 2). It is seen that the broad absorption band of the freshly prepared chelate was at 610 nm, whereas after 18 hr, a new absorption peak had evolved at 570 nm.

Fe(III)*ADR_3 ligand substitution.* The spectral changes observed during the aging of Fe(III)ADR_3 were considered to reflect a chemical change in the binding of iron to ADR. Therefore, the ability of an excess of chelating agents to remove Fe(III) from this chelate was examined. The substitution reaction of Fe(III)ADR_3 with desferrioxamine (DFO), EDTA or DTPA was followed spectroscopically at 602 nm, where Fe(III)ADR_3 absorbs but ADR, Fe(III)-EDTA, Fe(III)-DTPA and Fe(III)-DFO do not. Fe(III)ADR_3 was formed by mixing 1 mM ADR and 0.1 mM iron in 40 mM HEPES buffer, pH 7.4. The chelating agents were added at a 20 mM final concentration at various times after chelate preparation, and A_{602} was monitored. All three chelators gave practically identical results. The results obtained with DFO are presented in Fig. 3. It is seen that while DFO efficiently and rapidly removed iron from a freshly prepared Fe(III)ADR_3 chelate ($k = \sim 2 \times 10^{-2}\,sec^{-1}$), both the rate and the extent of iron removal decreased as the Fe(III)ADR_3 chelate aged. Thus, during a 10-min incubation DFO removed practically all the iron from the fresh chelate, but only 70 and 10% from 60-min and 22-hr-old chelates respectively. The rate of the ligand substitution was independent of chelator concentration over abroad range (data not shown).

Iron reduction by mild reductants. One of the important characteristics of ADR-bound iron is its ability to undergo redox cycling [4, 5]. Since this property is readily influenced by the character of the iron-ligand bonds, the effect of aging on the redox behavior of the chelates was investigated. The efficacy of mild biochemical reductants such as ascorbate, cysteine and glutathione to reduce the iron in Fe(III)ADR_3 was assayed by measuring the loss of 602 nm absorbance, after the addition of the reducing agents. The experiment was performed under anaerobic conditions, in order to prevent the immediate reoxidation of iron by molecular oxygen. Fe(III)ADR_3 was prepared as described in Materials and Methods, 2, 10 or 20 mM ascorbate, cysteine or glutathione was added under N_2 to reduce the iron, and the absorbance at 602 nm was monitored. The results obtained with cysteine are presented in Fig. 4. The Fe(III) was readily reduced by cysteine in the freshly prepared, but not in the 26-hr-old Fe(III)ADR_3 chelate. While glutathione was less effective than cysteine as a reducing agent, a similar effect of aging on the reduction of the iron was observed with glutathione (data not shown). Ascorbate failed to reduce either the fresh or the aged chelate to any appreciable degree.

Examination of Fe(III)*ADR_3 by ESR spectroscopy.* A simple explanation for the failure of the chelating agents to remove, and of cysteine to reduce, the iron of aged Fe(III)ADR_3, could be the formation of polynuclear iron-aggregates. It has been shown previously that certain ligands, such as citrate and fructose, are unable to maintain Fe(III) in solution in its monomeric form, unless present in large excess over the iron [19–21]. ADR could be similar in this respect, in which case the 5:1 ADR:iron ratio employed in the present study might be insufficient to prevent polymerization of the iron. To examine this possibility, electron spin resonance (ESR) spectroscopy was utilized. The typical ESR signal of monomeric iron would be broadened upon polymerization due to spin–spin interactions and become undetectable. To demonstrate this, Fe(III)–citrate at 1:25 and 1:1, both containing 1 mM Fe(III), were prepared as described in Materials and Methods, and their ESR spectra were recorded at 77°K. Monomeric Fe(III) (1:25 ferric–citrate) gave a strong signal (Fig. 5, trace A), whereas no signal was detected from 1:1 ferric–citrate (Fig. 5, trace B), which forms discrete spheres of polymeric iron [20]. Similarly, ESR spectra of fresh and aged Fe(III)ADR_3 were recorded at 77°K and compared. Fe(III)ADR_3 was prepared at 5 mM ADR, 1 mM Fe(III) in 40 mM HEPES buffer, pH 7.4. One sample of the

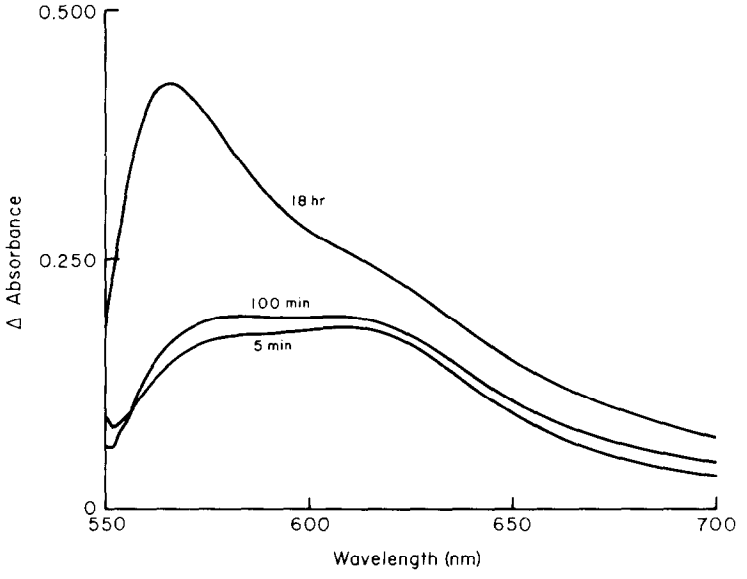

Fig. 2. Time-dependent changes in the Fe(III)ADR_3/ADR difference spectra. The 550–700 nm difference spectra of Fe(III)ADR_3 (0.05 mM iron and 1 mM ADR) vs ADR (1 mM) were recorded at various times after preparation of the chelate.

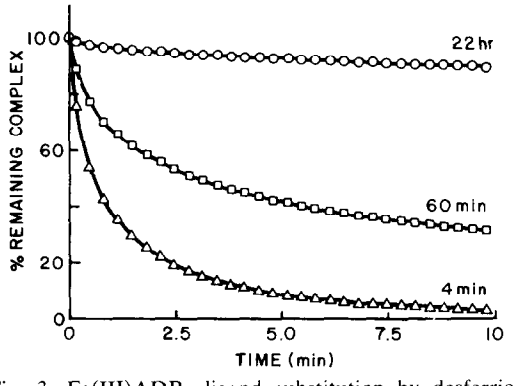

Fig. 3. Fe(III)ADR_3 ligand substitution by desferrioxamine. Fe(III)ADR_3 was prepared at 0.1 mM iron and 1 mM ADR, and 20 mM desferrioxamine (DFO) was added at various times after the preparation. The substitution of ADR by DFO was determined by following the decrease in A_{602}. The age of the preparation is indicated on the curves.

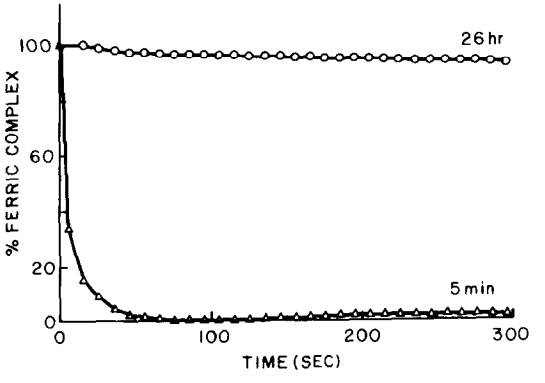

Fig. 4. Reduction of Fe(III)ADR_3 by cysteine. Fe(III)ADR_3 was prepared at 0.05 mM iron and 1 mM ADR, and made anaerobic by bubbling with N_2. Anaerobic cysteine was added to a final concentration of 20 mM, and the reduction of the iron was determined by following the decrease in A_{602}. The age of the chelate preparation is indicated.

Fe(III)ADR_3 chelate was frozen in liquid N_2 6 min after preparation, and a second sample was kept at room temperature for 26 hr before freezing. Practically no difference was noted between the ESR spectra of the fresh and the aged Fe(III)ADR_3 chelates (Fig. 6).

Degradation products of ADR. ADR degrades spontaneously at pH 7.4 as a function of temperature and concentration [22, 23]. Fe(III)ADR_3 is less stable than ADR under similar conditions, which may suggest iron-accelerated ADR-degradation. This possibility was investigated by examining fresh and aged samples using TLC. Solutions containing 2.5 mM ADR with or without 0.1 mM iron in 100 mM HEPES buffer, pH 7.4, were incubated at 30° for 0, 2, 8 and 23 hr; samples (1 μL) were loaded onto silica gel-60 TLC sheets and developed in three solvent systems. ADR and its products were detected by fluorescence. There was a rapid appearance of fluorescent degradation products from Fe(III)ADR_3, whereas the degradation of iron-free ADR was much slower. Three major degradation products—migrating with R_f 0.23, 0.66 and 0.86 in solvent system B—were common to the iron-bound and iron-free ADR, whereas two additional products, appearing in small quantities, were specifically produced, one in the presence and the other in the absence of iron. Charring revealed only

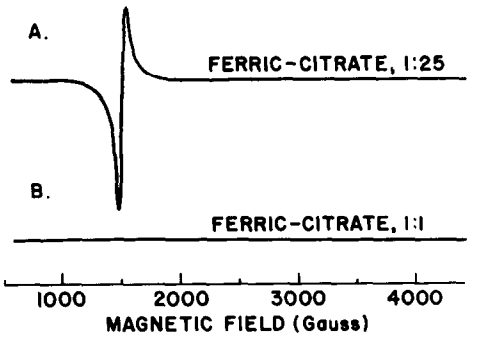

Fig. 5. ESR spectra of Fe(III)–citrate. Fe(III)–citrate (1:25 and 1:1) was prepared at 1 mM iron. The ESR spectra were obtained at 77°K with the following instrument settings: microwave frequency, 8.995 GHz; microwave power, 20 mW; modulation frequency, 100 KHz; and modulation amplitude, 25 G. Spectra are shown at identical gain.

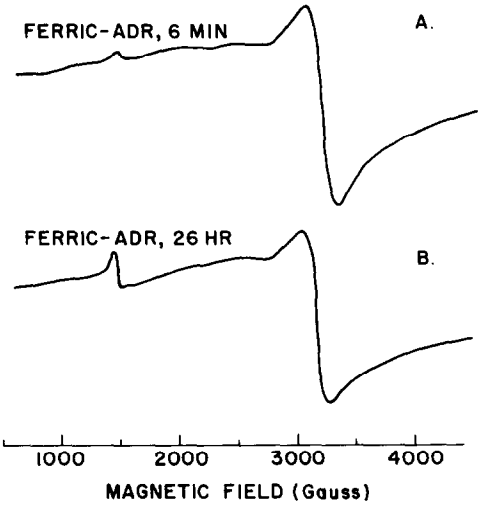

Fig. 6. ESR spectra of Fe(III)ADR_3. Fe(III)ADR_3 was prepared at 1 mM iron and 5 mM ADR. ESR spectra were obtained from a 6-min-old (trace A) and a 26-hr-old (trace B) chelate, as described in Fig. 5.

products coincident with the fluorescence (or comigrant with the lactose), suggesting that no non-fluorescent degradation products were produced. The degradation products did not comigrate with ADR-ol or the aglycones of ADR, ADR-ol and 7-deoxy-ADR included as markers.

Degradation under anaerobic conditions. The iron-accelerated degradation of ADR may involve metal-catalyzed oxidation of ADR. This possibility was examined by comparing degradation under aerobic and anaerobic conditions. Solutions of ADR and Fe(III)ADR_3 were prepared at 1 mM ADR with and without 0.02 mM Fe(III), bubbled with N_2 or air for 16 hr at room temperature, and their absorption spectra were recorded. No difference was noted between the aerobic and the anaerobic solutions, whether in the presence or absence of iron (data not shown).

Toxicity of aged ADR. Since the changes observed in Fe(III)ADR_3 were found to be related to ADR degradation, which also occurs in the absence of Fe(III), the toxicity of 48-hr-old ADR was determined (as described above). The toxicity of ADR decreased with time, even in the absence of iron, although at a slower rate (Fig. 1A).

DISCUSSION

The results reported above establish that Fe(III) accelerates ADR degradation to products with lower *in vivo* toxicity. As ADR degrades, the iron is gradually transferred from ADR to one of the degradation products. In the new complex(es) iron is less available to ligand substitution by strong chelators and to reduction by biological reducing agents. Iron-hydroxypolymers are not formed during this aging process.

The toxicity of the fresh Fe(III)ADR_3 chelate was comparable to that of free ADR (Fig. 1). This observation is consistent with the expected dissociation of the chelate upon its dilution in the blood stream, as previously discussed in detail [16]. As a consequence of the dissociation, the iron-accelerated ADR degradation ceases upon injection, and the released drug becomes subject to normal metabolic breakdown. Thus, the administration of a fresh chelate is essentially equivalent to providing a readily available source of free ADR. In contrast, the aged chelate has reduced toxicity. Since a major fraction of the ADR is degraded under the conditions employed, the remaining ADR alone cannot account for the observed toxicity. Thus, the degradation products must be considered as contributing to the overall toxicity, albeit to a lesser degree than ADR. In the absence of iron, ADR degradation proceeds at a much slower rate, as suggested by the toxicity of the drug to mice. The effect of the degradation process on the antitumor activity of ADR and Fe(III)-ADR is presently under investigation.

Both ADR and Fe(III)ADR_3 produced three major products: one with R_f 0.86 in solvent system B and the other two with R_f values 0.23 and 0.66 which are close to, but not identical with, those of ADR-ol and ADR-ol aglycone. The minor product which appeared only in the presence of iron may be 9-carboxy-ADR, in accordance with the report of Gianni *et al.* [17]. The small amount of this product may indicate that C_9-sidechain oxidation is a minor degradation pathway, or it may reflect an iron:ADR ratio dependence of this reaction. These possibilities and the true identity of the products are presently under investigation.

The ADR-degradation observed in the presence of iron (estimated from TLC) may be so extensive that the remaining ADR is insufficient to provide three ligands per Fe(III), in agreement with the data of Gianni *et al.* [17]. Consequently the iron must either bind to the degradation-products or remain unbound, which will result in hydrolysis. Since no hydrolysis was observed by ESR, it may be concluded that an iron-(ADR-degradation product) or a mixed iron-ADR-(ADR-degradation product) chelate was formed. The general term "iron-(ADR-product)" will be used for these chelates.

The shift of the absorbance peak from 610 nm to 570 nm, observed with aging of ADR or iron–ADR, most probably reflects the appearance of a degradation product with an absorbance maximum in that region. Beraldo *et al.* [14] reported spectral changes in Fe(III)–ADR preparations up to 24 hr after mixing, attributing these to conformational changes in the complex. While the present results indicate that a shift of iron-binding from ADR to one of its products occurred as degradation proceeded, it is more likely that the spectral change is due to the appearance of a certain degradation product, since it also occurred in the absence of iron, albeit to a lesser degree.

The binding of iron to the degradation products is stronger than its binding to ADR, as evidenced by the inability of DFO, EDTA and DTPA to remove iron from the aged chelate. This characteristic is probably reflected in the failure of EDTA to remove the iron from a 12-hr-old ternary DNA–Fe(III)–ADR chelate, as reported by Eliot *et al.* [9], whereas in a freshly prepared ternary chelate the iron was readily available to ligand exchange by chelating agents (unpublished results). The difference between the $Fe(III)ADR_3$ and iron-(ADR-product) chelates is also reflected in the discrepancy between the reports of Beraldo *et al.* [14], who found that apotransferrin is unable to remove iron from preformed $Fe(III)ADR_3$, and Demant and Nørskov-Lauritsen [10], who showed that at physiological pH ADR is unable to remove iron from transferrin. The latter report is in good agreement with the effective binding constants of $Fe(III)ADR_3$ and transferrin [16], whereas the results of the former, using a preformed "stable conformation" chelate implies that iron-(ADR-product) has a log $K_{eff} > 20$.

It was shown that the iron in $Fe(III)ADR_3$, but not in iron-(ADR-product), is readily reduced by cysteine. The toxicity of ADR has often been ascribed to free radical-induced damage [1, 9, 11, 15], caused by hydroxyl radicals produced through redox cycling of transition-metal ions associated with ADR. Consequently, the altered redox behavior of iron-(ADR-product) as compared to $Fe(III)ADR_3$ may explain the reduced toxicity of aged $Fe(III)ADR_3$. Specific types of damage, such as *in vivo* lipid peroxidation [8] may be similarly affected by the age of the chelate used. The difference in redox behavior between $Fe(III)ADR_3$ and iron-(ADR-product) may account for the discrepancy between reports of $Fe(III)ADR_3$-mediated free radical formation from reduction by thiols [9, 15] or by submitochondrial particles [24, 25] and the observation that $Fe(III)ADR_3$ (presumably the "stable conformation") does not mediate superoxide production by mitochondrial NADH dehydrogenase [14].

ADR degradation did not depend on molecular oxygen and occurred under anaerobic conditions. This suggests that an intermolecular disproportionation-type reaction may occur between ADR molecules, as previously suggested [26], producing ADR at altered oxidation states and giving rise to chemically modified ADR possessing the spectral characteristics and altered metal-binding properties observed. It has been reported that iron and ADR participate in a variety of redox reactions, including an intramolecular one-electron transfer between iron and ADR [4, 5, 17]. Furthermore, the reduction of ADR may cause chemical modifications of the molecule, such as cleavage of the glycosidic bond [1]. Several other redox reactions involving the components of the $Fe(III)ADR_3$ chelate have been reported as well. Thus, the role of iron in the acceleration of ADR-degradation may be to facilitate an iron-specific reaction, as previously suggested [17], or to catalyze electron transfer between ADR molecules. Details of the role of iron in the degradation of ADR are presently under investigation.

The possible involvement of iron-hydroxypolymer formation in the aging of $Fe(III)ADR_3$ was ruled out by the ESR study. It was shown that the monomeric and polymeric forms of ferric–citrate are readily distinguishable by their ESR signal (Fig. 5). The ESR spectra of fresh and aged $Fe(III)ADR_3$ were practically identical (Fig. 6), indicating that once the chelate is formed no polymerization occurs.

In the present study ADR was consistently kept in excess of Fe(III) to prevent the formation of iron-hydroxypolymers [21, 27] and consequent precipitation, and thus addressed only true chemical changes in the nature of the chelate and its components. In contrast, Bachur and co-workers [6] observed that, when injected in mice, mixtures of ADR with an excess of Fe(III) form aggregates that precipitate in certain organs. As the excess of iron is lowered, the mixtures must be aged to produce such precipitates. An ESR study of freshly prepared iron–ADR mixtures at various ratios showed that when the iron:ADR ratio was kept below 1:10 the iron was maintained in its monomeric form, and above 1:3 no monomeric iron was detected (unpublished results). Thus, the precipitable aggregates reported [6] apparently are iron-hydroxypolymers with superficially bound or entrapped ADR. When the excess of iron is lowered, the ADR-trapping capacity of the iron polymer is reduced. In view of the present results, it may be proposed that the aging is then required to produce ADR-degradation products with higher affinity for iron in order to form precipitates of iron-bound drug or drug-products. Consequently, the precipitates must be considered artifacts of the procedure for chelate preparation when [iron] approaches or exceeds [ADR], and of biological interest only inasmuch as trapping of the drug renders it unavailable to its ordinary targets and local effects may ensue due to vessel occlusion.

The iron-accelerated ADR-degradation with subsequent formation of an iron-(ADR-product) chelate has important practical implications, both for the interpretation of experimental results and for the biological use of Fe(III)–ADR mixtures. The striking difference in toxicity between fresh and aged chelates has extensive implications for the biological use of the $Fe(III)ADR_3$ chelate. Investigations involving mixtures of Fe(III) and ADR must take into account the time elapsed between formation of the chelate and its use in order to define the chemical species involved and to properly interpret the results. The present investigation thus dictates that Fe(III)–ADR should be used immediately following its preparation, since that is the only time the chelate is well defined.

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