tended: a fluorine labeling reagent for NMR studies and an azide reagent for photolysis studies have been attached to parvalbumin. The results indicate that the groups are selectively attached to cysteine-18 and that the aryl rings lie in a cleft formed by residues 16–22 and 61–73 of parvalbumin.

Acknowledgments

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Registry No. MFP, 84332-82-1; MPA, 84332-83-2; *p*-fluorophenol, 371-41-5; mercuric trifluoroacetate, 13257-51-7; *p*-(acetoxymercuri)aniline, 6283-24-5; *S*-[(2-hydroxy-5-fluorophenyl)mercuri]glutathione, 84332-84-3; *S*-[(4-azidophenyl)mercuri]glutathione, 84332-85-4.

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Direct Enzyme-Catalyzed Reduction of Anthracyclines by Reduced Nicotinamide Adenine Dinucleotide†

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ABSTRACT: The *Vibrio harveyi* NADH:flavin oxidoreductase forms ternary complexes with NADH and daunomycin and with NADH and aclacinomycin A. The anthracyclines occupy the flavin binding site of this enzyme. Within this complex, the enzyme affects the catalytic reduction of the anthracycline by NADH. Under aerobic conditions, the reduced anthracyclines are rapidly oxidized by molecular oxygen and are cycled catalytically. Under anaerobic conditions, reductive elimination of the glycoside occurs for both. For daunomycin, the only product obtained is 7-deoxydaunomycinone, showing the stereospecific incorporation of a solvent proton at C-7. Given the redox chemistry of NADH and the stereochemistry of this protonation, the suggested mechanism for this conversion is synchronous 2e, H^+ (hydride) transfer to provide the daunomycin hydroquinone that reductively eliminates to the quinone methide. Solvent proton (electrophile) trapping of the quinone methide occurs while the quinone methide remains enzyme bound. In contrast, enzymatic reduction of aclacinomycin provides two products, 7-deoxyaklavinone (28%) and the 7-deoxyaklavinone dimer (67%), identical with the dimer observed previously from chemical and enzymatic aclacinomycin reduction. This product composition indicates that aclacinomycin comproportionation successfully competes against a hydroquinone elimination pathway. The mechanism for aclacinomycin glycoside elimination is probably comproportionation to a semiquinone molecular complex and then glycoside loss followed by collapse of the semiquinone methide molecular complex to the dimer. It is not possible to trap the quinone methide from daunomycin (by thiol or thiolate nucleophiles) nor the radical pair from aclacinomycin (by thiol hydrogen atom donors). The data provide the following conclusions. Most importantly, it has been established that the anaerobic redox chemistries of daunomycin and aclacinomycin are significantly different, in that different pathways are followed regardless of whether a hydride or one-electron reductant is used. For daunomycin, a hydroquinone reductive elimination has been proven feasible, but the resulting quinone methide is either inaccessible or (most probably) unreactive to solution nucleophiles. For aclacinomycin, a semiquinone reductive elimination dominates but undergoes preferential dimerization within a molecular complex rather than hydrogen atom abstraction from solution donors. These studies suggest that in the absence of a specific target, the reductive elimination pathways of these two anthracyclines (although different) are chemically innocuous.

The anthracycline antitumor antibiotics (Arcamone, 1981; Young et al., 1981) are among several antitumor antibiotics that are activated in vivo by electron transfer. During reductive activation, the quinone functional group of the anthracyclines accepts either one or two electrons (depending on the reducing agent) to provide semiquinone or hydroquinone reduced states, respectively. The relationship of these reduced anthracycline redox states to the potent biological effects these antibiotics express is the focus of much current research. On the basis of in vitro study, several immediate consequences of anthracycline redox turnover have been identified; these include the inhibition of enzymes, the covalent labeling of macromolecules, and the initiation of oxygen-dependent lipid peroxidation and DNA degradation pathways (Bachur et al., 1979; Pan et al., 1981; Sinha & Gregory, 1981; Oki, 1977;

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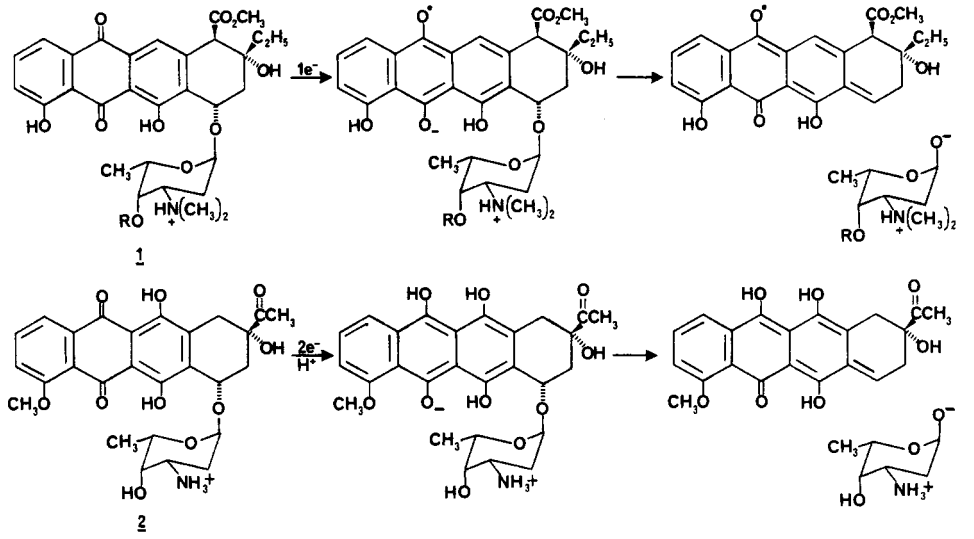
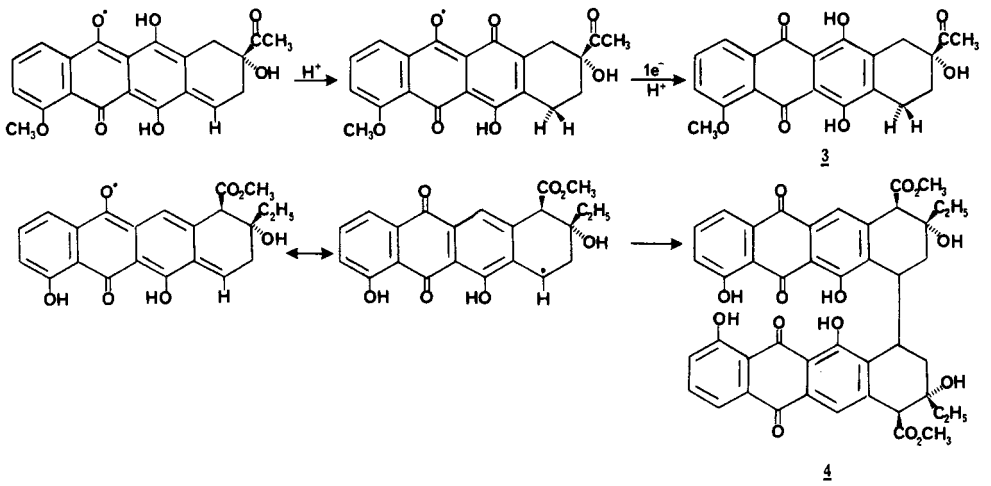
Scheme I: Possible Mechanisms for Glycoside Reductive Elimination:[a] (A) Semiquinone Pathway Illustrated with Aclacinomycin (1);[b] (B) Hydroquinone Pathway Illustrated with Daunomycin (2)



[a] See Komiyama et al. (1979a,b), Bachur et al. (1979), Moore (1977), and Moore & Czerniak (1981). [b] R = 2-deoxy-L-fucose-L-cinerulose.

Scheme II: Possible Fates of the Semiquinone Methide:[a] (A) Derived from Daunomycin; (B) Derived from Aclacinomycin



[a] See Komiyama et al. (1979b).

Komiyama et al., 1979a,b; Zwelling et al., 1981). Given a probable relationship between these molecular events and the cytotoxicity of anthracycline administration, necessary questions include the identity and location of the enzymes providing the electrons in the anthracycline reduction and the basic chemistry of electron intake and departure. The efforts of several research groups have brought attention to flavin coenzyme containing enzymes as important electron sources (Goodman & Hochstein, 1977; Komiyama et al., 1979a,b; Pan et al., 1981). These studies have also established that an intimate aspect of the reduction chemistry is the loss, by carbon–oxygen bond cleavage, of the anthracycline C-7 glycoside under anaerobic conditions. The resulting aglycons (7-deoxyanthracyclinones) are observed from both in vitro and in vivo anthracycline reduction. Two mechanistic suggestions have been made to connect quinone reduction, glycoside departure, and macromolecule degradation. In the first mechanism, one-electron reduction of the quinone provides a transient semiquinone that heterolytically eliminates the glycoside to yield a C-7 neutral benzylic radical (Scheme I). The radicals reduce molecular oxygen and may abstract hydrogen atoms or label susceptible nucleophiles (Komiyama et al., 1979b; Bachur et al., 1979; Sinha & Gregory, 1981; Pan et al., 1981). A second mechanistic possibility is two-electron reduction to a hydroquinone, followed by heterolytic glycoside loss providing a quinone methide product (Scheme I). The quinone methide is potentially electrophilic and provides an attractive intermediate in the covalent labeling of macromolecule nucleophiles ("bioreductive alkylation"; Moore, 1977; Moore & Czerniak, 1981; Lin et al., 1980). Although the glycoside loss is easily accomplished experimentally, it is more difficult to assign the correct mechanism of the two possibilities. A semiquinone may disproportionate or a hydroquinone comproportionate, resulting in an equilibrium among the redox states as the reaction proceeds. Further, there is evidence for both pathways. The clean conversion of daunomycin to 7-deoxydaunomycinone by chemical (dithionite) reduction (Smith et al., 1976) is most easily accounted for by a hydroquinone elimination followed by solvent protonation at C-7. In contrast, reduction of aclacinomycin provides a 7-deoxy dimer as the major product (Komiyama et al., 1979b), the structure of which would correspond to benzylic radical coupling. Oki and co-workers have suggested that glycoside loss occurs from the semiquinone for both, but with daunomycin the presence of a C-11 hydroxyl leads to a faster tautomerization relative to radical coupling at C-7. Scheme II

summarizes these postulated events.

A distinction between the semiquinone and hydroquinone pathways for glycoside elimination rests upon the judicious choice of reducing agent. Flavins, although a major enzymatic electron source, are not useful for mechanistic study as they possess freely accessible semiquinone and hydroquinone redox states, and hence all anthracycline redox states would be copresent by disproportionation–comproportionation equilibria. On the other hand, dihydronicotinamides, found in the redox coenzymes NADH and NADPH, act as exclusive hydride donors and thus should bypass the anthracycline semiquinone states. If it is assumed that anthracycline hydroquinone comproportionation could be precluded, the appearance of a 7-deoxyanthracyclinone could be due only to a hydroquinone precursor. Barone et al. (1981) have demonstrated the feasibility of chemical hydride ion reduction of anthracyclines, although in this chemical system it is not possible to exclude comproportionation to the semiquinone prior to glycoside loss. A similar experiment with NADH would also suffer this uncertainty; moreover, the velocity of NADH reduction of anthracyclines is insufficient for mechanistic study.[1] Only with the benefit of an enzyme catalyst can the NADH reduction velocity become satisfactory and the seclusion of the hydroquinone from comproportionation attained.

We have recently demonstrated (Fisher et al., 1982) that the egg white aporiboflavin binding protein complexes, within the riboflavin binding site, several anthracycline antibiotics. This anthracycline–binding protein complex may be chemically reduced with dithionite to the 7-deoxyanthracyclinone (with the first two electrons) and then to the 7-deoxyanthracyclinone hydroquinone (with the second two electrons). Although the circumstances of these titrations suggest that the facile loss of the glycoside during the first two-electron reduction occurs from the hydroquinone, the data interpretations were inconclusive. Nevertheless, the observation that anthracyclines may be accepted into a riboflavin binding site suggests a method by which NADH might be activated for anthracycline reduction. Numerous flavoenzymes contain a pyridine nucleotide binding site positioned for catalytic electron transfer. Thus, the potential substitution of an anthracycline for the flavin coenzyme would provide the opportunity for enzyme-catalyzed anthracycline reduction by NADH. This paper describes the outcomes of such experiments. These establish the feasibility of the hydroquinone route for glycoside loss for daunomycin, indicate the weak electrophilicities of the quinone methide for nucleophiles, and confirm the distinctly different redox behavior between the aclacinomycin and daunomycin anthracycline families.

Experimental Procedures

Reagents. Daunomycin was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute; aclacinomycin A was from Dr. A. Yoshimoto of the Sanraku-Ocean Co. NADH (grade III) was obtained from Sigma, and (4*R*)-[4-^{2}H]NADH was synthesized from NAD and ethanol-d_6 (Oppenheimer et al., 1971). Potassium xanthate was reagent grade, recrystallized from acetone.

Enzyme. For the initial experiments at the start of this work, the *Vibrio harveyi* oxidoreductase was purified to approximately 50% homogeneity by ion-exchange, gel filtration, and affinity chromatography (Jablonski & DeLuca, 1977). Due to unacceptably large decreases in total activity during purification, most of the experiments reported herein were done with enzyme having a specific activity of 0.25 μmol of NADH oxidized by riboflavin min^{-1} A_{280nm}^{-1} [0.10 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 30 °C]. The ratios of reduction velocities for daunomycin and riboflavin were independent of the relative enzyme purity.

Daunomycin and Aclacinomycin Reductions. Daunomycin reduction was carried out in anaerobic 0.10 M Tris-HCl–0.5 mM EDTA, pH 8.0, buffer, in the presence of excess NADH and the oxidoreductase catalyst. Aclacinomycin reduction was carried out similarly, but in 0.10 M imidazole hydrochloride–0.5 mM EDTA, pH 7.0, buffer due to the greater aqueous solubility of aclacinomycin at this pH. Quantitation of the velocity was determined by the initial rate of NADH consumption, measured at 340 or 365 nm in an anaerobic cuvette. Preparative reactions were carried out under anaerobic conditions in a Schlenck tube; a typical example is described in the following paragraph.

Preparative Isolation of 7-Deoxydaunomycinone. To a Schlenk tube were added 10.0 mL of Tris-HCl, pH 8.0, buffer, 0.5 mM EDTA, 25.0 mg of NADH, 5.0 mg of daunomycin, and 10 mg of glucose. The solution was degassed under vacuum and thorougly equilibrated under O_2-free nitrogen. An enzyme solution containing 3.2 μmol min^{-1} units (riboflavin reduction) of the oxidoreductase, 17 μmol min^{-1} glucose oxidase, and 20 μg of catalase was prepared and rendered oxygen free by gentle stirring under a nitrogen atmosphere. The enzyme solution (0.90 mL) was transferred to the Schlenk tube by syringe, and the reaction was initiated. After 24 h in the dark at ambient (25 ± 2 °C) temperature, the reaction was complete, as evidenced by a very pale red solution and the deep red precipitate of the 7-deoxydaunomycinone product. The precipitate was collected by centrifugation, washed with distilled water, and purified by silica gel chromatography in 3:1 chloroform:ethyl acetate. The identity of the precipitate as 7-deoxydaunomycinone was by comparison with an authentic sample (Smith et al., 1976) and by its spectroscopic properties. An analogous experiment in D_2O gave 7-deoxydaunomycinone showing 0.85 mol of deuterium incorporated (ratio of m/z 383/382 at 70 eV in an AE1 MS-30 mass spectrometer) at C-7 (by high-resolution decoupled ^{1}H NMR on a Nicollet NT-300 WD spectrometer), as described under Results. Experiments to look for possible nucleophilic trapping of the quinone methide intermediate were run in an identical fashion, with solid potassium xanthate or *N*-acetylcysteine added to yield a final concentration of 10 mM for these sulfur nucleophiles. Chemical reduction was carried out under similar conditions with sodium dithionite replacing the NADH and enzyme (Smith et al., 1976). Daunomycin is dissolved in anaerobic Tris buffer, and to this is added a 3–4 molar excess of dithionite, prepared in 0.1 M sodium bicarbonate. After the solution stood for 30 min at ambient temperature, it was extracted with CH_2Cl_2. Removal of the organic solvent provides 7-deoxydaunomycinone, uncontaminated by its leuco isomer (Fisher et al., 1982).

Stereochemistry of Hydrogen Transfer from NADH to Daunomycin. In agreement with previous determinations, a kinetic isotope effect on the V_{max} of riboflavin reduction by (4*R*)-[4-^{2}H]NADH of 5.2-fold was observed with the enzyme preparation used for these experiments (literature, 4.8; Fisher et al., 1976). The following experiment was performed in order to confirm that daunomycin reduction proceeded with an identical stereochemistry. To an aerobic solution of 1.5 μmol of daunomycin in 30 mL of 0.10 M Tris, pH 8.0, and 0.5 mM

[1] Anaerobic combination of NADH (0.43 mM) and daunomycin (0.15 mM) in 0.10 M Tris-HCl, pH 8.0, buffer, 30 °C, gave no detectable formation of 7-deoxydaunomycinone during a period of 2 days.

EDTA buffer were added (4*R*)-[4-^{2}H]NADH (85 μmol) and *V. harveyi* oxidoreductase (1.87 μmol min^{-1} activity, measured by riboflavin oxidation of NADH). Catalase (40 μg) was added to regenerate oxygen from hydrogen peroxide. After 15 h, no further decrease in the 340-nm absorbance was observed. The solution was concentrated 3-fold by lyophilization and thawed, and to this was added 50 mL of acetone. After 30 min at 0 °C, the NAD^+ had precipitated and was collected by filtration. The NAD^+ was washed with acetone and dried in vacuo, with a yield of 21 μmol. Examination of the NAD^+ by high-field proton magnetic resonance indicated that the protium hydrogen was retained at C-4 (Arnold et al., 1976). Therefore, the stereochemistry of daunomycin reduction by NADH is identical with that for riboflavin.

5-Carba-5-deazariboflavin Inhibition of Daunomycin Reduction. 5-Carba-5-deazariboflavin is a slow-reacting, tightly bound (K_m = 8 μM) substrate of the *V. harveyi* oxidoreductase (Fisher et al., 1976). As such, it was anticipated that were the oxidoreductase the catalyst for both flavin and anthracycline reduction, the copresence of the 5-carba-5-deazariboflavin would significantly diminish the rate of anthracycline reduction. This expectation was confirmed by the following experiment. To a solution of NADH (0.33 mM) and daunomycin (43 μM) in 3.0 mL of anaerobic 0.10 M Tris-HCl–0.5 mM EDTA, pH 8.0, buffer was added sufficient enzyme to quantitatively convert the daunomycin to 7-deoxydaunomycinone in approximately 45 min. In the presence of 50 μM 5-carba-5-deazariboflavin, over this period of time no detectable 7-deoxydaunomycinone product was formed.

Aclacinomycin reductions were carried out under very similar circumstances, with the exception that 0.10 M imidazole hydrochloride–0.5 mM EDTA, pH 7.0, buffer was used. Since the two products of enzymatic reduction (the dimer and 7-deoxyaklavinone; see Results) maintain reasonable aqueous solubility, at the conclusion of the reaction the aqueous solution was extracted with chloroform, and the two products were separated by silica gel chromatography using 3:1 chloroform:ethyl acetate. The reaction progress was monitored by silica thin-layer chromatography in the same solvent system (R_f anhydroaklavinone, 0.64; R_f 7-deoxyaklavinone, 0.46; R_f dimer, 0.19). Yields of the products were determined by integration of the 7-deoxyaklavinone and dimer peaks obtained upon liquid chromatography [monitoring at 445 nm by using an isocratic 90% methanol:10% water (1.25 mL min^{-1}) elution on an Altex C18 Ultrasphere column], giving retention times of 12 min for the dimer and 4 min for 7-deoxyaklavinone. The extinction coefficients used for 7-deoxyaklavinone and for the dimer were 8430 and 13 210 M^{-1} cm^{-1}, respectively, at 438 nm.

Characterization of the 7-Deoxyaklavinone Dimer. Two reductively deglycosylated products were obtained from enzymatic reduction of aclacinomycin. The less polar was identified as 7-deoxyaklavinone on the basis of its spectroscopic properties. The more polar product showed a melting point of 263–270 °C and an M^+ peak at m/z 396 in the electron-impact mass spectrum. These characteristics suggested that this product was the 7-deoxyaklavinone 7,7′ dimer (**4**), obtained previously by Oki et al. (1979) from their studies on the chemical and enzymatic reduction of aclacinomycin. The identity of this material as the dimer was established by the proton NMR spectrum in dioxane-d_8 (Oki et al., 1979), and by the following chemical ionization (NH_3) mass spectrum: (negative) 790 ($M + e^-$), 772 ($M + e^- - H_2O$); (positive) 414 ($M/2 + NH_4^+ + H^+$).

Conformation of the 7-Deoxyanthracyclinones. A stereochemical assignment for the face to which a proton is delivered during protonation of an anthracycline quinone methide requires that the conformation of the 7-deoxyanthracyclinone cyclohexene A ring be known. For anthracyclines and anthracyclinones having a 7,9-cis relationship of the oxygen substituents, it has been established that in chloroform a half-chair conformation is adopted, placing the C-7 oxygen and C-9 hydroxyl in pseudoaxial positions, within hydrogen bonding distances [see Tresselt et al. (1975), Wiley et al. (1977), and Swenton et al. (1981)]. In water, the ring puckers to permit solvent access (Brown et al., 1982). The proton NMR spectrum of 7-deoxyaklavinone in $CDCl_3$ shows an ABMN pattern for the C-7 and C-8 protons: δ 3.06 (ddd, J = 16.8, 6.9, 2.2 Hz, H_{7e}), 2.83 (ddd, J = 16.8, 9.6, 6.9 Hz, H_{7a}), 2.28 (ddd, J = 14.4, 9.6, 6.9 Hz, H_{8a}), 1.92 (m; upon decoupling at C-10 a ddd, J = 14.4, 6.9, 2.2 Hz, H_{8e}), and 3.94 (d, $J \sim 1$ Hz, H_{10}). This pattern and other NMR data, summarized below, indicate that 7-deoxyaklavinone also adopts the half-chair-like conformation having the C-9 hydroxyl in the pseudoaxial position, as shown in Figure 2. First, the chemical shifts for the C-10 hydrogen and C-10 carbomethoxy methyl group are similar to those of aclacinomycin derivatives (Tanaka et al., 1980) established to have a pseudoaxial placement of the C-10 carbomethoxy group. Decoupling experiments show a nuclear Overhauser effect between the C-10 and C-11 methines, and the C-10 methine and C-13 methylene, suggesting an approximately coplanar relationship among these three carbons. Finally, a long-range W coupling is seen between H_{8e} and H_{10e}, indicating a 1,3-diequatorial relationship (Krohn & Radeloff, 1978; Swenton et al., 1981). Thus, a proton delivered to the *re* face of the C-7 in an aklavinone methide will appear as the pseudoequatorial resonance in the chloroform proton spectrum.

The conformation of 7-deoxyduanomycinone is more problematical. The C-7 and C-10 benzylic hydrogens show the following pattern: δ 3.16 (apparent ddd, J = 18.8, 6.0, 2.5, 0.8 Hz, H_{7e}), 3.05 (dd, J = 18.4, 2.4 Hz, H_{10a}), 2.95 (m, not resolvable, H_{7a}), and 2.91 (apparent dd, J = 18.4, 1.1 Hz, H_{10e}). Simultaneous decoupling at C-8a and C-8e simplifies the C-7 resonances and also removes a 1,3-diequatorial coupling to H_{10e}. The couplings that remain to the δ 3.05 resonance (2.4 Hz) and δ 2.91 resonance (1.1 Hz) are assigned to H_{10a}, H_{7a} and H_{10e}, H_{7a} couplings, respectively. Although these 1,4 long-range couplings are not seen with 7-deoxyaklavinone, the similarity between 7-deoxyaklavinone and 7-deoxydaunomycinone in the C-7 and C-8 coupling and the preference for an axial C-9 hydroxyl regardless of C-10 substitution in the aclacinomycins (Tanaka et al., 1980) suggest similar conformations for 7-deoxydaunomycinone and 7-deoxyaklavinone. The proton delivered to the *re* face of a daunomycinone methide, therefore, should also appear as the pseudoequatorial hydrogen in the proton NMR spectrum.

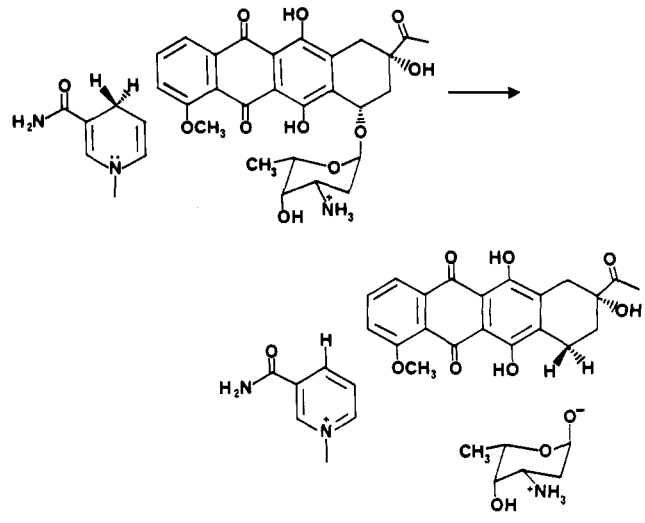
Results

Nature of the Enzymatic Catalyst. The *Vibrio harveyi* NADH:flavin oxidoreductase functions in this luminescent marine bacterium to provide a luciferase with substrate quantities of dihydroflavin for a dihydroflavin; aldehyde; and oxygen-dependent light emission process (Hastings & Nealson, 1977). The characteristics and protein chemistry of the oxidoreductase have been investigated by several research groups (Gerlo & Charlier, 1975; Duane & Hastings, 1975; Michaliszyn et al., 1977; Jablonski & DeLuca, 1977; Nefsky & DeLuca, 1982). With regard to its flavin substrate specificity, the oxidoreductase catalyzes electron transfer from NADH to a large number of flavins and flavin analogues (Fisher et al., 1976; Spencer et al., 1977; Walsh et al., 1978). Because

the tolerance of flavin structure for the oxidoreductase is similar to that for the egg white riboflavin binding protein (Becvar & Palmer, 1982), and the binding protein complexes anthracyclines well (Fisher et al., 1982), *V. harveyi* cell extracts containing oxidoreductase activity were examined for an ability to catalyze the anaerobic reduction by NADH of daunomycin. Such activity was indeed observed and was further found to copurify with the oxidoreductase through ion-exchange, gel filtration, and affinity column (Jablonski & DeLuca, 1977) chromatography, at a constant ratio of riboflavin to daunomycin velocities. The identity of the NADH:flavin oxidoreductase as the catalytic species involved in daunomycin reduction was confirmed by the (4*R*)-NADH stereospecificity for hydrogen transfer, as is the case for flavin reduction (Walsh et al., 1978), and by the potent inhibition of the daunomycin reaction by the copresence of 5-carba-5-deazariboflavin, a slow-reacting and tightly bound flavin substrate (Fisher et al., 1976).[2] Thus, by analogy with the mechanism for flavin reduction (involving a direct hydrogen transfer from the NADH to the flavin), the catalytic mechanism for the *V. harveyi* oxidoreductase with daunomycin involves binding NADH and daunomycin in a ternary complex (Jablonski & DeLuca, 1977), with the daunomycin occupying the flavin binding site, that activates NADH to hydride reduction of the anthracycline.

Kinetics and Product Identification of Daunomycin Reduction. An aerobic mixture of NADH, daunomycin, and the oxidoreductase results in NADH consumption, measured by the loss in absorbance at 340 nm. This loss requires both daunomycin and the enzyme. Under these circumstances, oxygen acts as the ultimate electron acceptor, and its rate of oxidation of the reduced daunomycin species exceeds the rate for reductive glycoside elimination. Thus, no reduced product is obtained, and the daunomycin functions catalytically. An identical aerobic outcome has been observed by others with different enzyme catalysts (Pan et al., 1981). Relative to the normal enzymatic reactions with riboflavin as an electron acceptor, the daunomycin reaction is 730 times slower. From the known turnover velocity for riboflavin with this enzyme at saturation of 15.5 s^{-1} (Jablonski & DeLuca, 1977), this corresponds to a daunomycin velocity of 2.1×10^{-2} s^{-1}. On the basis of the overall velocities, the K_m for daunomycin is estimated to be no greater than 5 μM. Under anaerobic conditions, an identical initial velocity is observed, but with the formation of a product corresponding to reductive glycosidic elimination. This deglycosylated product was identified as 7-deoxydaunomycinone (**3**) on the basis of chromatographic comparisons (thin-layer and liquid chromatography) with an authentic sample obtained by aqueous dithionite titration (Smith et al., 1976), and by its spectroscopic properties (electron-impact mass spectroscopy and nuclear magnetic spectroscopy). Use of (4*R*)-[4-^{2}H]NADH (corresponding to the known stereochemistry for this enzyme; Fisher & Walsh, 1974) in the anaerobic reaction yields 7-deoxydaunomycinone without detectable deuterium incorporation (mass spectrometry), at a velocity identical with that obtained for the protio reagent. Hence, hydrogen transfer is not rate determining for daunomycin turnover, in contrast to the case for flavins over a wide range of reaction velocities (Walsh et al., 1978). The only remaining source for the proton incorporated at C-7 in the 7-deoxy product is solvent. This was confirmed by running the reaction in D_2O, resulting in the incorporation of 0.85 equiv of deuterium at C-7 (vide infra) in the isolated 7-deoxydaunomycinone product.

Scheme III: Overall Reaction Catalyzed by the Oxidoreductase with NADH and Daunomycin[a]



[a] An arbitrary facial orientation for daunomycin is shown.

Overall Summary of the Reaction. The oxidoreductase catalyzes the NADH-dependent conversion of daunomycin to 7-deoxydaunomycinone (Scheme III). This is a two-electron reduction, resulting in glycoside elimination and subsequent solvent protonation at C-7. When the visible absorption spectrum of daunomycin is monitored as the reaction proceeds, a subtle change in shape and in intensity is seen as 7-deoxydaunomycinone is formed. At the completion of the reaction (and occasionally during its course), the 7-deoxydaunomycinone begins to precipitate, ultimately providing a clear supernatant and a fine, blood-red precipitate at the bottom of the vessel. The 7-deoxydaunomycinone is not further reduced to the 7-deoxyhydroquinone; either it does not bind or it is too poor an oxidant. Although we have not attempted to differentiate between these possibilities, there is reason to believe that both explanations contribute. A poor affinity of 7-deoxydaunomycinone is suggested by the smooth, complete conversion of daunomycin to product under preparative conditions. Its presence in no way diminishes the forward velocity, as might be expected were competitive product inhibition occurring. There are data indicating that it is a less efficient oxidant. Although its redox potential is slightly more positive than that of daunomycin (Rao et al., 1978), slower rates of 7-deoxy reduction have been observed for chemical (Barone et al., 1981) and enzymatic reductions (Pan et al., 1981). This suggests the lower efficiency is kinetic rather than thermodynamic: possibly the protonated amino group of the glycoside provides electrostatic stabilization of the transition state for electron transfer.

Mechanism of the Reductive Glycosidic Elimination from Daunomycin. Catalytic electron transfer by the *V. harveyi* oxidoreductase proceeds within a ternary complex of the reductant (NADH) and oxidant (Jablonski & DeLuca, 1977). Given a chemical mechanism for electron transfer from NADH of synchronous (or near synchronous) movement of the hydrogen nucleus with the electrons [hydride ion; most recently discussed by Roberts et al. (1982)], it is most probable that daunomycin is directly converted to its hydroquinone without semiquinone intermediates. The overall conversion

[2] Surprisingly, neither the V_{max} nor the K_m for riboflavin reduction by NADH is affected by the copresence of either daunomycin or aclacinomycin. This suggests that these two anthracyclines observe a different kinetic mechanism than riboflavin. Nefsky & DeLuca (1982) have established for riboflavin that NADH and riboflavin add in an ordered fashion to provide the reactive ternary complex.

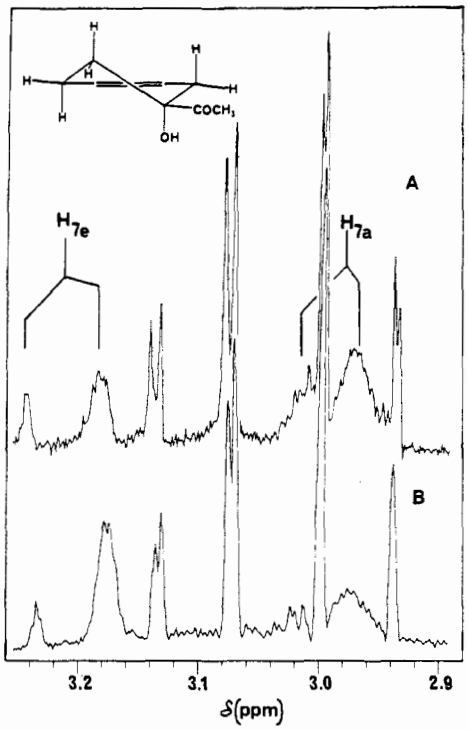


FIGURE 1: Proton NMR spectra of the C-7 and C-10 benzylic hydrogens of 7-deoxydaunomycinone, obtained with decoupling of the C-8 hydrogens (300 MHz, $CDCl_3$). Spectrum A shows 7-deoxydaunomycinone obtained from anaerobic dithionite reduction in D_2O. Deuterium incorporation has occurred primarily (2-fold) into the H_{7e} position. Spectrum B shows 7-deoxydaunomycinone obtained from anaerobic enzymatic reduction in D_2O. Deuterium incorporation has occurred primarily into the H_{7a} position. In this spectrum, the most shielded doublet (H_{10e}) lacks the 1,4 splitting due to the predominance of deuterium at H_{7a}.

of daunomycin to 7-deoxydaunomycinone is a 2e, H^+ process; consistent with this is the observed stoichiometry of 1.0 (±0.1) mol of NADH consumed per mol of aglycon formed. A hydroquinone-initiated elimination (Scheme I) also provides for solvent protonation at C-7. Since none of the six hydrogens of the cyclohexene A ring of 7-deoxydaunomycinone is equivalent, nuclear magnetic resonance spectroscopy allows the stereochemical determination of the isotopically labeled product. Although the benzylic region encompassing the four hydrogens of C-7 and C-10 is complex, decoupling at C-8 simplifies and permits assignment of the resonances. The C-8 decoupled, benzylic hydrogen resonances for the deuterated 7-deoxydaunomycinone obtained from enzymatic reduction in D_2O are shown in Figure 1. Within the C-7 pair, the more deshielded resonance has been assigned to the pseudoequatorial hydrogen in a half-chair conformation having an axial C-9 hydroxyl. Integration of the C-7 resonances was made relative to the C-10 pseudoaxial resonance set equal to 1.00; control experiments with the protio material showed no nuclear Overhauser effect on the C-7 integration. Thus, for the enzymatic, solvent-deuterated 7-deoxydaunomycinone, the equatorial C-7 showed 0.86 protium and the axial C-7 0.35 protium. The deuterium content determined by this integration method (0.79 mol) agrees well with the value from mass spectroscopy of 0.85 mol. The deuterium stereochemistry indicates a 4-fold preference for deuteron approach to the *si* face, cis to the hydroxyl. Also shown in Figure 1 is the matching region for 7-deoxydaunomycinone, deuterated at C-7 as a consequence of dithionite reduction in D_2O. Integration of the C-7 resonances for this sample shows that chemical reduction leads to the opposite stereochemical outcome for deuteron approach to the methide. A preferential *re* approach has also been seen with solution electrophiles reacting at C-7 (Kende & Rizzi, 1981). These data provide unequivocal evidence for solvent trapping of a C-7 quinone methide.

There are two explanations for the opposite stereochemistries; either the mechanisms for dithionite and enzymatic reduction are different and C-7 protonation occurs for different intermediates or the mechanisms are identical and the stereochemical outcome in the enzymatic case is determined by the chiral nature of an enzyme–quinone methide complex. Although there is no experiment that differentiates these possibilities, our preference is for the latter explanation. In contrast to the enzymatic mechanism, it is most probable that the dithionite reduction proceeds through a daunomycin semiquinone anion intermediate. This intermediate may choose between two pathways. It may undergo reduction by a second electron to the hydroquinone, or it may eliminate the glycoside and then protonate (ketonize) prior to obtaining the second electron. In the first pathway, protonation occurs to a hydroquinone methide and in the second to a semiquinone methide. The stereochemical outcome of the two mechanisms should be identical, assuming that the A-ring conformation is identical for both quinone methides and that the direction of protonation is determined by the C-9 substituents. Hence, we interpret the experimental observation of different stereochemical courses as favoring glycoside elimination and solvent protonation as events occurring from enzyme-complexed intermediates.

Attempted Nucleophile Trapping of the Hydroquinone Methide. As formulated by Moore (1977; Moore & Czerniak, 1981), the quinone methide intermediate obtained from glycoside loss from daunomycin is a potential electrophile that may be anticipated to react with cellular nucleophiles to achieve covalent labeling of critical cellular macromolecules. An important goal at the onset of this study was to critically evaluate the efficiency of this potential process. Using the oxidoreductase as a catalyst for the generation of the hydroquinone methide, we determined the product outcome in the presence of potential nucleophiles. The first investigated was the enzyme itself. The oxidoreductase is very sensitive to sulfhydryl reagents and requires the presence of dithiothreitol to sustain activity (Fisher et al., 1976; Jablonski & DeLuca, 1977). Following anaerobic turnover of daunomycin, the catalytic activity of the enzyme was assayed. Small decreases (less than 10%) were observed for both the control and the daunomycin enzyme. Thus, repeated generation of the quinone methide at the enzyme active site has no effect on the oxidoreductase activity. Further, oxidoreductase-catalyzed daunomycin turnover in the presence of either 10 mM *N*-acetylcysteine or 10 mM potassium xanthate provides only 7-deoxydaunomycinone; within the limits of detection, no nucleophile adduct is obtained. The failure of potassium xanthate is particularly telling, since this thiolate nucleophile is able to quantitatively trap the electrophilic quinone methides obtained upon mitomycin c reductive activation (Hornemann et al., 1979; Fisher & Olsen, 1982). Two possible conclusions may be made. The hydroquinone methide obtained during oxidoreductase turnover is either inaccessible to these nucleophiles or unreactive to these nucleophiles. Two experiments in our laboratory favor the latter hypothesis. Neither the quinone methide obtained during spinach ferredoxin reductase reduction[3] nor that obtained from dithionite titration of daunomycin is trapped by xanthate anion. Although the possibility

[3] K. McLane, unpublished experiments.

that these last two reductants yield semiquinone methides—which would certainly be less electrophilic than hydroquinone methides—cannot be excluded, it appears in any case that the electrophilicity of the quinone methides obtained by daunomycin activation by various reductants is not expressed by a rapid reaction with solvent nucleophiles. Rather, this electrophilicity is dissipated by solvent proton assisted tautomerization to the more stable ketone isomer.

Aclacinomycin. The extensive investigations by Oki and co-workers (Oki, 1977; Oki et al., 1979; Komiyama et al., 1979ab) have established that the redox behavior of aclacinomycin is fundamentally different from that of daunomycin. Under conditions of chemical (catalytic hydrogenation) and enzymatic reduction (flavin-requiring oxidoreductases), the major reductively deglycosylated product is a dimer, having the structure shown in Scheme II. An attractive mechanism for dimer formation is a semiquinone elimination to a neutral radical pair that collapses with bonding between the two C-7 carbons. Given the mechanistic outcome with daunomycin, it was our expectation that with NADH as reductant the semiquinone state would be bypassed, resulting in a significantly different product composition.

The ability of aclacinomycin to function as an oxidoreductase substrate and to undergo catalytic NADH reduction was established by the zero-order consumption of NADH, only in the presence of enzyme. Aerobic turnover yielded only aclacinomycin; anaerobic turnover provided reductively deglycosylated aglycons. The relative initial velocity for aclacinomycin turnover was 1.2% of that for riboflavin, under both aerobic and anaerobic conditions, and with either NADH or (4*R*)-[4-^{2}H]NADH. Under anaerobic reaction conditions, NADH and the enzyme catalyst quantitatively convert aclacinomycin into three reduction products. All are aglycons. The one obtained in smallest quantities (approximately 5%) is 9,10-anhydro-7-deoxyaklavinone. The second product is 7-deoxyaklavinone, present in approximately 28% yield. The mechanistic origins of these two aglycons remain uncertain. Anhydro-7-deoxyaklavinone might reasonably be though to derive from 7-deoxyaklavinone, although the latter is stable under the reaction conditions. Possibly, anhydro-7-deoxyaklavinone is formed incidentally by the enzyme during, or following, the reduction by NADH. 7-Deoxyaklavinone is the analogous product to 7-deoxydaunomycinone. Both are two-electron-reduction products. As is the case for 7-deoxydaunomycinone, 7-deoxyaklavinone is formed with the incorporation of a solvent proton at C-7 (0.95 mol of deuterium in D_2O solvent; Figure 2). The proton nuclear magnetic resonance spectrum indicates a slight preference (1.2-fold) for deuteration trans to the C-9 hydroxyl. This is different in direction and in magnitude from that observed for the enzyme-mediated deuterium incorporation into 7-deoxydaunomycinone. A mechanistic interpretation of the stereochemical outcome with 7-deoxyaklavinone is elusive at present. Chemical reduction of aclacinomycin with dithionite in D_2O provides only trace amounts of 7-deoxyaklavinone; we have not been able to isolate sufficient quantities for a stereochemical comparison. Further, the approach of electrophiles to the aklavinone methide in solution is preferentially cis to the C-9 hydroxyl (Kende & Rizzi, 1981). Thus, deuteron approach from both chemical and enzymatic reduction would have been expected to occur preferentially to the C-7 *si* face. Consequently, all possibilities remain for the formation of 7-deoxyaklavinone. Following hydride transfer to produce the aclacinomycin hydroquinone, glycoside loss may occur at the active site, or following hydroquinone release to solution or

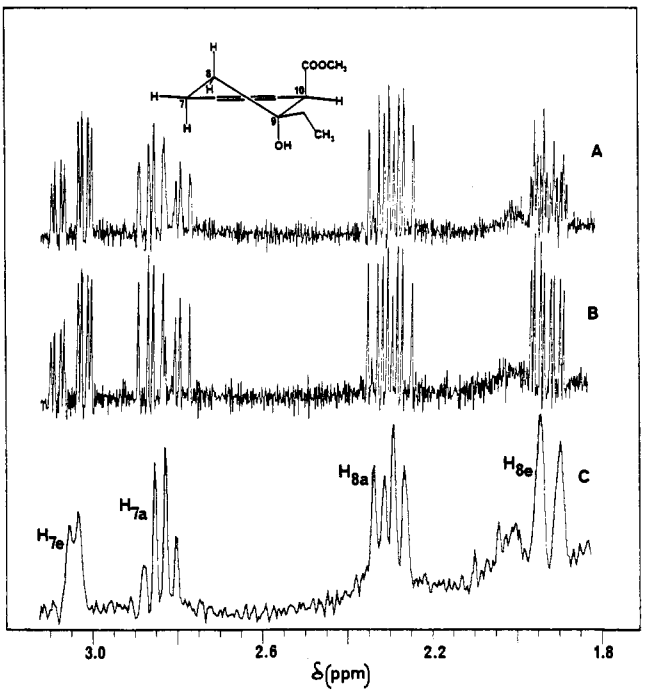


FIGURE 2: Proton NMR spectra of the C-7 and C-8 hydrogens of 7-deoxyaklavinone (300 MHz, $CDCl_3$). Spectrum A is 7-deoxyaklavinone, and spectrum B is 7-deoxyaklavinone with decoupling of the C-10 hydrogen. With this decoupling, the H_{8e} resonance (δ 1.92) simplifies to a ddd. Spectrum C is 7-deoxyaklavinone from anaerobic enzymatic reduction in D_2O. By integration, 0.55 mol of deuterium is present at H_{7e} and 0.45 mol present at H_{7a}. Thus, this spectrum corresponds to a near-equimolar mixture of diastereomers, epimeric at C-7.

following comproportionation to the semiquinone. In contrast to the less equivocal circumstances of 7-deoxydaunomycinone formation, a choice among these three may not be made.

The major aglycon product is obtained in approximately 70% yield. It has physical properties (melting point, proton nuclear magnetic resonance, electron-impact mass spectrum) that are identical with those reported for an aglycon originally isolated and characterized by Oki and co-workers, and to which was assigned a dimeric 7,7′-deoxyaklavinone structure (Scheme II). A dimeric structure is also supported by the chemical ionization mass spectrum (see Experimental Procedures). Given the known redox chemistry of aclacinomycin (Komiyama et al., 1979a,b), and the certain evidence for a dimer, the 7,7′ linkage is the only plausible assignment. The appearance of this dimer as the major product leads to several conclusions. First, the anticipation that NADH would alter the product outcome was not justified. The behavior of aclacinomycin with the oxidoreductase and its hydride reductant is very similar to that previously observed for chemical (catalytic hydrogenation) and enzymatic (sequential one electron) reduction (Komiyama et al., 1979a,b). Thus, the outcomes of both daunomycin and aclacinomycin anaerobic reduction are independent of the chemical nature of the reducing agent. While 7-deoxyaklavinone and the 7,7′ dimer must share an identical origin—the acceptance of electrons from NADH by aclacinomycin—at some subsequent point the pathways diverge. The mechanistic question to be settled with aclacinomycin is the intermediate that corresponds to this juncture.

Probable Mechanism of Dimer Formation. The likely intermediate for the parititioning is the initial reduction product, aclacinomycin hydroquinone. This intermediate is chemically competent to produce 7-deoxyaklavinone, by direct elimination

either on or off the enzyme, and the dimer. The following experimental observations pertain to dimer formation. First, the product stoichiometry indicates that less than 1 equiv of NADH should be needed to consume the aclacinomycin. A value of 0.65 ± 0.1 mol of NAD^+ formed is predicted; 0.5 ± 0.1 mol is observed. This small discrepancy arises from uncertainties in the extinction coefficients, the correction for nonenzymatic NADH decomposition, and experimental error. The dimer obtained from reaction in D_2O shows no deuterium incorporation, precluding solvent participation in its formation. Examination of the relative product composition at both early and late points in the reaction indicates that the ratio of dimer to 7-deoxyaklavinone is constant. Thus, the two are formed independently and do not interconvert. Lastly, the overall velocity of aclacinomycin turnover is approximately 10-fold faster than that for daunomycin, although neither has, apparently, hydride transfer as a rate-limiting step. This suggests that the participation of the second aclacinomycin molecule results in a different rate-limiting step for aclacinomycin than for daunomycin. Possibly, the slow step for daunomycin occurs after daunomycin hydroquinone formation (glycoside loss, protonation, or product dissociations), and for aclacinomycin, the second molecule intervenes and bypasses this slow step. Within these constraints, the following suggestion is made to account for dimer formation. Aclacinomycin and NADH are bound and react to provide a ternary product complex of NAD^+ and aclacinomycin hydroquinone. At this point, the aclacinomycin turnover differs from that of daunomycin by the association of a second aclacinomycin molecule to the aclacinomycin hydroquinone, probably after hydroquinone release from the enzyme. Comproportionation ensues to provide semiquinone anions that heterolytically eliminate their glycosides. The resulting neutral radicals (Scheme II) dimerize at the benzylic carbon. Although this mechanism is not proven, all possible mechanisms must originate with aclacinomycin hydroquinone and procede to a one-electron reduced form. On the basis of available information, the above mechanism appears most probable.

The negative outcome of two trapping experiments provides further information on the 7-deoxyaklavinone radicals. The presence of neither potassium xanthate (sulfur nucleophile) nor *N*-acetylcysteine (here, a hydrogen atom donor) alters the dimer yield. Xanthate anion might reasonably be expected to intercept the semiquinone methide; its failure to do so indicates the unreactivity of this methide to solution nucleophiles. Among common biological functional groups, the sulfhydryl is the best hydrogen atom donor. The failure of either the semiquinone methide or the neutral radical to abstract a hydrogen atom, and thus diminish the dimer yield, is surprising. Thus, it is improbable that these two radical intermediates will abstract a hydrogen atom in vivo. In this regard, we note that the dimer is an in vivo metabolite of aclacinomycin (Oki, 1977). Also, for this reason, the pathway leading to 7-deoxyaklavinone formation from the oxidoreductase-catalyzed NADH reduction is most probably a hydroquinone elimination of the glycoside, as is observed for daunomycin hydroquinone, rather than a hydrogen atom abstraction. Xanthate anion is apparently also incapable of intercepting the resulting quinone methide. The behavior of aclacinomycin is identical in this respect with daunomycin behavior.

Conclusions

The *V. harveyi* NADH:flavin oxidoreductase is the second flavoprotein that accepts anthracyclines into its flavin binding site. As with the first, the chicken egg riboflavin binding protein, the anthracycline binds at concentrations that are typical of that attained following anthracycline administration. The identification of two flavoproteins that complex anthracyclines lends credence to the hypothesis that an aspect of anthracycline cytotoxicity may be competition against the flavin coenzyme for its binding site (Fisher et al., 1982). The catalysis by the oxidoreductase of electron transfer from NADH to anthracyclines provides further insight into the redox chemistry of daunomycin and aclacinomycin. For daunomycin, the feasibility of hydroquinone reductive deglycosylation has been proven. This conclusion results from the experimental design and outcome. First, NADH has been used as a hydride (H^-) donor that bypasses anthracycline semiquinones. This mechanistic presumption has been verified in dihydronicotinamide self-exchange (Roberts et al., 1982) and carbonyl reduction (MacInnes et al., 1982; Chung & Park, 1982). Dihydronicotinamide reduction of flavins also appears to be a hydride transfer (Hemmerich et al., 1977; Powell et al., 1982). While the anthracyclines may represent an exception, this is not likely. Second, the different stereochemical outcome at C-7 for the enzyme-mediated reductive deglycosylation of daunomycin, as compared to chemical reduction, is interpreted to reflect enzyme participation in glycoside loss and in the methide protonation. Together, these indicate the hydroquinone pathway. Other than Moore (1977; Moore & Czerniak, 1981), the hydroquinone elimination has not been seriously considered as a facet of anthracycline redox chemistry. While a semiquinone pathway has by no means been excluded for other enzymes and other circumstances, it is now required that both the hydroquinone and semiquinone pathways be considered in the evaluation of anthracycline redox data. The complexity that is present is seen in the contrast between daunomycin and aclacinomycin. As first described by Oki and co-workers (Oki, 1977), these two anthracyclines behave quite differently following anaerobic reduction. It is demonstrated here that this difference persists even with an enzyme having NADH as the direct reductant. Regardless of the reducing agent, daunomycin yields 7-deoxydaunomycinone, while aclacinomycin partitions between 7-deoxyaklavinone and the 7,7′ dimer. The decided preference of aclacinomycin for reductive dimerization was interpreted by Komiyama et al. (1979a) as a reflection of a higher spin density at C-7 than in daunomycin, due to the absence of a competing ketonization reaction (Scheme II). Although this remains a viable (and attractive) explanation, the data here suggest that other factors contribute. These may be either a faster rate of aclacinomycin release from this enzyme, a faster rate of comproportionation in solution, or a more favorable equilibrium for dimeric self-association than for daunomycin association ($K \sim 1500\ M^{-1}$; Chaires et al., 1982). The relative contributions of these may not as yet be determined. Under conditions where dimer association is precluded—the chemical reduction of riboflavin binding protein bound aclacinomycin (Fisher et al., 1982)—7-deoxyaklavinone is the only product produced.

What is the electrophilicity of the quinone methides encountered in these conversions? Apparently with regard to solution reagents, there is none. In the absence of a *specific* target, the reductive eliminations produce methides that, although different, are innocuous. The extent to which covalent labeling of macromolecules represents an important chemical attribute of anthracycline activity will depend on two circumstances. It may be necessary for the activating enzyme to act upon the anthracycline–target macromolecule complex, such that the susceptible nucleophile has a kinetic advantage over the mechanisms for the dissipation of the methide elec-

trophilicity (protonation for daunomycin, dimerization and protonation for aclacinomycin). Second, the circumstances of nucleophile addition to the methide may require the presence of an oxidant, to oxidatively trap the adduct. Otherwise the equilibrium favors the expulsion (reductive elimination) of the nucleophile. Neither of these features is present in this study; they remain goals for future experimental design.

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