Complexation of Anthracycline Antibiotics by the Apo Egg White Riboflavin Binding Protein†

Jed Fisher,* K. Ramakrishnan, and Kathryn E. McLane

ABSTRACT: The apo chicken egg white riboflavin binding protein complexes several anthracycline antitumor antibiotics and their metabolites. The K_d value for three important anthracycline glycosides (adriamycin, daunomycin, aclacinomycin A) is approximately identical at 0.5 μM. The anthracycline occupies the flavin binding site in this complex, having its D–B rings overlaying the region normally occupied by the riboflavin A–C rings, respectively. The glycoside of the anthracycline, attached to C-7 of the A ring, is exposed to the solvent; consequently, the binding protein discriminates poorly between anthracycline A ring geometric isomers. Anthracyclinones, metabolites lacking the C-7 glycoside, are bound about 10-fold more tightly. The basis for the occupancy by anthracyclines of this flavin binding site is a steric homology (both ligands contain planar, linear conjugated rings) permitting the energetically favorable displacement of water from the hydrophobic pocket. The binding protein–anthracycline complex has been used for anaerobic aqueous redox titrations. Dithionite reduction of the anthracycline glycoside provides, in a two-electron process, the 7-deoxyanthracyclinone, via reductive elimination. The bound 7-deoxyanthracyclinone is then further two-electron reduced to the bound hydroquinone. Semiquinone radical intermediates are observed transiently; these are neither stable in solution nor complexed to the binding protein. Oxidation of the hydroquinone is accomplished by several reagents (oxygen, hydrogen peroxide, ferricyanide, cytochrome *c*). In the case of 7-deoxydaunomycinone hydroquinone, a mixture of two products is produced upon oxidation; these are the *chromo*-7-deoxydaunomycinone (identical with the first 2-e$^-$ reduction intermediate) and its leuco isomer, 8-acetyl-7,8,9,10-tetrahydro-5,8,12-trihydroxy-1-methoxy-6,11-naphthacenedione (iso-7-deoxydaunomycinone). These studies provide useful information concerning the redox properties of the anthracyclines and suggest that these antitumor antibiotics may be capable of functioning as riboflavin antagonists in vivo.

Given the importance of riboflavin as a coenzyme to numerous essential enzymatic transformations, it is surprising that the current knowledge concerning its uptake, transport, and incorporation into proteins is so poor (Merrill et al., 1981). One of the few areas where information at the molecular level is available is riboflavin transport, during gestation, to the developing embryo. In both chickens (Winter et al., 1963) and rats (Murty & Adiga, 1982) a riboflavin carrier (binding) protein is induced that is essential for the development of the embryo. In the chicken, the riboflavin binding protein is deposited within the egg while in the rat it circulates in the maternal bloodstream and assists in riboflavin transfer through the placental membrane. The chicken and rat proteins are immunologically related (Murty & Adiga, 1982). Of the two, the chicken egg protein is the better characterized (Rhodes et al., 1959). The binding protein is found in both the yolk and the white of the egg. The yolk and white proteins possess identical molecular weights (32 000) and flavin binding sites but differ in carbohydrate content (Nishikimi & Kyogoku, 1973; Steczko & Ostrowski, 1975; Blankenhorn, 1978). The egg white protein exists in the egg white as a complex with a thiamin binding protein (Muniyappa & Adiga, 1979; Miller et al., 1981); this complex dissociates upon purification. As a consequence of its exceptional stability, its abundance, and its ease of isolation, the chicken egg white binding protein has been extensively studied with regard to riboflavin binding. The experimental approaches that have been used include fluorescence quenching (Becvar, 1973; Choi & McCormick, 1980), resonance Raman infrared spectroscopy (Nishina et al., 1978; Kitagawa et al., 1979; Dutta et al., 1980; Schopfer & Morris, 1980), nuclear magnetic resonance spectroscopy (Yagi et al., 1976), protein chemistry (Kumoski et al., 1982), protein modification (Blankenhorn, 1978; Kozik, 1982), flavin chemical reactivity (Schopfer et al., 1981), calorimetry (Nowak & Langerman, 1982), and the determination of equilibrium constants for binding for an impressive array of flavins and flavin analogues (Becvar, 1973; Walsh et al., 1978; Choi & McCormick, 1980). This protein will stabilize flavin tautomers and flavin semiquinones and has been used to determine their physical properties (Spencer et al., 1977; Walsh et al., 1978; Choi & McCormick, 1980). Consequently, the egg white riboflavin binding protein is a protein of choice for the study of flavin binding to proteins, for which a considerable knowledge of its binding site exists.

The anthracycline glycosides are amongst the most efficacious antitumor antibiotics (Arcamore, 1981; Young et al., 1981). They possess a diverse spectrum of biological activities at the molecular level, being able to (amongst other things) inhibit enzymes, intercalate DNA, initiate oxygen-dependent lipid peroxidation, and reductively alkylate macromolecules. How these capabilities relate to events manifested at the cellular level—acute and chronic toxicities, mutagenicity, and tumor cytotoxicity—is not known. There is a consensus, however, that an important feature of the anthracyclines is an ability to accept electrons into their quinone functional group. These electrons are either passed to molecular oxygen (Bachur et al., 1979) or are used in a reductive elimination of the glycoside, providing an electrophilic quinone methide (Lin et al., 1980; Moore & Czerniak, 1981). For the enzymologist, several questions pertaining to these redox processes may be asked: What are the susceptible enzymes? What variables determine how the rates, yields, and products balance between the many competing reactions? Is the enzyme catalyst sensitive to the inactivation pathways it initiates? The

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importance attached to the answers to these questions is widely appreciated. It is now known that flavin-containing enzymes, in particular NADPH–cytochrome P-450 reductase, are important electron sources for the reductive activation of quinone antitumor antibiotics (Oki, 1977; Goodman & Hochstein, 1977; Pan et al., 1981; Powis & Appel, 1980; Kharasch & Novak, 1981b).

Our own interest in this general problem is a possible broader relationship between flavins and the anthracyclines. The aspect of this relationship that we discuss in this paper is an ability of anthracyclines to mimic flavins sufficiently to compete with them for protein binding sites. We have found that several anthracycline glycosides and their 7-deglycosylated metabolites are bound to the egg white riboflavin binding protein with surprisingly high affinity and that this protein is useful in aqueous redox titration studies of the anthracyclines. The ability of anthracyclines to compete against flavin for this protein binding site suggests that this may well occur for flavin-dependent enzymes.

Experimental Procedures

Materials. Adriamycin, daunomycin, AD-32, and 1,4-dihydroxy-5,8-bis[[[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione ("dihydroxyanthracenedione", DHAQ; NSC-279836) were provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute. Nogalamycin was provided by Dr. Paul Wiley, The Upjohn Co. (Wiley et al., 1977, 1979); aclacinomycin A was provided by Dr. Akihiro Yoshimoto, Sanraku-Ocean Co., Ltd (Oki et al., 1979); racemic aklavinone was provided by Dr. Andrew Kende, University of Rochester (Kende & Rizzi, 1981); and 4-demethoxydaunomycin and 7,9-diepi-4-demethoxydaunomycin were provided by Dr. Federico Arcamone, Farmitalia Carlo Erba (Zunino et al., 1976). Iso-7-deoxydaunomycinone was prepared by air oxidation of 7-deoxydaunomycinone hydroquinone followed by preparative reverse-phase liquid chromatography. The isolation, structure proof, and properties of this isomer will be presented shortly.[1] Quinacrine, tetracycline, and chlorpromazine were obtained from Sigma. Carminic acid was obtained from Tridom-Fluka.

Apo riboflavin binding protein was purified to homogeneity from the white of indigenous eggs according to the procedure of Becvar (1973). The purified protein was stored frozen at −20 °C as a 0.49 mM solution in 10 mM potassium phosphate–1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.0 buffer. The protein concentration was determined by riboflavin titration and by absorbance ($\epsilon_{H_2O}^{282nm}$ = 48 800 M^{-1} cm^{-1}; Nishikimi & Kyogoku, 1973); both methods were in agreement.

Solutions of the anthracyclines were made in the following fashion. Working solutions of adriamycin and daunomycin were obtained by dissolution in warm buffer, followed by cooling. These aqueous solutions show an extinction coefficient of 8550 M^{-1} cm^{-1} at 23 °C and 8640 M^{-1} cm^{-1} at 30 °C (references to $\epsilon_{CH_3OH}^{480nm}$ = 11 400 M^{-1} cm^{-1}). Aclacinomycin A was dissolved in dimethylformamide and then diluted into buffer to give a final dimethylformamide concentration of 1% or less. Prepared in this fashion, aclacinomycin A has a λ_{max} at 435 nm and an $\epsilon_{H_2O}^{435nm}$ = 8140 M^{-1} cm^{-1} (references to $\epsilon_{CH_3OH}^{431nm}$ = 10 550 M^{-1} cm^{-1}). 4-Demethoxydaunomycin and its epimer were dissolved directly in water ($\epsilon_{H_2O}^{485nm}$ = 9200 M^{-1} cm^{-1}; Zunino et al., 1976). Nogalamycin ($\epsilon_{CH_3CH_2OH}^{480nm}$ = 15 590 M^{-1} cm^{-1}), aklavinone ($\epsilon_{CH_3OH}^{431nm}$ = 11 870 M^{-1} cm^{-1}), and 7-deoxydaunomycinone ($\epsilon_{CH_3OH}^{467nm}$ = 12 750 M^{-1} cm^{-1}) were made as concentrated solutions in dimethylformamide and the solution concentrations detemined by dilution into alcohol. *leuco*-Iso-7-deoxydaunomycinone was dissolved in dimethylformamide and diluted into buffer, giving ϵ^{442nm} = 15 280 M^{-1} cm^{-1}. In general, the molar absorptivities of the anthracyclines are lower in water than in methanol and sensitive to the presence of organic cosolvents. For quantitative determinations, it is essential that the extinction coefficient be determined for each solvent system.

Fluorometric determinations of the K_d values for the fluorescent anthracyclines were obtained on a Varian SF-330 fluorometer, with 3-nm excitation slits and 20-nm emission slits. The wavelengths used for excitation and emission were 480 and 550 nm (nogalamycin), 500 and 585 nm (adriamycin and daunomycin), 420 and 575 nm (aclacinomycin A), 485 and 560 nm (4-demethoxydaunomycin), 470 and 575 nm (7-deoxydaunomycinone), 425 and 560 nm (aklavinone), 421 and 498 nm (quinacrine), and 418 and 480 nm (iso-7-deoxydaunomycinone). All determinations made at neutral pH were in 0.035 M potassium phosphate pH 7.0 buffer, containing 0.15 M NaCl and 0.5 mM EDTA, at ambient temperature (24 ± 2 °C). A representative procedure is described. To a 3.0-mL solution containing 3 μM adriamycin was added 5.0-μL increments of the stock binding protein solution (0.49 mM) until at least 85% of the anthracycline was bound (approximately 45 μL of binding protein solution total). From the observed decrease in fluorescent intensity and the known concentrations of the reagents, a Scatchard plot was constructed. All plots were linear and showed horizontal intercepts of 0.97 ± 0.03. The K_d was obtained as the inverse of the vertical intercept. The K_d for nonfluorescent materials was determined by difference spectrum (carminic acid, DHAQ) or competitive fluorescence (chlorpromazine) titrations.

Spectrophotometric titrations were made on a Cary 219 spectrophotometer. Anaerobic titrations were done with gas-tight syringes, with the deoxygenated solutions maintained under a positive pressure of O_2-free nitrogen. Dithionite was freshly prepared in O_2-free buffer and standardized by anaerobic riboflavin titration.

Results

Complexation of Adriamycin. This anthracycline glycoside was chosen for detailed analysis due to its major position in cancer chemotherapy (Young et al., 1981). Since complexation of the fluorescent flavins to the egg binding protein results in the complete quenching of the flavin fluorescence (Becvar & Palmer, 1982; Choi & McCormick, 1980), it was anticipated that the anthracycline fluorescence would also be quenched were these molecules to be bound. This proved to be the case. Addition of a slight excess of apo binding protein to an aqueous solution of adriamycin (20 μM) gave an almost complete loss of the adriamycin fluorescence. This property was used to determine the stoichiometry and equilibrium constant, by Scatchard analysis of an apo binding protein titration. A linear plot was obtained corresponding to 1 mol of adriamycin bound per mol of binding protein with an equilibrium constant for dissociation K_d = 0.5 ± 0.1 μM (0.035 M sodium phosphate, 0.15 M NaCl, 0.5 μM EDTA, pH 7.0, 24 ± 2 °C). Occupancy of the flavin binding site by the adriamycin was confirmed by the addition of 1 equiv of riboflavin (K_d = 0.002 μM), resulting in the immediate and total restoration of the adriamycin fluorescence. The nature of this binding was further examined by difference absorption spectrum titration (Figure 1). Adriamycin binding results in a bathochromic shift of its chromophore concomitant with vi-

[1] K. E. McLane, unpublished experiments.

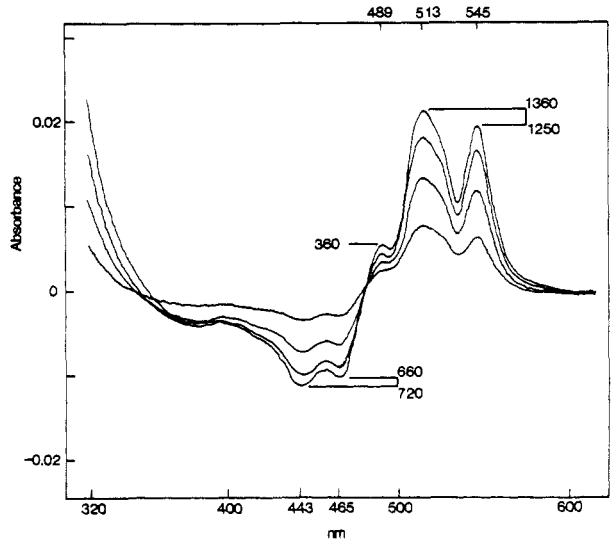


FIGURE 1: A difference spectrum titration of adriamycin with apo binding protein. Two identical cuvettes containing 1.50 mL of 21.2 μM adriamycin in 0.035 M sodium phosphate pH 7.0 buffer containing 0.15 M NaCl and 0.5 mM EDTA were placed in the sample and reference beams of the spectrometer and equilibrated to 30 °C. These were titrated with successive increments of 15, 25, 50, and 60 μL of a 0.49 mM apo binding protein solution and buffer, respectively. The final volume was 1.65 mL, and the final concentrations were 19.3 μM (adriamycin) and 44.5 μM (protein). At these concentrations the ligand is 96% bound. These spectra are uncorrected for dilution. The extinction coefficients given are from extrapolation to complete binding and are further corrected for protein absorbance.

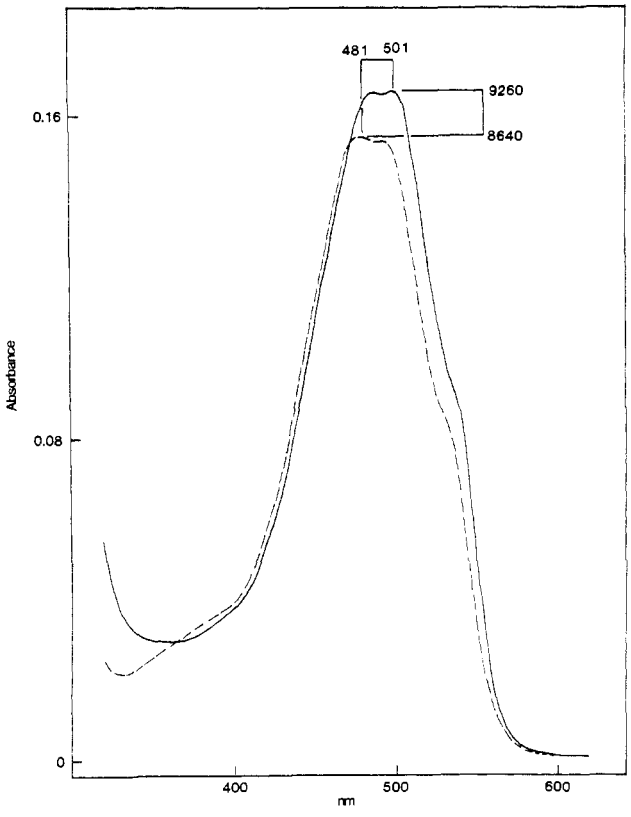
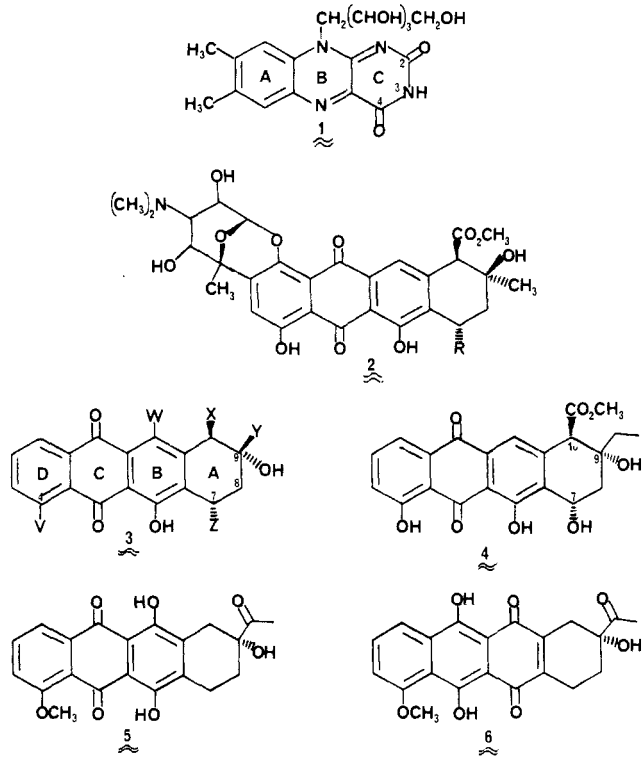


FIGURE 2: Visible absorption spectra of free (--) and binding protein bound (—) adriamycin at 30 °C. The free spectrum is 18.1 μM adriamycin in 0.035 M sodium phosphate pH 7.0 buffer containing 0.15 M NaCl and 0.5 mM EDTA. The bound spectrum is an identical concentration of adriamycin in the presence of 65.3 μM apo binding protein; under these conditions the anthracycline is 98% bound.

brational resolution, quite analogous to what is observed with typical flavins (Becvar, 1973). The absorption spectrum of

Chart I: Structures of Anthracyclines and Anthracyclinones Bound to the Binding Protein[a]



[a] (1) Riboflavin; (2) nogalamycin (R = nogalose); (3) AD-32 [V = OCH_3, W = OH, X = H, Y = $COCH_2OCO(CH_2)_4CH_3$, Z = *N*-(trifluoroacetyl)daunosamine], aclacinomycin A (V = OH, W = H, X = CO_2CH_3, Y = CH_2CH_3, Z = L-rhodosamine-2-deoxy-L-fucose-L-cinerulose A), adriamycin (V = OCH_3, W = OH, X = H, Y = $COCH_2OH$, Z = L-daunosamine), daunomycin (V = OCH_3, W = OH, X = H, Y = $COCH_3$, Z = L-daunosamine), and 4-demethoxydaunomycin (V = X = H, W = OH, Y = $COCH_3$, Z = L-daunosamine); (4) aklavinone; (5) *chromo*-7-deoxydaunomycinone; (6) *leuco*-iso-7-deoxydaunomycinone.

the bound adriamycin (Figure 2) is consistent with the hydrophobic character of the flavin binding site (Nishikimi & Yagi, 1969) as it mimics the changes seen in the adriamycin chromophore upon transfer to methanol as a solvent. This spectrum of the binding protein–adriamycin complex is very different from the spectrum obtained for adriamycin intercalated between the DNA base pairs (DiMarco & Arcamone, 1975; Wiesehahn et al., 1981) where a pronounced decrease in the chromophore intensity is observed, most reasonably reflecting a more polar character to the adriamycin–DNA complex (Patel et al., 1981). The pH dependence of the adriamycin–binding protein complex is similar to that seen for riboflavin, showing an increase in K_d at acidic pH (Choi & McCormick, 1980). The equilibrium constants obtained were as follows at the indicated pH: pH 4.0, 21 μM (formate buffer); pH 5.0, 1.6 μM (acetate); pH 6.0, 0.6 μM; pH 7.0, 0.5 μM; pH 8.0, 0.5 μM (phosphate buffers). The equilibrium constant calculated from a spectrophotometric titration of adriamycin at 30 °C was K_d = 1.0 μM.

General Complexation of Anthracycline Glycosides. The general ability of the binding protein to complex anthracycline glycosides was determined with the anthracycline glycosides nogalamycin, aclacinomycin A, daunomycin, 4-demethoxydaunomycin, 7,9-diepi-4-demethoxydaunomycin, and *N*-(trifluoroacetyl)-14-valeryladriamycin (AD-32). Chart I contains the structures of these compounds and Table I their dissociation constants. With the exception of nogalamycin, these analogues are bound as well as or better than adriamycin. The

Table I: Equilibrium Constants for Dissociation (K_d) for Anthracyclines from the Apo Egg White Riboflavin Binding Protein[a]

	K_d (μM)
anthracycline glycoside	
nogalamycin[b]	>1000
AD-32[c]	
aclacinomycin A	1.0
adriamycin	0.5
daunomycin	0.4
4-demethoxydaunomycin	0.3
7,9-diepi-4-demethoxydaunomycin	0.08
anthracyclinones	
7-deoxydaunomycinone	0.08
(±)-aklavinone[d]	0.02
iso-7-deoxydaunomycinone	0.02

[a] 0.035 M sodium phosphate, 0.15 M NaCl, and 0.5 mM EDTA, pH 7.0, 24 ± 2 °C. The K_d uncertainty is approximately 20%. [b] Does not bind. [c] Binds well, but aqueous insolubility precludes quantitative determination. [d] No detectable difference between enantiomers.

different stereochemical features of these analogues permit an assignment of the relative orientation of anthracycline in the flavin binding site.

The most important observations bearing on this issue are the tight binding of AD-32 and aclacinomycin A. AD-32 is a diacylated derivative of adriamycin with distinctly different pharmacological properties (Krishnan et al., 1981). It is also insoluble in water. Nonetheless the addition of AD-32 as a concentrated solution in dimethylformamide to an aqueous solution of a slight molar excess of the apo binding protein, to give final concentrations of each of approximately 20 μM, results in the quantitative formation of the AD-32–binding protein complex. In contrast, dispersal of the AD-32 dimethylformamide solution in the same buffer without the binding protein results in the instantaneous precipitation of the AD-32. Due to this insolubility, titration studies are not feasible. Since it is possible, however, to form this complex at 20 μM concentrations, the two atoms of adriamycin that are acylated (valeryl at the C-14 hydroxyl and trifluoroacetyl at the C-4′ amino) must lie outside the binding site. This notion is also supported by the inconsequence to binding of the C-7 substituent, which is a trisaccharide for aclacinomycin A but a monosaccharide for adriamycin. Since large substituents on the cyclohexene A ring of the anthracycline do not influence the anthracycline binding, it is probable that this ring lies outside of the protein and thus within the aqueous solvent. Also consistent with the anthracycline A ring being solvent exposed is the small difference between 4-demethoxydaunomycin (having the natural 7*S*,9*S* configuration) and its diepi, 7*R*,9*R* diastereomer. Placement of this anthracycline ring outside of the binding site establishes the relative orientation of the anthracycline within the binding site.

Riboflavin association with the binding protein places the flavin A ring within the protein interior and the flavin pyrimidine C ring such that the C-2 carbonyl is solvent accessible (Blankenhorn, 1978; Schopfer et al., 1981). Fluorescence quenching occurs by energy transfer to a tryptophan (and possibly tyrosine) residue that lines the binding site cavity (Blankenhorn, 1978). If one presumes that the quenching of the anthracycline occurs by a similar process, a picture is constructed where the anthracycline D–B rings overlay the space occupied by the flavin A–C rings, respectively. This proposed orientation accounts for the failure of nogalamycin to bind since this anthracycline is glycosylated at D ring positions that correspond to protein regions of clear steric

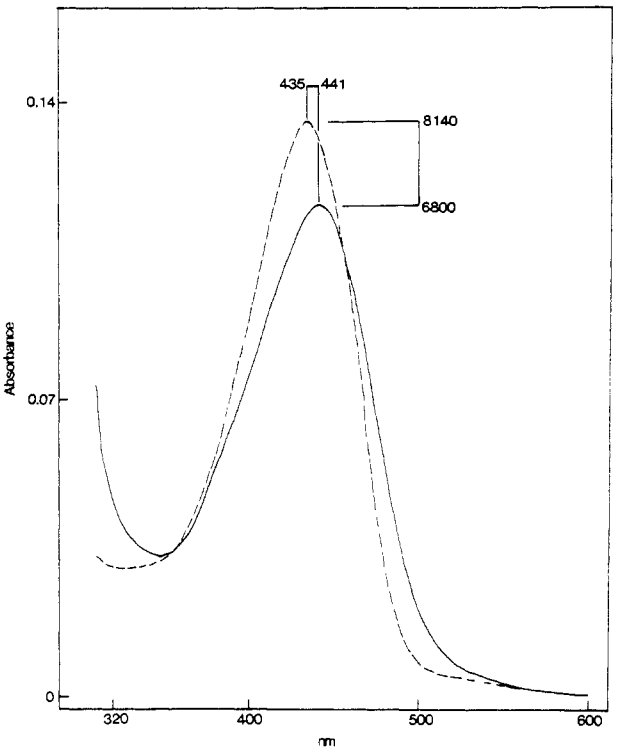


FIGURE 3: Visible absorption spectra of free (– –) and binding protein bound (—) aclacinomycin A at 30 °C. The free spectrum is that of 16.8 μM aclacinomycin A in 0.035 M sodium phosphate pH 7.0 buffer containing 0.15 M NaCl, 0.5 mM EDTA, and 1.0% dimethylformamide. The bound spectrum is an identical concentration of aclacinomycin A in the presence of 23.3 μM apo binding protein; the anthracycline is 90% complexed. The extinction coefficients given are from extrapolation to complete binding.

limitation to the flavin A ring. The 4-methoxyl substituent is unimportant as it coincides with the flavin C-6, an open region of the protein interior. No data bear on a preferred facial orientation of the anthracycline relative to the flavin when bound.

Some variability amongst the anthracyclines within this orientation is suggested by the absorption spectrum changes expressed upon complexation of the second anthracycline chromophore tested, that of aclacinomycin A (Figures 3 and 4). We have found that the absorption intensity of free anthracyclines in water is lower than in less polar solvents such as methanol. Consequently, the increase in extinction coefficient for binding protein bound adriamycin is an expected indication of the hydrophobicity of the binding cavity. While aclacinomycin A is also bathochromically shifted and vibrationally resolved, its absorption intensity decreases upon binding. This decrease is not due to distortion within the site, induced by the bulky C-7 trisaccharide, as the loss of this substituent yields a virtually identical bound absorption spectrum (for 7-deoxyaklavinone). No explanation is apparent for the different response of the two chromophores to the binding site environment.

General Complexation of Anthracyclinones. The major metabolic pathways for the anthracyclines are C-13 carbonyl reduction to the alcohol (Bachur & Felsted, 1980), C-7 hydrolytic deglycosylation, and C-7 reductive deglycosylation (Arcamone, 1981; Andrews et al., 1980; Schwartz & Parker, 1981). The products of these processes are anthracyclinols, anthracyclinones, and 7-deoxyanthracyclinones, respectively; a significant portion of the metabolites show both C-13 carbonyl reduction and loss of glycoside. In order to complete this study of anthracycline binding, it was necessary to de-

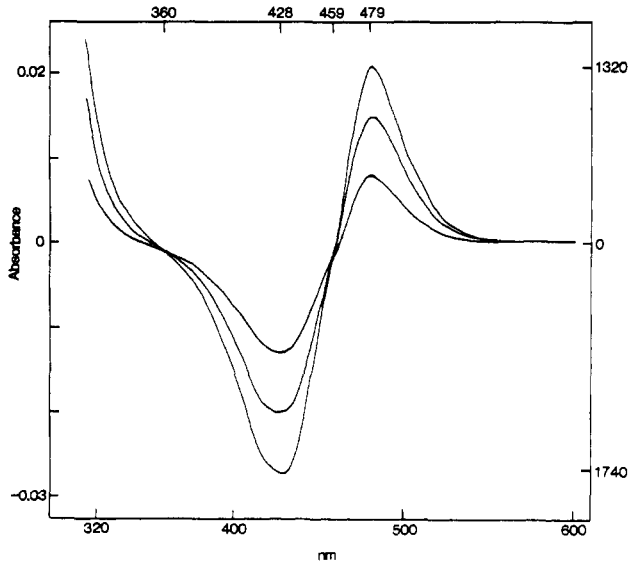


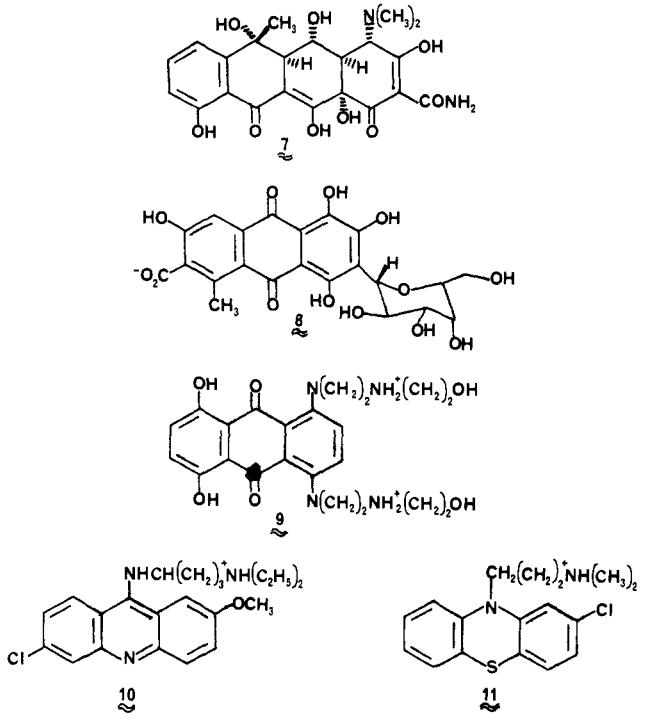
FIGURE 4: A difference spectrum titration of aclacinomycin A with apo binding protein. Two identical cuvettes containing 1.50 mL of 17.6 μM aclacinomycin A in 0.035 M sodium phosphate pH 7.0 buffer containing 0.15 M NaCl, 0.5 mM EDTA, and 1.0% dimethylformamide were equilibrated to 30 °C. These were titrated with successive increments of 15, 15, and 25 μL of a 0.49 mM apo binding protein solution and buffer, respectively. The final volume was 1.555 mL, and final concentrations were 17.0 μM (aclacinomycin A) and 17.4 μM (binding protein). At these concentrations the ligand is 85% bound. These spectra are uncorrected for dilution. The extinction coefficients given are from extrapolation to complete binding and are corrected for protein absorbance.

termine the binding protein affinity of three anthracyclinones: (±)-aklavinone, 7-deoxydaunomycinone, and iso-7-deoxydaunomycinone. These were found to be bound better than the parent anthracycline glycosides (Table I). Although their dissociation constants are somewhat uncertain, due to the sensitivity limit of the fluorometer, it is clear that these anthracyclinones are bound as well as several isoalloxazines (Becvar & Palmer, 1982; Choi & McCormick, 1980). The tighter binding of the anthracyclinones relative to the anthracyclines is a likely reflection of the very low aqueous solubility of the anthracyclinones, resulting in a more favorable partitioning into the hydrophobic binding site relative to the aqueous solvent, as well as the possible loss of a sterically unfavorable interaction between the glycoside and the protein.

Within the limits of detection, both enantiomers of the racemic aklavinone (7*S*,9*R*,10*R*, and 7*R*,9*S*,10*S*) are equally well bound. These A ring chiral centers lie outside of the protein and do not affect binding. Through comparison of aklavinone with 7-deoxydaunomycinone, the C-11 hydroxyl may be added to the C-7, C-9, and C-10 substituents as unnecessary to binding protein recognition. Also of interest is the high affinity of the binding protein for iso-7-deoxydaunomycinone (8-acetyl-7,8,9,10-tetrahydro-5,8,12-trihydroxy-1-methoxy-6,11-naphthacenedione). This compound is a kinetic product of 7-deoxydaunomycinone hydroquinone oxidation (vide infra). Although not stable in aqueous buffer to isomerization to 7-deoxydaunomycinone, this material is stable in organic solvents and when complexed to the binding protein. A possible reason for the binding protein preference for this isomer is that it may accept a hydrogen bond normally directed to the flavin C-4 carbonyl. This would improve its mimicry of the flavin and account for the strengthened binding protein association.

Complexation of Anthraquinones and Related Molecules. Although a comprehensive survey of the binding protein's

Chart II: Structures of Anthraquinone-like Molecules Examined for Complexation by the Binding Protein[a]



[a] (7) Tetracycline; (8) carminic acid; (9) DHAQ; (10) quinacrine; (11) chlorpromazine.

capabilities was not undertaken, it remained of interest to ascertain whether the anthracyclines were specific or adventitious residents of the flavin binding site. This question has been probed with the several anthraquinones and anthraquinone-like molecules given in Chart II. Within the limits of spectrophotometric detection (K_d >250 μM) neither tetracycline nor carminic acid (an insect feeding deterrent; Eisner et al., 1980) binds. For tetracycline, this is a probable result of its nonplanar ring system and its numerous out-of-plane substituents. Carminic acid possesses an ionized carboxylate (negative charge strongly destabilizes binding protein association) and a glycoside, which may result in unfavorable steric interactions with the protein. The interesting antitumor antibiotic DHAQ is bound weakly (K_d = 60 μM); its affinity may be decreased by unfavorable steric interactions between the protein and its bis amino side chains. The high affinity of the binding protein for chlorpromazine (K_d = 1 μM) and quinacrine (K_d = 0.2 μM) is significant as both these are known to complex to flavin-dependent enzymes. A possible similar relationship for the anthracyclines is elaborated in the discussion.

Spectrophotometric Redox Titrations of Bound Anthracyclines. Aqueous redox titrations of anthracyclines cannot be done. Although anthracycline glycosides are water soluble, loss of the glycoside (with its positive charge) provides the almost completely water-insoluble 7-deoxyanthracyclinones. Unfortunately, glycoside loss is an integral aspect of anthracycline redox chemistry. Two mechanisms for the reductive cleavage of the glycosidic bond have been proposed. Moore has suggested heterolytic cleavage of the hydroquinone (Moore & Czerniak, 1981) while Bachur et al. (1979) propose a heterolytic cleavage of the semiquinone. Recent data presented by Barone et al. (1981) and obtained from our own laboratory[1,2] have demonstrated the competence of the heterolytic

[2] K. Ramakrishnan, unpublished experiments.

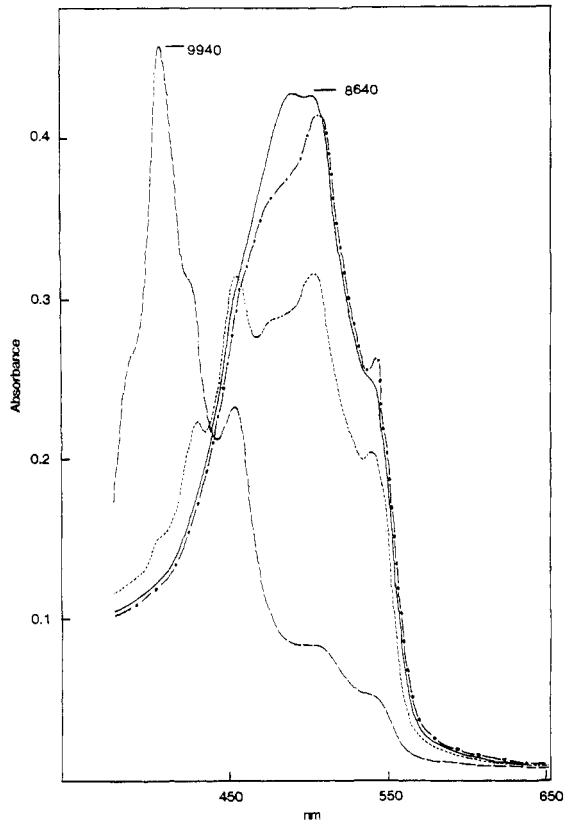


FIGURE 5: Visible absorption spectra obtained during redox titration of binding protein bound daunomycin. Daunomycin (75 μM) in 1.30 mL of 0.035 M sodium phosphate pH 7.0 buffer containing 0.15 M NaCl, 0.5 mM EDTA, and 1 equiv of binding protein (—) was titrated with two-electron equiv of dithionite to yield bound 7-deoxydaunomycinone (–·–) and a further two-electron equiv of dithionite to yield bound 7-deoxydaunomycinone hydroquinone (– –) and oxidized by air to a mixture of bound *leuco*- and *chromo*-7-deoxydaunomycinone (- - -). This last spectrum is indefinitely stable.

mechanism. On the other hand, in vitro enzymatic reduction of aclacinomycin A produces 7,7′ dimers of the anthracyclinone (Oki, 1977), anticipated products of a radical coupling process. The homolytic pathway may not as yet be excluded. To investigate this and other possible features of the anthracyclines as oxidizing agents, we have used the binding protein as a specific, aqueous-soluble complexing reagent for redox titration of the anthracyclines. The reducing agent used was dithionite anion. Dithionite has been used extensively in flavin systems (Mayhew, 1978) and has the virtue of the facile delivery of low-potential electrons.

Dithionite has been also used for the preparative conversion of anthracycline glycosides to the 7-deoxyanthracyclinones (Smith et al., 1976). The outcome of an anaerobic dithionite titration of the daunomycin–binding protein complex is shown in Figure 5. Equilibration with dithionite is rapid, generally within mixing time. Daunomycin is first converted in a two-electron process to 7-deoxydaunomycinone, with reductive elimination of the glycoside. Assignment of structure to this titration intermediate was confirmed by its isolation and chromatographic comparison with an authentic sample. Further dithionite addition reduces the bound 7-deoxydaunomycinone to a new chromophore (λ_{max} = 407 nm). This is the 7-deoxyhydroquinone on the basis of the electron stoichiometry (two electrons) and by its similarity to the absorption spectrum reported for the pentacetyl derivative [λ_{max} 417 (CH_3OH), ϵ = 19950 M^{-1} cm^{-1}; Barone et al., 1981]. The structures of the three species observed in this four-electron total reduction process are shown in Scheme I. The hydroquinone is not further reduced but is very easily oxidized upon mixing with oxidants such as oxygen, hydrogen peroxide, ferricyanide, and cytochrome *c*. The absorption spectrum obtained following complete oxygen oxidation of the hydroquinone is shown in Figure 5 and is different from any previous spectrum. Although the longer wavelength features are easily assigned to the presence of 7-deoxydaunomycinone, a new chromophore (λ_{max} approximately 445 nm) is also present. This chromophore appears (to varying extents) with each oxidant (Table II). The structure of the compound corresponding to this chromophore is iso-7-deoxydaunomycinone on the basis of its spectroscopic data.[1] In neutral aqueous buffer in the absence of the binding protein, this compound promptly isomerizes to 7-deoxydaunomycinone ($k = 6 \times 10^{-4}$ s^{-1}). As the binding protein complexed species, it is, however, stable for days. Binding protein stabilization is also reflected in an estimated 10-fold slower rate of dithionite reduction of the iso-7-deoxydaunomycinone compared to that of either 7-deoxydaunomycinone or daunomycin. Of these three oxidized quinones it is the one preferentially bound by the riboflavin binding protein.

Scheme I: Structures of Products Observed during Dithionite Titration of the Daunomycin–Binding Protein Complex

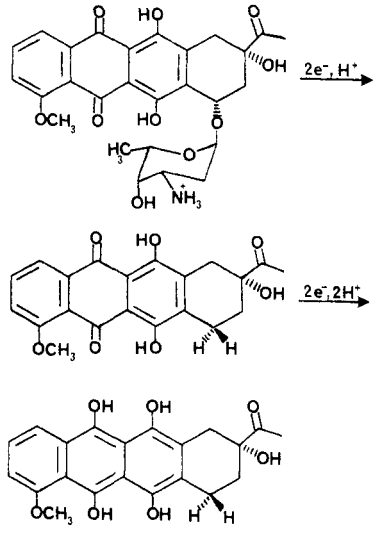


Table II: Ratio of 7-Deoxydaunomycinone and Iso-7-deoxydaunomycinone Produced upon Oxidation of Binding Protein Bound 7-Deoxydaunomycinone Hydroquinone[a]

oxidant	7-deoxy (%)	iso-7-deoxy (%)
O_2	76	24
ferricyanide	76	24
H_2O_2	52	48
cytochrome *c*	53	47

[a] Determined spectrophotometrically following addition of excess oxidant. The relative yields are variable from experiment to experiment but are generally within 5% of the given values.

Titration experiments with aclacinomycin A gave very similar results (Figure 6). Bound aclacinomycin is smoothly and quantitatively two-electron reduced anaerobically to the 7-deoxy species in the first spectral conversion. This contrasts to dithionite titration in the absence of the binding protein, where the major product obtained is a 7-deoxyaklavinone dimer,[2] identical with that obtained from enzymatic reduction (Oki, 1977). The failure to observe the dimer suggests a direct reduction of the aclacinomycin–protein complex, as opposed to reduction of free aclacinomycin followed by recomplexation.

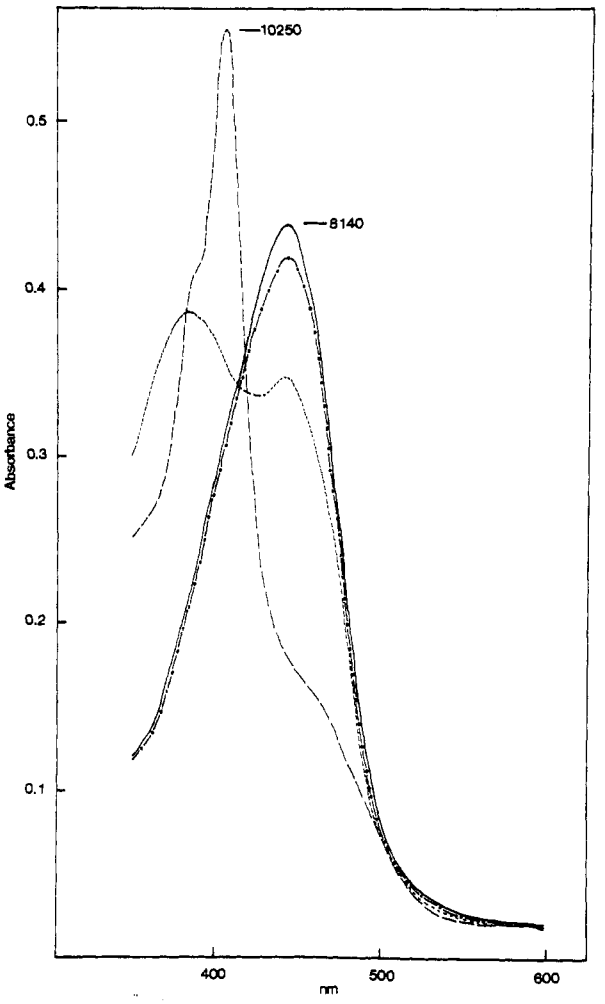


FIGURE 6: Visible absorption spectra obtained during redox titration of binding protein bound aclacinomycin A. Aclacinomycin (100 μM) in 1.30 mL of 0.035 M sodium phosphate pH 7.0 buffer containing 0.15 M NaCl, 0.5 mM EDTA, and 1 equiv of binding protein (—) was titrated with two-electron equiv of dithionite to yield bound 7-deoxyaklavinone (–•–) and a further two-electron equiv of dithionite to yield bound 7-deoxyaklavinone hydroquinone (– –) and oxidized with air to a mixture of bound 7-deoxyaklavinone isomers (- - -). This last spectrum converts within minutes to one identical with that for bound 7-deoxyaklavinone.

Further two-electron reduction gives the hydroquinone, which may then be two-electron oxidized by various oxidants. Again, a different spectrum is obtained upon oxidation that is suggestive of a mixture of 7-deoxy tautomers, as was the case for daunomycin. The structure of the contributing tautomer(s) remains unknown as these quickly (minutes) convert to protein-bound 7-deoxyaklavinone.

Under titration conditions both the reductive and oxidative conversions occur without detectable intermediates. Given the chemical nature of the reductant and the oxidants, it is likely that the redox reactions occur in one-electron increments. In order to ascertain whether semiquinone intermediates were produced, we quickly (1–2 s) mixed anaerobic solutions of the daunomycin and aclacinomycin binding protein complexes with a single equivalent of dithionite. For both, this gave a transient (seconds) blue color to the solution, as expected for the semiquinones, but in neither case was the intensity or the duration of the color appreciably different from that obtained in the absence of the binding protein. Thus the semiquinones are not stabilized by the binding protein. This is in contrast to the behavior with flavins, where the blue zwitterionic flavin semiquinone is bound and stabilized. This discrepancy may result from the radical anion nature of the anthracycline semiquinones at neutral pH and the intolerance of the binding protein for negative charge (Blankenhorn, 1978; Walsh et al., 1978). The transient nature of the semiquinones indicates that the one-electron hydroquinone–semiquinone potential is more positive than the semiquinone–quinone potential, and thus rapid disproportionation occurs. The ability of near stoichiometric quantities of dithionite anion to reduce the quinone places the semiquinone–quinone potential above that for dithionite (–660 mV at pH 7.0 under titration conditions; Mayhew, 1978). This is consistent with electrochemical estimates of anthracycline reduction potentials of about –300 mV (Rao et al., 1978; Svingen & Powis, 1981).

There are two aspects to the dithionite reduction of the complexed anthracyclines that are uncertain. It is not known whether dithionite transfers electrons to the equilibrium concentration of anthracycline in solution or directly to the anthracycline–binding protein complex. Dithionite reduction of the riboflavin–binding protein complex is significantly slower than that of free riboflavin, but the kinetic studies required to rule in favor of a direct reduction pathway have not been done. All that may be said with certainty is that the electron transfer is rapid and that the apparent binding protein preference for the quinone over the semiquinone is not that large to lower the redox potential of the bound anthracycline below that of dithionite at pH 7.0 (–660 mV). Nor is it possible to decide between the semiquinone and the hydroquinone as the immediate precursor to glycoside loss. Lastly, regardless of mechanism, the resulting quinone methide does not label the protein, as the bound 7-deoxyanthracycline obtained at the end of the redox titration is easily displaced by riboflavin. Either no susceptible nucleophile is present, or the quinone methide is unreactive.

Discussion

No flavoprotein has been more comprehensively examined for its ability to bind flavins and flavin analogues than the egg riboflavin binding protein. By the correlation of equilibrium constant with structure, a good (albeit indirect) model of the binding site has been obtained. This model was first described by Becvar (1973) and most recently reviewed by Choi & McCormick (1980) and Becvar & Palmer (1982). The following description is summarized from these references. No compound is bound more tightly than riboflavin (K_d = 0.002 μM; Becvar, 1973; Nishikimi & Kyogoku, 1973). Riboflavin binding occurs primarily by the energetically favorable displacement of water from a hydrophobic cavity, supplemented by particularly favorable hydrophobic contacts, hydrogen bonds, and steric compatibility. Thus, hydrophobic contact is made to the flavin 7,8-dimethyl groups; hydrogen bonding is made to the C-2′ hydroxyl and C-4 carbonyl; and steric limitations exist at C-4, C-8, and N-10. The binding site prefers neutral molecules or zwitterions, tolerates cations, and eschews anions. The flavin rests between a tyrosine residue and a typtophan residue, having the dimethylbenzene ring in the protein interior and the pyrimidine ring near the protein surface. Within these limitations, this protein might be anticipated to bind a variety of compounds having a flavin-like structure. However, few have been tested. A cationic, substituted indole (indocyanine green) was found to bind with a K_d of 1 μM and with large spectral changes consistent with transfer to a hydrophobic site (Steczko & Ostrowski, 1975; Blankenhorn, 1978). Choi & McCormick (1980) demonstrated binding protein recognition of flavin "fragments". In this context, it is more easily understood why anthracyclines are bound. Like flavins, they are linear, planar conjugated

molecules, although tetracyclic rather than tricyclic. While they do not duplicate features that are advantages to the flavins, neither do they possess substituents that would intrude on sterically forbidden regions. Thus the anthracyclines can insert into the binding site with their D–B rings overlaying the space normally occupied by the flavin A–C rings. This orientation is, coincidentally, identical with that for the flavin–adriamycin molecular complex formed in solution (Kharasch & Novak, 1981a). The conclusion to be made is that the resemblance of anthracyclines to flavins is sufficient to realize a good deception of the binding protein.

The complexation of anthracyclines by the binding protein raises two questions. The first is whether the anthracycline binding is specific or fortuitous. From the preceding discussion it is apparent that there is an element of both to the binding. Three molecules that superficially resemble riboflavin as well as anthracyclines—tetracycline, carminic acid, and DHAQ—are weakly or not at all bound. In contrast, anthracycline glycosides are bound with an equilibrium constant of approximately 0.5 μM. The 3 orders of magnitude difference from riboflavin might be easily rationalized with reference to the obvious points of difference. Yet glycoside loss provides a product—an anthracyclinone—that is bound with an affinity quite comparable to several isoalloxazines, such as lumiflavin. Given the specific binding of anthracyclines and anthracyclinones to the riboflavin binding protein, the second question to evaluate is the potential significance to other flavoprotein systems. This is not a question with a simple answer. To begin with, it must be appreciated that there is no typical flavoprotein. The flavin coenzyme is, perhaps more than any other coenzyme, subjected to the influence of the protein environment to optimize its reactivity for the catalysis of the reaction at hand (Massey & Hemmerich, 1980). Furthermore, the flavin orientation used by the egg binding protein is by no means universal; many flavoenzymes have the dimethylbenzene ring at the protein surface (Schopfer et al., 1981). Such an enzyme would be very unlikely to bind anthracyclines, due to their A ring and its substituents. A reasonable hypothesis is that the binding protein is a model for *some* flavin-dependent enzymes. For such enzymes it may be possible for anthracyclines (or more likely anthracyclinones) to compete against the flavin coenzyme for the protein site. If this were to occur, the catalytic ability of the enzyme would suffer. The anthracyclines have a lower intrinsic redox potential (Rao et al., 1978; Svingen & Powis, 1981) and would certainly have different mechanistic requirements for electron entry and departure. There is circumstantial evidence in support of flavoenzyme inhibition by anthracyclines. Two chemotherapeutic agents known to antagonize flavin coenzyme biosynthesis and flavin coenzyme activity, quinacrine (Weinbach, 1981) and chlorpromazine (Rivlin, 1979), are bound to the binding protein with an affinity equal to that of the anthracyclines. Further, there are several reports of decreased flavoenzyme activity in the presence of anthracyclines or anthraquinones (Taylor & Hochstein, 1978; Pan et al., 1981; Kharasch & Novak, 1981b). The mechanism of these inhibitions has yet to be established and may involve flavin displacement from the active site.

There are two conclusions that may be drawn from the observation of anthracycline complexation by the egg riboflavin binding protein. First, anthracyclines and their metabolites may be capable of antagonizing flavoenzyme function by competition against the coenzyme for its binding site. A very similar suggestion to this has been made by Kharasch & Novak (1981a) on the basis of their own investigations in this area. Second, it suggests a mechanism for anthracycline transport and entry into cells, by usurpation of the carrier proteins present for riboflavin. Should this be the case, coadministration of riboflavin with anthracycline might have a significant effect on the anthracycline pharmacodynamics. It may be anticipated that further study will clarify the possible relationship between anthracycline cytotoxicity and flavoenzyme function.

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