Anthracycline Antibiotic Reduction by Spinach Ferredoxin–NADP$^+$ Reductase and Ferredoxin†

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ABSTRACT: Spinach NADPH:ferredoxin oxidoreductase (EC 1.6.7.1) catalyzes the NADPH-dependent reduction of the anthracyclines daunomycin, aclacinomycin A, and nogalamycin and their respective 7-deoxyanthracyclinones. Under anaerobic conditions, the endogenous rate of O_2 reduction by NADPH catalyzed by ferredoxin reductase (0.12 s^{-1} at pH 7.4) is augmented by the anthracyclines and 7-deoxyanthracyclinones. The catalytic constants are approximately equivalent for this augmentation for all substrates (approximate V of 2 s^{-1} and K_M of 75 μM). Both O_2^- and H_2O_2 are made. Under anaerobic conditions, anthracycline reduction catalyzed by ferredoxin reductase results in the elimination of the C-7 substituent to provide a quinone methide intermediate. Following tautomerization by C-7 protonation, 7-deoxyanthracyclinones are obtained. Under appropriate conditions these may be further reduced to the 7-deoxyanthracyclinone hydroquinones. For daunomycin, the quinone methide is formed rapidly after reduction and is easily monitored at 600 nm. It may react with electrophiles other than H^+, as demonstrated by its competitive trapping by *p*-carboxybenzaldehyde. It may also react with nucleophiles, as demonstrated by its competitive trapping by *N*-acetylcysteine. For aclacinomycin, quinone methide formation is also rapid although no distinct transient near 600 nm occurs. In addition to protonation, it reacts with itself providing the 7,7′-dimer. With ethyl xanthate as a thiolate nucleophile, adducts derived from addition to C-7 are obtained. For nogalamycin, quinone methide formation is not rapid. Nogalamycin is reduced to its hydroquinone, which slowly converts in a first-order process [$k = (1.2 \pm 0.2) \times 10^{-3}$ s^{-1}, pH 8.0, 30 °C] to the quinone methide, which is then quenched by protonation. Spinach ferredoxin in its reduced form is chemically competent for anthracycline reduction. Its effect on both the aerobic and anaerobic reactions catalyzed by ferredoxin reductase is to increase severalfold the overall velocity for anthracycline reduction. In conclusion, the aerobic reaction pathways for the anthracyclines as mediated by ferredoxin reductase are remarkably similar, while the anaerobic reactions are remarkably different. If these anthracyclines exert their antitumor activity by a common anaerobic pathway, it is most likely that the pathway is determined by the properties of the anthracycline as complexed to its in vivo target. The behavior of ferredoxin further suggests that not only low-potential flavin centers but also iron–sulfur centers should be regarded as important loci for anthracycline reductive activation.

The anthracycline glycosides represent an important class of antitumor antibiotics (Arcamone, 1981, 1984; Young et al., 1981). Several of the biological effects of anthracycline administration are presumed to derive from their quinone moiety acting as an oxidant. Whether this ability contributes more to the general toxicity or to the antineoplastic activity is as yet unknown. In any case, quinone reduction is unquestionably a major route of primary metabolism, both in bacteria (Marshall et al., 1976; Rueckert et al., 1979) and in mammals (Schwartz & Parker, 1981; Bachur et al., 1982). In spite of the potential importance of these oxidizing processes to the in vivo chemistry of the anthracyclines, the chemical mechanisms involved are incompletely characterized.

All studies indicate that the chemical mechanism taken after quinone reduction is determined by the presence or absence of molecular oxygen (Bachur et al., 1979; Komiyama et al., 1979). With O_2 present, rapid electron transfer from the reduced anthracycline to O_2 occurs, regenerating the quinone oxidation state. With O_2 absent, intramolecular elimination of the C-7 glycoside occurs with a rate constant of at least 1 s^{-1} (Rao et al., 1978). The present evidence suggests that this elimination is reserved to the hydroquinone and not to the semiquinone oxidation state (Kleyer & Koch, 1984). The resulting quinone methide expresses a diversity of chemical behavior. It may act as a nucleophile to undergo solvent protonation at C-7 (Moore, 1977; Moore & Czerniak, 1980; Fisher et al., 1982; Kleyer et al., 1984) or react with other electrophiles (Kleyer & Koch, 1984). If C-11 is unsubstituted, it may act as both electrophile and nucleophile with respect to itself to provide a 7,7′-dimer (Kleyer et al., 1984) or in the presence of an oxidant to react with other nucleophiles (Ramakrishnan & Fisher, 1983). Since these reactions exhibit a dependence on the anthracycline structure, and as several anthracyclines are presently undergoing in vivo antitumor antibiotic evaluation, a systematic investigation of the aerobic and anaerobic processes of the anthracyclines is warranted.

A set of anthracyclines that span a range of substitution, yet retain antitumor efficacy, is examined here (Chart I). Daunomycin (**1a**) is a class I anthracycline and is structurally quite similar to adriamycin; these two are the primary anthracyclines in clinical use. Anaerobic reduction of daunomycin yields 7-deoxydaunomycinone (**1b**) as the only product (Smith et al., 1976). Aclacinomycin (**2a**) is a class II anthracycline with excellent in vivo activity; its anaerobic reduction provides a mixture of 7-deoxyaklavinone (**2b**) and the 7,7′-dimer (**3**) (Komiyama et al., 1979). Nogalamycin (**4a**) differs from the preceding anthracyclines both by a second

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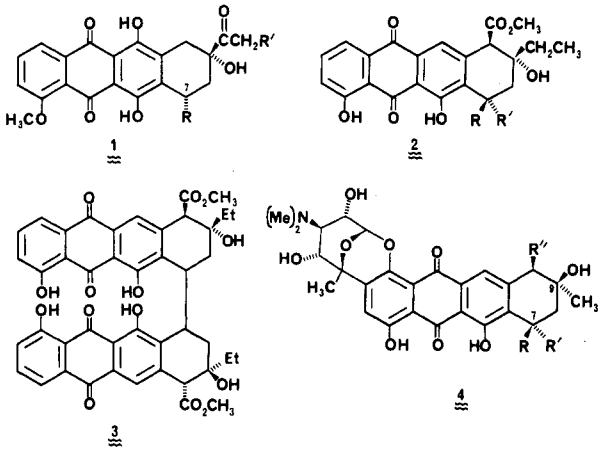
Chart I: Anthracyclines and Anthracyclinones[a]



[a] Daunomycin family (1): daunomycin (1a, R = L-daunosamine; R′ = H); 7-deoxydaunomycinone (1b, R = R′ = H); daunomycinone (1c, R = OH; R′ = H); adriamycin (1d, R = L-daunosamine; R′ = OH); epirubicin (1e, R = 4′-epi-L-daunosamine; R′ = OH). Aclacinomycin family (2): aclacinomycin A (2a, R′ = L-rhodosamine-2-deoxy-L-fucose-L-cinerulose A; R = H); 7-deoxyaklavinone (2b, R = R′ = H); 7(*R*)-[ethoxy(thiocarbonyl)]-thio-7-deoxyaklavinone (2c, R = $SCSOCH_2CH_3$; R′ = H); 7(*S*)-[ethoxy(thiocarbonyl)]thio-7-deoxyaklavinone (2d, R = H; R′ = $SCSOCH_2CH_3$). Bi(7-deoxyaklavinon-7-yl) (3). Nogalamycin family (4): nogalamycin (4a, R = H; R′ = nogalose; R″ = CO_2CH_3); 7-deoxynogalarol (4b, R = R′ = H; R″ = CO_2CH_3); (7*R*)-nogamycin (4c, R = nogalose; R′ = R″ = H); menogarol (4d, R = OCH_3; R′ = R″ = H); epimenogarol (4e, R′ = OCH_3; R = R″ = H); 7-deoxynogarol (4f, R = R′ = R″ = H).

D-ring glycoside and by its unusual 7*S*,9*S*,10*R* stereochemistry (Wiley et al., 1977; Arora, 1983). Anaerobic reduction of nogalamycin provides as product 7-deoxynogalarol (**4b**) (Marshall et al., 1976; Wiley et al., 1977). Menogarol (**4d**) is prepared by displacement of the C-7 nogamycin glycoside by methoxide, with concomitant improvement in the in vivo activity (Wiley et al., 1982). Its anaerobic reduction yields 7-deoxynogarol (**4f**). These anthracyclines provide the requisite set for mechanistic study.

Anthracycline reduction in vivo occurs under enzymatic aegis. Bachur and co-workers have found several low-potential flavin-containing enzymes that are excellent in vitro catalysts (Pan et al., 1981; Gutierrez et al., 1983). This observation and several additional criteria were used to identify the enzymatic catalyst for this study. The enzyme sought must have the ability to pass electrons at low potential to a broad variety of substrates, be available in homogeneous form in large quantities, and offer experimental versatility. In particular, it was desired to explore the possibility that enzymatic prosthetic groups other than flavin—such as metal centers—were competent catalysts for anthracycline reduction. From these criteria the choice of spinach NADPH:ferredoxin reductase (EC 1.6.7.1) as catalyst is made. This enzyme has also been used by Gutteridge & Toeg (1982) in their study of the redox reactions of the iron–adriamycin complex.

Ferredoxin reductase possesses a low-potential flavin (E_m = −442 mV, pH 8; Batie & Kamin, 1981) more than sufficient to reduce anthracyclines ($E_m \sim -300$ mV; Rao et al., 1978; Land et al., 1983). Further, it catalyzes the NADPH reduction of a broad variety of acceptors (Zannetti & Curti, 1980) and is therefore reasonably expected to also mediate anthracycline reduction. Further, ferredoxin reductase reversibly transfers electrons to ferredoxin, a low-potential (E_m = −420 mV; Tagawa & Arnon, 1968) Fe_2S_2-containing protein, through formation of a 1:1 enzyme–protein complex (Batie & Kamin, 1981). Both the enzyme and protein are readily available. Thus by use of ferredoxin reductase alone and ferredoxin reductase with ferredoxin, a quantitative comparison of the flavin and iron–sulfur groups as catalysts of anthracycline reduction may be made.

Experimental Procedures

Materials. Sources for the reagents and the instrumentation used are provided in the supplementary material (see paragraph at end of paper regarding supplementary material).

Spinach NADPH:Ferredoxin Oxidoreductase (EC 1.6.7.1). Ferredoxin reductase is purified from spinach to near homogeneity by published procedures (Zanetti & Curti, 1980; Batie & Kamin, 1981). The enzyme has an absorbance ratio at 275–456 nm of 10.0 (literature value 8.3; Zanetti et al., 1982) and a visible absorption spectrum indicating that no other chromophoric enzyme is present. This permits the determination of this enzyme's concentration by use of its known ϵ_{456nm} = 10700 M^{-1} cm^{-1} (Zanetti et al., 1982). The specific activity of the ferredoxin reductase is measured in 0.10 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0 buffer, with 1.0 mM NADPH and 1.0 mM ferricyanide as electron acceptors, and is found equal to 330 s^{-1}. The enzyme is stored frozen in 0.10 M Tris-HCl buffer at −20 °C.

Spinach ferredoxin is likewise purified to near homogeneity by its recovery from the first DEAE column (Zanetti & Curti, 1980), followed by gradient elution from a smaller DEAE column and gel permeation chromatography. The purified protein is stored frozen in 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0, buffer. Its concentration is determined from ϵ_{420nm} = 9700 M^{-1} cm^{-1} (Batie & Kamin, 1981). Reduced ferredoxin is produced by anaerobic photoreduction [15 mM ethylenediaminetetraacetic acid (EDTA) in the presence of 0.1 equiv of 5-deaza-5-carbariboflavin, using a 2-min sungun illumination; Massey & Hemmerich, 1978]. The reduced protein is stored under positive N_2 pressure in a Schlenk tube, and transfers are made by gas-tight syringe into the rapidly stirred, anaerobic solutions of the anthracyclines.

Ferredoxin reductase activity during anthracycline reductions is measured by removal of an appropriate portion to the standard NADPH and ferricyanide assay. Recovery of the enzyme at the reaction conclusion is accomplished by dilution of the reaction with distilled water and absorption of the enzyme on a small (1-cm^3) DEAE column. The enzyme is removed with high salt and then passed through a 20 cm × 1 cm G25 column (Zanetti & Curti, 1980). Fractions containing enzyme are identified by assay and by the absorption at 278 or 456 nm.

Aerobic anthracycline reduction is quantitated by monitoring O_2 loss in an oxygen electrode chamber. The electrode is calibrated by using the endogenous oxidase activity of ferredoxin reductase, independently measured by NADPH consumption spectrophotometrically. This oxidase activity is 0.12 s^{-1} (0.10 M Tris-HCl, pH 7.4 buffer, 30 °C) and 0.18 s^{-1} (0.10 M Tris-HCl, pH 8.0 buffer, 30 °C). Experiments that systematically compare the anthracyclines are done at pH 7.4, due to the insolubility of aclacinomycin at higher pH. Individual experiments are done at pH 8.0, due to the higher limiting velocity for ferredoxin reductase at this pH. Assays are initiated by injection of an appropriate quantity of a dimethylformamide solution of the anthracycline or 7-deoxyanthracyclinone into a vigorously stirred solution containing 0.5 mM NADPH and 23 nM ferredoxin reductase. The final dimethylformamide concentration is 1% (v/v) for the anthracyclines and 5% (v/v) for the 7-deoxyanthracyclinones. Substantial precipitation of the aglycons occurs shortly after addition, but nevertheless an estimate of the initial velocity

is possible. Control experiments establish that dimethylformamide does not alter the initial velocity. For assays containing ferredoxin, this protein is added to give a final concentration equal to 2 μM. All velocities are corrected for the endogenous oxidase activity and plotted as v vs. $v/[S]$ for the calculation of V and K_M.

Anaerobic anthracycline reductions are accomplished by using standard anaerobic techniques. Buffers are made O_2 free by boiling, followed by cooling under a positive N_2 flow. Enzyme solutions are made anaerobic by passing a moist N_2 flow over a gently stirred solution for at least 30 min. All transfers are by cannula or gas-tight syringe. The anaerobic 10.0-mm path length cell contains a stir bar and a septum cover to allow reagent transfer. Reactions are performed in the cuvette under positive N_2 flow and are initiated by rapid injection of the ferredoxin reductase. The mixing time under these conditions is <2 s. Kinetic determinations are made at 30 °C in 0.10 M Tris-HCl, pH 7.4 buffer for aclacinomycin and in 0.10 M Tris-HCl, pH 8.0 buffer for daunomycin, nogalamycin, and menogarol. These solutions contain NADPH (approximately 1.2 mM), anthracycline (approximately 75 μM), and ferredoxin reductase (approximately 0.5 μM) in a total volume of 2.65 mL. Ferredoxin (when present) is at 1.2 equiv to ferredoxin reductase. Glucose (5 mM), glucose oxidase (40 units), and catalase (10000 units) are present to further ensure anaerobicity. Preparative scale reactions also contain Mn^{2+}, isocitrate, and isocitrate dehydrogenase to regenerate NADPH, as $NADP^+$ is a strong competitive inhibitor (K_i = 10 μM; Dykes & Davies, 1982) of ferredoxin reductase.

Kinetic data are collected by using a microcomputer interfaced to the spectrometer. Initial velocities are estimated from the slope over the first 5 s. First-order decays were analyzed by Guggenheim's equation, choosing a stepping interval of approximately $t_{1/2}/8$ and a separation interval of approximately $t_{1/2}$, such that a set of 8–10 points from the first half-life are paired with a matching set from the third half-life. Correlation coefficients routinely exceeded 0.998.

Discontinuous assay of the anaerobic reactions is done by removal of a portion of the reaction by syringe and mixing under aerobic conditions with $CHCl_3$ to extract the anthracyclines and anthracyclinones from the aqueous layer. Product identification is made by comparison with known samples by thin-layer chromatography.

Mn^{2+} association to daunomycin and nogalamycin is observed by the spectral changes upon titration of Mn^{2+} in 0.05 M Tris-HCl, pH 7.4 buffer. The calculated K_d is 24 ± 4 mM for daunomycin and 29 ± 4 mM for nogalamycin, indicating that Mn^{2+} should not interfere with the outcomes of the preparative experiments.

Preparative Isolation of 7-Deoxydaunomycinone from Quinone Methide Protonation in D_2O. A solution of Tris base, Tris-HCl, glucose, isocitrate, and $MnCl_2$ in 3 mL of D_2O is adjusted to pD 7.65 (glass electrode corrected) by addition of 10% DCl/D_2O by syringe. The solution is freeze-dried, reconstituted with 2 mL of D_2O, and freeze-dried a second time. To this, daunomycin and NADPH are added in 13 mL of D_2O, and the entire solution is made anaerobic by vigorous passage of an N_2 stream (wetted with D_2O) with stirring and sonication. Final reagent concentrations are as follows (after enzyme addition, below): Tris buffer, 0.125 M; glucose, 60 mM; isocitrate, 25 mM; $MnCl_2$, 2.5 mM; daunomycin, 1.5 mM; NADPH, 2.0 mM. An enzyme solution consisting of ferredoxin reductase, isocitrate dehydrogenase, glucose oxidase, and catalase in 1.0 mL of 0.10 M Tris, pH 8.0 buffer/2.0 mL of D_2O is ultrafiltered to approximately 1 mL, diluted with 3.0 mL of D_2O, and again ultrafiltered to approximately 1-mL volume. This solution is made O_2 free by gentle stirring in an N_2 atmosphere for 30 min and then transferred to the reagent solution by syringe. The final enzyme concentrations are as follows: ferredoxin reductase, 2.3 μM; isocitrate dehydrogenase, 4.2 units; glucose oxidase, 200 units; catalase, 300000 units. After enzyme addition the reaction turns brown-black rapidly, followed by fading of this color and precipitation of 7-deoxydaunomycinone. After 60 min almost complete conversion has occurred. The solution is exposed to O_2 and centrifuged to collect the 7-deoxydaunomycinone. This precipitate is carefully washed 3 times with distilled water and is dissolved in 2 mL of CH_3OH. This solution is diluted with $CHCl_3$ (15 mL), extracted with distilled water, and dried (Na_2SO_4), and the solvent is removed. Analysis for deuterium content is made by both electron impact mass spectrometry (EIMS) and ^1H NMR spectroscopy. For EIMS, the spectrometer is calibrated for M/(M + 1) ratios at m/z 382/383 and 339/340 with undeuterated 7-deoxydaunomycinone immediately before analysis of the deuterated sample; this latter sample's deuterium content is then determined by M/(M + 1) correction at m/z 382/383/384 and 339/340/341. Both peak clusters indicate a deuterium content of 0.84 ± 0.05 atom. The location of the ^2H is determined from the ^1H NMR at 300 MHz, using an aquisition time of 3.2 s and a pulse delay of 2.0 s, with resolution enhancement of the free induction decay. (Identical areas, but with greater error due to peak overlap, were estimated from integration of the unenhanced spectrum's peaks). With the $H_{10,ax}$ dd at δ 3.025 as the integration standard, the $H_{7,eq}$ cluster at $\sim\delta$ 3.1 integrates to 0.46 atom of ^1H and the $H_{7,ax}$, $H_{10,eq}$ clusters at $\sim\delta$ 2.9 integrate to 1.70 ^1H atoms. Subtracting 1.0 ^1H atom from this latter integration (assuming the two H_{10} integrations are equal) gives a total of 0.84 atom of ^2H and a 0.54/0.30 ratio of ^2H at $H_{7,eq}$ to $H_{7,ax}$. This corresponds to a 1.6-fold preference (estimated error, 25%) for *re* face protonation of the quinone methide by D^+.

Aldehyde Trapping of the Daunomycin Quinone Methide. To an anaerobic 35-mL solution of 0.10 M Tris-HCl, pH 8.0 buffer are added the following reagents: glucose (24 mM), $MnSO_4$ (0.6 mM), NADPH (0.52 mM), daunomycin (0.33 mM), lithium *p*-carboxybenzaldehyde (30 mM), isocitrate (10 mM), glucose oxidase (200 units), isocitrate dehydrogenase (4.5 units), and catalase (300000 units). The reaction is initiated by ferredoxin reductase to a final concentration of 0.56 μM. The solution rapidly turns from dark red to brown and then slowly to a lighter red, with precipitation of deoxyanthracyclinone products evident within 3 min after starting. After 45 min, the reaction is worked up by exposure to O_2 and by the addition of an equal volume of CH_2Cl_2 with vigorous stirring. The CH_2Cl_2 layer is separated, and from it is recovered 3.3 μmol of 7-deoxydaunomycinone. Adjustment of the solution to pH 2 and further CH_2Cl_2 extraction recover 1 μmol of 7-deoxydaunomycinone (total yield, 37%) and 4 μmol of the aldehyde adduct **6** (34% yield), as well as substantial quantities of *p*-carboxybenzaldehyde. Remaining in the aqueous layer is approximately 3 μmol of unreacted daunomycin.

The aldehyde adduct is purified on silica by elution with 70/30 ethyl acetate/hexanes to remove the 7-deoxydaunomycinone, followed by 85/15 ethyl acetate/hexanes to separate the adduct from most of the *p*-carboxybenzaldehyde. The crude adduct is esterified (anhydrous dimethylformamide, sodium bicarbonate, methyl *p*-toluenesulfonate) and the resulting methyl ester purified on silica eluting with 80/20

Table I: Kinetic Constants for Anthracycline-Stimulated Oxygen Reduction Catalyzed by Ferredoxin Reductase[a]

substrate	K_M (mM)	V (s^{-1})
none		0.12
daunomycin	0.06	2.7
7-deoxydaunomycin	0.08	1.9
aclacinomycin A	0.07	2.1
7-deoxyaklavinone	0.10	2.3
nogalamycin	0.07	3.4
7-deoxynogalarol	0.07	4.2

[a] Oxygen-saturated 0.10 M Tris-HCl buffer (pH 7.4, 30 °C) containing 0.5 mM EDTA, at 0.48 mM NADPH. Solutions contained 0.1% (v/v) (for anthracyclines) or 0.5% (v/v) (for 7-deoxyanthracyclinones) dimethylformamide.

chloroform/ethyl acetate containing 1% v/v acetic acid. The structure of the adduct is analogous to that of the benzaldehyde adduct of Kleyer & Koch (1984), as determined by its spectral properties: mass spectrum [fast atom bombardment (FAB), glycerol, negative ion] m/z 546 (73, M), 547 (100, M + 1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 13.44 (s, 1 H), 12.92 (s, 1 H), 8.01 (dd, J = 7.7 and 1.0 Hz, 1 H), 7.76 (d, J = 8.4 Hz, 2 H), 7.73 (app t, $J \sim$ 7.9 Hz, 1 H), 7.04 (d, J = 8.3 Hz, 2 H), 5.45 (d, J = 2.2 Hz, CH-7′), 4.01 (s, 3 H), 3.93 [app dd, $J \sim$ 5.6 and 3.0 Hz (by decoupling, ddd, J = 3.2, 3.2, and 2.2 Hz), CH-7], 3.85 (s, 3 H), 3.49 (dd, J = 19.3 and 1.2 Hz, CH-10_{eq}), 2.75 (d, J = 19.6 Hz, CH-10_{ax}), 2.72 (app ddd, $J \sim$ 11.8, 3.0, and 2.0 Hz, CH-8_{eq}), 2.57 (br s, OH), 1.88 (dd, J = 11.9 and 3.4 Hz, CH-8_{ax}), and 1.65 (s, 3 H).

Xanthate Trapping of the 7-Deoxyaklavinone Quinone Methide with H_2O_2. To a 1.0-mL solution of aclacinomycin (0.1 mM), NADPH (0.3 mM), and potassium ethyl xanthate (15 mM) in 35 mM Tris-HCl, pH 7.4 buffer containing 1 mM EDTA is added ferredoxin reductase (final concentration, 1.2 μM). The solution is initially air-saturated and remains open to the atmosphere throughout. The reaction proceeds at ambient temperature for approximately 30 min, at which time some precipitation of products has occurred. These products are extracted (CH_2Cl_2), the solution is dried (Na_2SO_4), and the solvent is removed by a nitrogen stream. The residue is dissolved in the mobile-phase solution (below), and the product analysis is made with an Alltech Spherisorb phenyl column (5 μm; 4.6 × 250 mm) using 48.4% tetrahydrofuran, 51.5% H_2O, and 0.1% trifluoroacetic acid (v/v/v) as the mobile phase. Effluent monitoring is at 440 nm, with peak assignments made by comparison with the retention times of authentic samples.

Results

A complete description of the anthracycline redox behavior requires the characterization of the aerobic and anaerobic pathways. The following sections provide this characterization for daunomycin, aclacinomycin, nogalamycin, and menogarol with NADPH as the reductant with ferredoxin reductase, and ferredoxin reductase with ferredoxin, as catalysts.

Aerobic Reduction by Ferredoxin Reductase. In oxygen-saturated 0.10 M Tris-HCl buffer (pH 7.4, 30 °C) at saturating NADPH, spinach ferredoxin reductase exhibits an endogenous oxidase activity of 0.12 s^{-1}. Upon addition of an anthracycline, this rate of O_2 reduction increases. A compilation of the kinetic constants for the anthracyclines and 7-deoxyanthracyclinones is in Table I. The limiting velocity and K_M values are all similar and occur near 2 s^{-1} and 75 μM, respectively. The product of anthracycline glycoside-stimulated O_2 reduction is the glycoside itself, consistent with previous observations that electron transfer to O_2 occurs more quickly than glycoside elimination (Bachur et al., 1979; Komiyama et al., 1979). Further experiments indicate that both one- and two-electron reductions of oxygen occur. The ultimate product of O_2 reduction is H_2O_2, as seen by a half-fold recovery of the O_2 upon catalase addition to the completed reaction. An involvement of O_2^- was examined with ferricytochrome c as an oxidant trap for O_2^- by using nogalamycin-stimulated O_2 reduction. Addition of nogalamycin to a solution of NADPH and ferredoxin reductase results in an increased rate of cytochrome c reduction.[1] The dependence of this reaction on [nogalamycin][1] parallels that observed for overall O_2 reduction (Table I). Superoxide dismutase reduces this stimulation by a maximum of 60%. This indicates that cytochrome c may be directly reduced by the reduced anthracycline, as has been observed for other quinones (Bates & Winterbourn, 1981). Assuming that O_2^- is quantitatively trapped by the cytochrome c, the difference between total O_2 reduction and cytochrome c reduction provides an estimate of the ratio of O_2 reduced to O_2^- and to H_2O_2 in the presence of nogalamycin; this difference indicates that O_2^- comprises approximately 20% of the reduced oxygen yield. Lastly, it is noted that the 7-deoxyanthracyclinones are comparable to the parent glycosides in stimulating O_2 reduction. Ferredoxin reductase is similar in this respect to other flavoenzymes (Pan et al., 1981), and its behavior supports the generalization that both anthracyclines and deoxyanthracyclinones may contribute in vivo to O_2^- and H_2O_2 production.

Ferredoxin Reductase Inactivation during Anthracycline Aerobic Turnover. Ferredoxin reductase is sensitive to sulfhydryl reagents. Modification of an essential thiol results in an immediate loss in catalytic activity and is followed by a slower, irreversible denaturation process accompanied by coenzyme dissociation (Zanetti et al., 1982). As the redox cycling of anthracyclines in oxygenated solution yields hydrogen peroxide, the possibility exists that the hydrogen peroxide might oxidize the catalytically essential thiol. This possibility was confirmed by combination of excess NADPH and daunomycin with ferredoxin reductase in aerobic buffer, resulting in the loss of 26% of the ferredoxin reductase activity at the completion of the aerobic reaction. The final [H_2O_2] under these conditions is determined by the initial [O_2] and is approximately 0.20 mM. A direct involvement of H_2O_2 in this loss is indicated by several experiments. Addition of H_2O_2, to a final concentration of 0.20 mM, to ferredoxin reductase gives a 22% loss in activity over a 30-min period. This is comparable to the loss observed above, when the H_2O_2 is generated in situ. Similar activity decreases are observed when either the anthracycline or 7-deoxyanthracyclinone is used to generate the H_2O_2, precluding an involvement of the C-7 glycoside (leading to a potentially electrophilic quinone methide). Finally, ferredoxin reductase is completely protected from this activity loss by either dithiothreitol or catalase.

Aerobic Reduction by Ferredoxin. Ferredoxin and ferredoxin reductase form a stable 1:1 complex, within which intermolecular e transfer occurs (Batie & Kamin, 1981). Although electron transfer from reduced ferredoxin reductase to oxidized ferredoxin is thermodynamically unfavored, with saturating NADPH and an oxidant trap a substantial velocity for e transfer from NADPH to the trap, catalyzed by ferredoxin reductase and ferredoxin, may be sustained (Forti & Sturani, 1968; Nagai & Yoneyama, 1983). Among the traps that may be used is O_2 (Hosein & Palmer, 1983). Thus, addition of 2 μM ferredoxin to an aerobic solution of NADPH

[1] A figure that illustrates this experiment is included in the supplementary material. This information will also be provided with reprint requests.

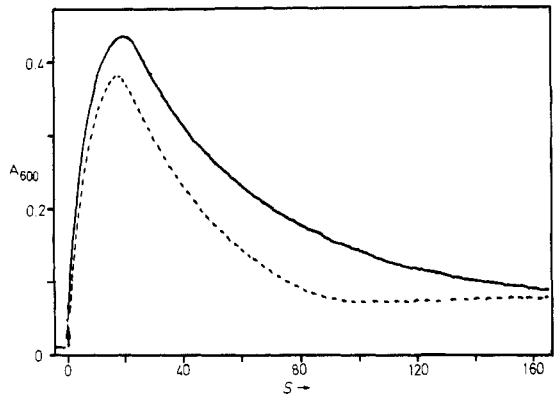


FIGURE 1: Representative kinetics of formation and decay of the 7-deoxydaunomycinone quinone methide, observed at 600 nm against time (seconds) upon addition of ferredoxin reductase (at arrow, final concentration of 3.7 μM) to an anaerobic solution of daunomycin (74 μM) and NADPH (1.3 mM). The quinone methide is observed in 0.10 M Tris-HCl, pH 8.0 buffer (---) and 0.05 M Tris-DCl, pD 8.0 (glass electrode corrected) buffer (—). The initial zero-order velocity with respect to enzyme for quinone methide formation is approximately 2 s^{-1} for both, and the observed first-order rate constant for quinone methide decay is 0.046 s^{-1} (H_2O) and 0.023 s^{-1} (D_2O).

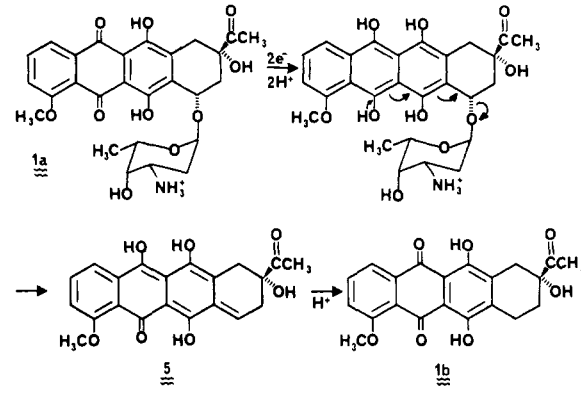
and 23 nM ferredoxin reductase increases the initial velocity for O_2 reduction from 0.12 to 15 s^{-1} (pH 7.4, 30 °C). If electron transfer from reduced ferredoxin to the anthracyclines is likewise more rapid than from ferredoxin reductase, an even greater rate of O_2 reduction should ensue. This is observed. Addition of 0.10 mM anthracycline increases the initial velocity to 25 s^{-1}, using daunomycin, aclacinomycin, and nogalamycin. This increase, while modest, provides evidence for reduced ferredoxin as a direct anthracycline reductant. This supposition is directly confirmed by a separate experiment, as discussed below.

Anthracycline Reduction by Reduced Ferredoxin. Ferredoxin in its reduced state is a powerful reductant, and electron transfer from it to the anthracyclines is favored by approximately 100 mV (Berg et al., 1982; Land et al., 1983; Tagawa & Arnon, 1968). The chemical competence of reduced ferredoxin in anthracycline reduction is established by a straightforward titration, under anaerobic conditions, of the anthracyclines by reduced ferredoxin obtained by photoreduction. It is observed that electron transfer occurs within the mixing time (<2 s). For daunomycin, the spectral conversions are to 7-deoxydaunomycinone (2 equiv of reduced ferredoxin) and then to 7-deoxydaunomycinone hydroquinone.[1] These two products are identified by their spectra (Fisher et al., 1982; Kleyer & Koch, 1984). Equilibrium concentrations of the semiquinone are not seen (no absorption above 600 nm). Likewise, nogalamycin and aclacinomycin are similarly reduced by reduced ferredoxin. These titrations indicate a facile ability of reduced ferredoxin to transfer electrons to these quinones.

Anaerobic Anthracycline Reduction by Ferredoxin Reductase: (*A*) *Daunomycin.* The anaerobic behavior of the anthracyclines is much more complex than the aerobic behavior. Whereas daunomycin, aclacinomycin, and nogalamycin have essentially identical abilities to mediate electron flow to O_2, anaerobically different pathways emerge that are unique to each anthracycline. In order to indicate these contrasts, a detailed presentation will be made for daunomycin, to which the others are then compared.

The essential feature of anaerobic daunomycin reduction is the appearance and decay of the quinone methide tautomer (Figure 1). This reaction is absolutely dependent on the presence of ferredoxin reductase as the catalyst. Immediately

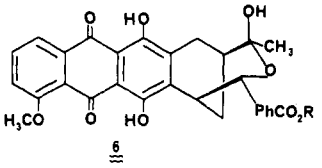
Scheme I



following the initiation of the reaction, a blue intermediate ($\lambda_{max} \sim 590$ nm) is seen. The intermediate then converts to 7-deoxydaunomycinone, appearing as a fine red precipitate within the cuvette. Several experiments establish the blue intermediate as the quinone methide **5**, obtained by intramolecular elimination of daunosamine after reduction (Scheme I). This quinone methide has been previously observed as the obligatory intermediate in the nonenzymatic anaerobic reductive conversion analogous to this enzymatic reaction and possesses $\epsilon_{618nm}^{CH_3OH} = 9400$ M^{-1} cm^{-1} (Kleyer & Koch, 1984). By use of this extinction coefficient, the initial velocity of the intermediate's appearance in the ferredoxin reductase reaction is estimated as 1.6 s^{-1}, very close to the initial velocity for O_2 reduction under identical, but aerobic, conditions (2.5 s^{-1}).[2] The rate of decay of the intermediate from the enzymatic reaction is reasonably approximated as a first-order process having $k = 0.046$ s^{-1} (pH 8.0), a somewhat larger rate constant (as expected) than that for quinone methide protonation by CH_3OH ($k = 0.013$ s^{-1}; Kleyer & Koch, 1984). In D_2O solvent, the rate of the intermediate's decay is diminished at least 2-fold (Figure 1). This solvent deuterium isotope effect[3] is less than that in CH_3OH (Kleyer & Koch, 1984) but is similar to the aqueous solvent deuterium effect for enol ether protonation (Leinhard & Wang, 1969). Examination of the 7-deoxydaunomycinone from the enzymatic reaction in D_2O shows 0.75 atom of deuterium preferentially incorporated into the H_{re} of C-7 (by 1.6-fold) as observed previously with chemical reduction (Fisher et al., 1983). Kleyer and Koch have further observed that the quinone methide may be competitively trapped by electrophiles other than H^+, such as benzaldehyde. This has likewise been found true for the blue intermediate in this enzymatic reaction. Generation of this intermediate in the presence of 37 mM *p*-carboxybenzaldehyde attenuates the intermediate's concentration by increasing its rate of decay ($k_{obsd} = 0.095$ s^{-1}; Figure 2). This increased decay indicates a bimolecular rate constant for aldehyde trapping of 1.3 M^{-1} s^{-1} and predicts that under the conditions of Figure 2 the quinone methide is as equally well trapped at C-7 by the aldehyde as by the proton. This is observed. Isolation and purification of the reaction mixture provide two products in equal quantities within experimental error. The first is 7-deoxydaunomycinone (**1b**) and the second the 7*R* aldehyde adduct **6**, characterized as the methyl ester. The

[2] Since the extinction coefficients of anthracyclines in water are generally lower than those in methanol, it is probable that 1.6 s^{-1} is a lower limit. Our best estimate for the quinone methide extinction coefficient is $\epsilon_{590nm}^{H_2O} \sim 6000$ M^{-1} cm^{-1}, which would change the initial velocity to 2.0 s^{-1}.

[3] From the ratio of k_{obsd} for H_2O to that for D_2O of 2.0 and incomplete deuteration at C-7 (0.75 atom of ^2H incorporated), the observed solvent isotope effect may be corrected to $k_{H_2O}/k_{D_2O} = 3.0 \pm 0.5$.



6

structure of this adduct is established by its spectral properties, which are found to be completely analogous to those reported for the benzaldehyde adduct of the quinone methide obtained under nonenzymatic conditions (Kleyer & Koch, 1984).

As the conditions of Figure 1 represent a convenient means of observing the daunomycin-derived quinone methide, an examination of its reactivity toward the ubiquitous biological electrophile, oxidized glutathione, is made. Observation of the quinone methide in the presence of 13 mM oxidized glutathione shows no decrease in its intensity nor in its duration. 7-Deoxydaunomycinone is obtained quantitatively. It may be concluded that the bimolecular reactivity of this quinone methide as a nucleophile is limited to more reactive electrophiles.

The difficulty in identifying reaction conditions allowing bimolecular reaction of the 7-deoxydaunomycinone quinone methide as an electrophile, with solution nucleophiles, is previously discussed (Fisher et al., 1983; Ramakrishnan & Fisher, 1983). However, it is now observed that quinone methide generation in the presence of excess *N*-acetylcysteine, with limiting NADPH, leads to appreciable trapping of the quinone methide and adduct formation.[4] Thus anaerobic reduction of daunomycin (0.3 mM) by NADPH (0.15 mM) catalyzed by ferredoxin reductase, with 20 mM *N*-acetylcysteine present, yields 50% adduct, as a 7:3 diastereomeric mixture (at C-7). A preliminary assignment of a 7*S* configuration to the major adduct is made possible by the coupling constants in the ^{1}H NMR spectrum. Hence, the quinone methide derived from daunomycin behaves either as an electrophile or as a nucleophile depending on reaction circumstances.

An examination of daunomycinone with ferredoxin reductase under conditions similar to those of daunomycin was made to determine the effect of the leaving group on the quinone methide generation. Both molecules provide the identical methide, but in the case of daunomycinone by expulsion of a potentially poorer leaving group (pK_a of the conjugate acid of OH^- is 15.74, compared to the pK_a of the conjugate acid of daunosamine, estimated as approximately 12). Observation of the anaerobic reduction of daunomycinone by NADPH, catalyzed by ferredoxin reductase, at 600 nm shows an absorbance increase followed by a decay that is qualitatively similar to those for daunomycin.[1] The decay is much more poorly defined due to competitive precipitation of the 7-deoxydaunomycinone product. Discontinuous assay ($CHCl_3$ quench) indicates that the overall forward velocity to 7-deoxydaunomycinone is comparable for daunomycinone relative to daunomycin. This indicates that the formation of the quinone methide is not sensitive to the leaving group pK_a in this system, confirming the original observation of Rao et al. (1978). In this regard it is noted that leaving group pK_a provides a poor estimate of nucleofugalities for E2-like eliminations (Stirling, 1979).

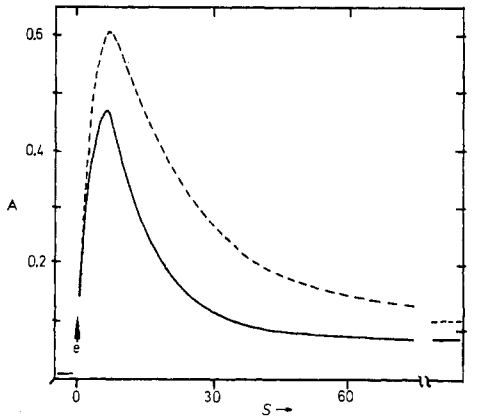


FIGURE 2: The appearance and decay of the 7-deoxydaunomycinone quinone methide, monitored at 600 nm against time (seconds), obtained by ferredoxin reductase catalyzed reduction of daunomycin (85 μM) by NADPH (1.2 mM). The enzyme is added to a final concentration of 4.7 μM to initiate the reaction (arrow). The two curves are obtained in the presence (—) and absence (- - -) of *p*-carboxybenzaldehyde as an alternative electrophile (to H^+) for the quinone methide. Both reactions are in anaerobic 0.10 M Tris-HCl, pH 8.0 buffer containing 1 mM EDTA at 30 °C.

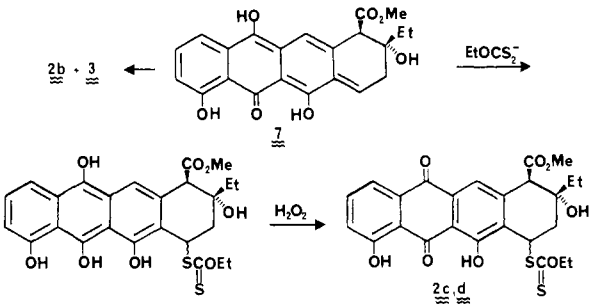
Adriamycin (**1d**) behaves quite similarly to daunomycin upon reductive activation. Quinone methide formation is observed at 600 nm,[1] having a zero-order rate with respect to enzyme of 3.4 ± 0.4 s^{-1} for its formation, at 68 μM adriamycin. Its decay occurs in a first-order fashion (k = 0.030 ± 0.005 s^{-1}, pH 8.0) and provides 7-deoxyadriamycinone. Epirubicin (**1e**) is a stereoisomer of adriamycin (epimeric at the C-4′ of daunosamine) with improved antibiotic activity (Arcamone, 1984). Although its quinone methide is the same as that from adriamycin, it is conceivable that the quinone methide's formation would be altered due to an effect of the epimerization either on the enzyme association or on the epidaunosamine nucleofugality. When an epirubicin concentration identical with that for adriamycin (68 μM) is used, the quinone methide appears with an initial zero-order rate of 4.4 ± 0.4 s^{-1} and decays with a first-order rate constant of 0.030 ± 0.005 s^{-1}. Hence the epimerization has no effect on this reaction.

(*B*) *Aclacinomycin.* In contrast to daunomycin, anaerobic reduction of aclacinomycin (pH 7.4) gives a weak, nonreproducible transient when it is monitored at the λ_{max} of the 7-deoxyaklavinone quinone methide tautomer (548 nm; Kleyer et al., 1984). Nonetheless, discontinuous assay of the reaction indicates that the aclacinomycin is consumed at a velocity comparable to that seen for daunomycin. This requires either that the quinone methide is not made or that it is made but its decay occurs faster than the analogous quinone methide derived from daunomycin. Examination of the reaction products supports the latter conclusion. Two products are seen, 7-deoxyaklavinone (**2b**, 40%) and the 7,7′-dimer (**3**, 60%). As **3** has been shown to derive from quinone methide dimerization (Kleyer et al., 1984), its appearance provides excellent evidence for quinone methide formation in the course of the enzymatic reaction. Also contributing to an increased rate of quinone methide decay is the lower pH (7.4), since the velocity for quinone methide protonation to 7-deoxyaklavinone may reasonably be presumed to be directly proportional to $[H^+]$ (Kleyer et al., 1984). Either or both of these processes may account for the weak quinone methide transient.

Several unsuccessful experiments were made in an effort to trap the aclacinomycin-derived quinone methide with *p*-carboxybenzaldehyde (the use of more electrophilic aldehydes

[4] K. Ramakrishnan, unpublished experiments. The negative results of our previous studies were obtained with ethyl xanthate as a nucleophile, on the presumption that its bimolecular rate constant toward the quinone methide would be exceptionally high. While this assumption is vindicated for the 11-deoxyanthracyclines, it is not here: Under identical conditions where *N*-acetylcysteine successfully traps the quinone methide, ethyl xanthate does not. A complete discussion and proof of structure for the adducts will be reported in due course.

Scheme II



is precluded by their rapid abolition of ferredoxin reductase activity). A conclusion that 11-deoxy quinone methides are unreactive is not yet warranted. Preliminary experiments[5] indicate that an aldehyde adduct is obtained from the quinone methide derived from 11-deoxydaunomycin, where intramolecular hemiacetal formation remains possible. This suggests that hemiacetal formation may influence adduct stability. The reactivity of 11-deoxyanthracycline-derived quinone methides to thiol nucleophiles has been previously noted (Ramakrishnan & Fisher, 1983). The salient aspect to the formation of a stable adduct is the copresence of an oxidizing agent, to convert the initial adduct from its hydroquinone oxidation state to the quinone oxidation state. As the hydroquinone, elimination of the C-7 group to re-form the quinone methide remains favored, and the quinone methide is eventually irrevocably lost to solvent protonation and dimerization. In the initial studies oxidation is achieved by coproportionation with the anthracycline glycoside. In the present studies the circumstances of ferredoxin reductase catalyzed anthracycline reduction offered an alternative route to these adducts. Consider the first events upon arrival of an anthracycline to an enzyme. Catalytic O_2 consumption will commence, proceeding to anaerobicity, at the expense of NAD(P)H and with the production of O_2^- and H_2O_2. The first anaerobic turnover provides the quinone methide. If a willing nucleophile is available, addition to the quinone methide **7** will occur, and should hydrogen peroxide remain from the aerobic reaction phase, it may act as the requisite oxidant (Scheme II).

This hypothesis has been examined by the initiation of aclacinomycin reduction, catalyzed by ferredoxin reductase, by NADPH in buffer that is initially air saturated. The nucleophile provided for the quinone methide is ethyl xanthate, as the adducts have known structures. Examination of the reaction products indicates formation of substantial quantities of the C-7 diastereomers **2c** and **2d**.[1] These are present in a ratio of 7*S* to 7*R* of 4:1, similar to previously observed data (Ramakrishnan & Fisher, 1983). Inclusion of catalase reduces the yield measurably. Although it is not possible to differentiate H_2O_2 acting directly as an oxidant or indirectly by initiating coproportionation, this simple experiment suggests a circumstance by which similar adducts may form in vivo. Furthermore, it offers a mechanistic union between the aerobic and anaerobic anthracycline chemistry.

(*C*) *Nogalamycin.* The nature of anaerobic nogalamycin reduction differs even yet from that for daunomycin and aclacinomycin. No long-wavelength transient is observed during the initial course of the reaction; rather, nogalamycin converts to a yellow intermediate (λ_{max} = 428 nm).[1] The spectral characteristics indicate this intermediate to be a hydroquinone. The hydroquinone then decays slowly [k = (1.2 ± 0.2) × 10^{-3} s^{-1}, pH 8.0] to an oxidized anthracycline spectrum (Figure 3). The spectral conversions suggest that nogalamycin undergoes rapid enzymatic reduction to its hydroquinone, followed by a slower conversion to 7-deoxynogalarol in an enzyme-independent process. This sequence is proven by two simple experiments. First, termination of the reaction at the point of maximal 428-nm absorption, by $CHCl_3$ extraction and air oxidation, results in the near quantitative recovery of nogalamycin. Second, the spectrum obtained from decay of the nogalamycin hydroquinone is found to be identical with that for 7-deoxynogalarol, and 7-deoxynogalarol is indeed isolated upon reaction workup. Hence, for nogalamycin the *formation* of quinone methide from the hydroquinone is rate limiting. A comparison of nogalamycin hydroquinone to daunomycin hydroquinone indicates a rate difference of ~10^3 for quinone methide formation (Rao et al., 1978).

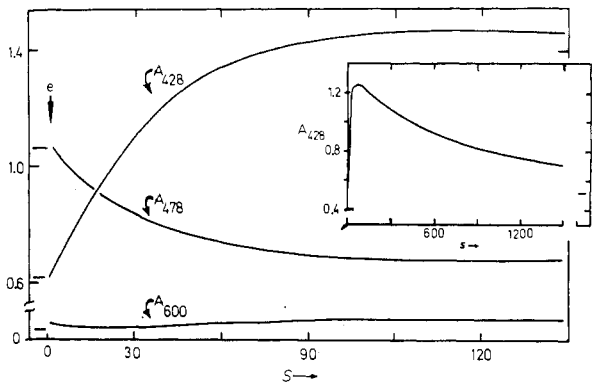


FIGURE 3: The kinetics of nogalamycin reduction to nogalamycin hydroquinone catalyzed by ferredoxin reductase, monitored against time (seconds) at 428 (hydroquinone formation), 478 (quinone reduction), and 600 nm (quinone methide or semiquinone presence). The solution contains at the time of enzyme addition (at arrow, final concentration of 0.70 μM) NADPH (1.2 mM) and nogalamycin (95 μM), in anaerobic 0.10 M Tris-HCl, pH 8.0 buffer containing 1 mM EDTA. The inset shows an identical experiment where the absorbance at 428 nm is monitored for a longer time, showing the decay of nogalamycin hydroquinone to 7-deoxynogalarol (NADPH, 1.2 mM; nogalamycin, 85 μM; ferredoxin reductase, 0.88 μM).

(*D*) (*7R*)-*Nogamycin.* A reasonable explanation for the unexpectedly large difference in hydroquinone glycoside stability may be found in the stereoelectronic relationship between the leaving group and the displacing moiety, the adjacent π orbitals. In both the daunomycin and aclacinomycin families, the 7*S*,9*S* configuration renders the leaving group pseudoaxial and presumably more disposed to the intramolecular reaction. Nogalamycin, on the other hand, being epimeric at C-9, may favor a conformation having the C-7 glycoside pseudoequatorial [the solution chemistry observed by Wiley et al. (1977) suggests this to be the case, although it is noted that nogalose is pseudoaxial in the crystal; Arora, 1983]. An experimental examination of this hypothesis is offered by (7*R*)-nogamycin. This derivative retains the same leaving group, nogalose, but is epimeric at C-7. It also lacks the C-10 carbomethoxy group, but while this group is expected to strongly influence anthracycline A ring conformation equilibria, independent observations do not support a strong influence of this group on the factors that determine the rate of quinone methide formation (compare aclacinomycin to daunomycin and the menogarols, below, to nogalamycin). Since nogamycin achieves a 7,9 cis relationship and a greater conformational flexibility by its lack of a C-10 substituent, one might expect

[5] Anaerobic activation of 11-deoxydaunomycin in the presence of 40 mM *p*-carboxybenzaldehyde gives a new product in ~10% yield, having mass spectral characteristics expected for the hemiacetal adduct (FAB, glycerol, negative ion) *m/z* 516 (63, M), 517 (100), and 518 (30). Unfortunately, we are unable to prepare sufficient quantities to undertake a proof of structure.

its behavior to now parallel the daunomycin and aclacinomycin families in giving rapid quinone methide formation from its hydroquinone. This turns out not to be the case. Ferredoxin reductase anaerobically reduces (7*R*)-nogamycin to its hydroquinone, which again slowly eliminates nogalose [$k = (1 \pm 1) \times 10^{-3}$ s^{-1}, pH 8, 30 °C]. Following this decay and reaction workup, 7-deoxynogarol (**4f**) is obtained as the product.

(*E*) *Menogarol Diastereomers.* In order to further explore the structural effects on quinone methide formation, both menogarol (**4d**) and its C-7 epimer (**4e**) were examined. Both undergo ferredoxin reductase catalyzed NADPH reduction and provide a sequence qualitatively identical with that of nogalamycin. At saturating NADPH, menogarol (66 μM) is reduced to its hydroquinone[1] with an initial velocity of 3.8 s^{-1}. The hydroquinone then undergoes first-order decay to 7-deoxynogarol with $k = (3.4 \pm 0.2) \times 10^{-3}$ s^{-1} (pH 8.0). Likewise, (7*S*)-epimenogarol (**4e**) is reduced to its hydroquinone (initial velocity at 83 μM **4e** of 6.2 s^{-1}); this in turn decays to 7-deoxynogarol (**4f**) with $k = (1.0 \pm 0.2) \times 10^{-3}$ s^{-1} (pH 8.0). Lastly, as a test of the overall anaerobicity and as a control, 7-deoxynogalarol is smoothly reduced to its hydroquinone (initial velocity at 72 μM of 4.0 s^{-1}). In contrast to the hydroquinone glycosides, this hydroquinone remains stable as long as anaerobicity is maintained.

The slow rate of leaving group elimination to the quinone methide from the nogalamycin hydroquinone glycosides thus appears to be a *general* phenomenon of this anthracycline family. Examination of the present data provides no simple explanation for this fact. The similarity of the rate constants for all four derivatives examined suggests that the slow rate derives not from any stereochemical effect but rather from some fundamental electronic effect present in the nogalamycins yet not found in either the daunomycin or aclacinomycin family. At this time a more satisfactory description of this effect is not possible.

Effect of Anthracycline Turnover on Enzyme Activity. A curious aspect of the above experiments with the nogalamycin-derived anthracyclines is the conversion of the hydroquinone glycosides to the 7-deoxy aglycon *quinones*. Since the NADPH is in excess, the enzymatic reduction is fast relative to quinone methide formation, and the quinones are competent oxidants for the enzyme, it would be anticipated that the 7-deoxy aglycon quinones obtained by quinone methide protonation should be rapidly reduced to the hydroquinone. That this does not occur requires either that anaerobicity has been lost or that ferredoxin reductase has lost its catalytic ability. Control experiments exclude the former possibility. The required answer, loss of enzyme activity, may derive from several explanations. The most obvious possibility is activity loss as a consequence of covalent modification of the enzyme by the quinone methide. In such a case a time-dependent and turnover number dependent decrease in the activity is required. Several straightforward experiments exclude this possibility. Generation of the quinone methide from the three anthracyclines (daunomycin, aclacinomycin, and nogalamycin), with removal and assay of enzyme portions at time points corresponding to the appearance and decay of the quinone methide, indicates that under these conditions (about 100 turnovers) no loss of enzyme activity occurs. Use of limiting quantities of NADPH, which would enhance adduct stability, gave an identical result. Hence ferredoxin reductase is not alkylated by these quinone methides under these conditions. The correct explanation for the loss in enzymatic activity presented itself unexpectedly, during several late runs with (7*R*)-nogamycin, where conversion to the 7-deoxy hydroquinone *did* occur. It is observed that certain lots of NADPH result in a substantial, yet reversible, decrease in ferredoxin reductase activity after partial conversion to $NADP^+$, possibly due to a variable $NADP^+$ content initially, as $NADP^+$ is a strong competitive inhibitor (Dykes & Davies, 1982) of ferredoxin reductase.

Anaerobic Anthracycline Reduction Mediated by Ferredoxin. On the basis of the aerobic kinetics and the anaerobic titration with reduced ferredoxin, it was anticipated that the presence of ferredoxin during anaerobic turnover of the anthracyclines by ferredoxin reductase would result in an increased velocity. This anticipation is confirmed. Indeed, with manual mixing techniques it is not possible to saturate anthracycline reduction with ferredoxin at a fixed anthracycline concentration. For comparative purposes, initial velocity determinations were made at modest ferredoxin concentrations, comparable to those described for the aerobic reactions. A representative experiment is described. To an anaerobic solution of 100 μM daunomycin and 1.6 mM NADPH containing 84 nM ferredoxin at pH 7.4 is added 70 nM ferredoxin reductase, to initiate the reaction. Without ferredoxin under these conditions the initial velocity is 0.7 s^{-1}; with ferredoxin it is 3.8 s^{-1}. Likewise, the initial velocities in the absence of ferredoxin for nogalamycin (1.0 s^{-1}) and aclacinomycin (1.9 s^{-1}) are also increased approximately 5-fold with ferredoxin present. This suggests that electron movement from NADPH to ferredoxin reductase, to ferredoxin, and then to the anthracycline is a more expedient route than direct transfer from ferredoxin reductase.

Discussion

The present experiments represent the first effort to systematically compare anthracycline reductive chemistry. As such they provide a mechanistic foundation upon which more detailed investigation—for example, dealing with other anthracycline structures, metal ion effects, and nucleic acid interactions—may be built. From the present vantage several conclusions and inferences may be drawn, and these are summarized.

To begin, spinach NADPH:ferredoxin oxidoreductase and ferredoxin have proven themselves as versatile and powerful *catalysts* of anthracycline reduction. (There is no evidence that either materially influences the anthracycline reactivity following reduction.) Ferredoxin reductase thus fits well into the pattern identified by Bachur and colleagues (Pan et al., 1981) that low-potential, flavin-dependent electron transferases (notably NADPH:cytochrome P-450 oxidoreductase) are generally capable of mediating anthracycline reduction. Under roughly identical conditions, the initial velocities for daunomycin reduction catalyzed by cytochrome P-450 reductase are approximately 10-fold faster than those for ferredoxin reductase alone (Kharasch & Novak, 1983). Ferredoxin reductase remains at least severalfold faster than the other enzymes for which velocities are available (xanthine oxidase, EC 1.2.3.2; nitrate reductase, EC 1.6.6.3; NADH cytochrome *c* reductase, EC 1.6.99.3; Pan et al., 1981). Although the detailed aspects of cytochrome P-450 reductase mediated anthracycline reduction have yet to be examined, there are clearly several fundamental differences between this enzyme and ferredoxin reductase. Most notably, cytochrome P-450 reductase exhibits the greater substrate selectivity. Several investigations have determined a strong correlation between the velocity for quinone reduction by cytochrome P-450 reductase and the quinone's oxidizing ability, measured by the redox potential (Powis & Appel, 1980; Kharasch & Novak,

1983). In the present instance even though aclacinomycin has an E_m significantly more positive than daunomycin (Berg et al., 1982), the two possess a comparable velocity with ferredoxin reductase. Additionally, Mimnaugh et al. (1982) have taken the failure of nogalamycin to initiate lipid peroxidation, catalyzed by cytochrome P-450 reductase under aerobic conditions, as presumptive evidence for it being a poor substrate of this enzyme. Again, ferredoxin reductase does not make this distinction. A third difference derives from the observation by Pan et al. (1981) that cytochrome P-450 reductase is unable to reduce 7-deoxydaunomycinone to the hydroquinone. This reduction may be accomplished effortlessly with ferredoxin reductase, perhaps reflecting the more powerful reducing ability of this enzyme. In brief, the present data suggest ferredoxin reductase as the enzyme of the two with broader anthracycline specificity and reduction ability.

Recently Youngman and co-workers (Paur et al., 1984; Youngman & Elstner, 1984) have examined anthracycline reduction by the *Euglena gracilis* ferredoxin reductase. Similarly to the spinach enzyme, adriamycin stimulates O_2 reduction producing O_2^- and H_2O_2 and in the presence of Fe^{3+} initiates peroxidation processes. Accompanying this reaction however are spectral transitions for adriamycin that are dissimilar to the transitions observed here. The data these authors provide are insufficient for a mechanistic interpretation of their transitions; at present we conclude that the two ferredoxin reductases may lead to different outcomes, perhaps as a result of differently matched redox potentials.

The presumed moiety that the anthracyclines receive the electrons from in ferredoxin reductase is its flavin cofactor. After reduction, the anthracyclines behave independently of the enzyme's control. The anthracycline behavior in the presence of oxygen is remarkably similar, reflecting both ferredoxin reductase's broad tolerance of anthracycline structure and the ease with which the anthracyclines mediate oxygen reduction. With oxygen removed, few reaction similarities remain. Daunomycin and aclacinomycin are similar only with respect to the ease with which the C-7 glycoside is lost. It has been presumed that this elimination occurs from the anthracycline semiquinone rather than from the hydroquinone (Gutierrez et al., 1983). Yet there is irrefutable evidence as to the chemical competence of the hydroquinones for this process (Fisher et al., 1983; Kleyer & Koch, 1984; Kleyer et al., 1984), and in the particular instance of the nogalamycin anthracyclines, the hydroquinone is the required intermediate. The correlation between semiquinone concentration and 7-deoxyanthracyclinone formation (Gutierrez et al., 1983) may simply reflect the semiquinone as an intermediate redox state between the quinone and hydroquinone. Thus while the semiquinone elimination is not precluded, neither do the data speak for it, and by the strong analogies between the quinone methides obtained enzymatically here and those obtained by Kleyer and co-workers (Kleyer & Koch, 1984; Kleyer et al., 1984)—which *are* 7-deoxyanthracyclinone tautomers—it is concluded that the glycoside elimination occurs from the hydroquinone state.

Nonetheless, the quinone methides from daunomycin and aclacinomycin retain individual identities. The 7-deoxydaunomycinone quinone methide acts as a discriminating nucleophile and electrophile, while the 7-deoxyaklavinone quinone methide acts primarily as a discriminating electrophile. The 7-deoxynogalarol quinone methide—regardless of its intrinsic reactivity—is presumed not to have a sufficient steady-state concentration to afford significant bimolecular reactivity. If then the anaerobic generation of quinone methides from anthracyclines is a *common* mechanism for the expression of antitumor activity, this common mechanism must be enforced by circumstances beyond mere solution reactivity. Two obvious circumstances are metal ion chelation (Spinelli & Dabrowiak, 1982; Eliot et al., 1984) and the different properties anticipated for a redox-active anthracycline–DNA complex.

The final discussion addresses the relevance of ferredoxin reductase and ferredoxin to mammalian anthracycline metabolism. A general role for these two in vivo has not been previously considered. Although mammalian ferredoxin reductases are not abundant, they appear in several tissues (Goswami & Rosenberg, 1981) with the best characterized enzyme being the adrenodoxin and adrenodoxin reductase electron transport system to the cytochrome $P\text{-}450_{scc}$ of the adrenal cortex. Of possibly greater import is the observation that spinach ferredoxin is an excellent anthracycline reductant. The importance of ferredoxin in the reductive activation of the antiparasitic nitroimidazoles is established [most recently discussed by Marczak et al. (1983) and Moreno et al. (1984)]. Its behavior certainly indicates that both low-potential flavin *and* iron–sulfur centers may represent loci at which anthracycline reduction may occur. In this regard it may be noted that the mitochondrial NADH dehydrogenase, established by Doroshow (1983a,b) as the major catalyst of anthracycline reduction in this organelle, contains both such centers. Iron–sulfur centers (of various redox potentials) are found in numerous other mitochondrial enzymes (such as NADH: ubiquinone oxidoreductase and succinate dehydrogenase). It is perhaps not surprising that mitochondrial damage is an early event following anthracycline administration. A general ability of quinones to disrupt mitochondrial function (beyond acting as ubiquinone antagonists) is known [for recent discussions, see Egerer et al. (1982) and Fry et al. (1984)]. The susceptibility of anthracyclines to reduction by low-potential flavin and iron–sulfur centers and the abundance of these in the mitochondrion offer strong circumstantial evidence that the mitochondrion is a particular and important target for the anthracyclines. This hypothesis is separately defended by Nudd & Wilkie (1983). In addition to the acute toxicity associated with a disruption of the respiratory chain, damage to the genetic integrity of the cell is also possible. The mitochondrion contains simultaneously DNA that is particularly susceptible to covalent modification by mutagens (Backer & Weinstein, 1980) and that is in genetic communication with the nuclear DNA (Fox, 1983; Reid, 1983). Thus although there remains an interest in locating anthracycline-reducing enzymes in the nucleus, there is no compelling reason that such enzymes be found in order to account for anthracycline-mediated damage to the nuclear DNA. Whether this conjecture is true and whether ferredoxin and ferredoxin reductase possess a relevance beyond being extraordinarily convenient catalysts for the study of anthracycline reductive activation await even further experimental creativity.

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SUPPLEMENTARY MATERIAL AVAILABLE

Sources of reagents, enzymes, and anthracyclines, description of the instrumentation used and eight figures illustrating

experiments described in the text (7 pages). Ordering information is given on any current masthead page.

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