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Molecular Association between Doxorubicin (Adriamycin) and DNA-Derived Bases, Nucleosides, Nucleotides, Other Aromatic Compounds, and Proteins in Aqueous Solution

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SUMMARY

Doxorubicin (adriamycin) forms molecular associations with other aromatic and planar molecules (hetero-association) and with other doxorubicin molecules (self-association) in aqueous solution. The ability of doxorubicin to form complexes was demonstrated in a nonbiological system by measuring the doxorubicin partition coefficient. A decreased apparent doxorubicin activity coefficient in the presence of complex formation was also demonstrated in a biological system by measuring the transmembranous doxorubicin transport and the doxorubicin distribution at equilibrium in human red blood cells and their suspending medium. Doxorubicin formed complexes in aqueous solution at 37° (pH 7.3) with (a) DNA-derived bases, nucleosides, and nucleotides; (b) amino acids such as tryptophan; (c) proteins such as human serum albumin and hemoglobin; and (d) a broad range of biologically active compounds such as NAD, propanthelline, caffeine, chloroquine, imipramine, and propranolol. The apparent thermodynamic quantities of the complex formation with adenosine 5′-triphosphate were ΔH^0, −9.5 kcal·mole^{-1}; ΔS^0, −19 eu·mole^{-1}; and ΔG^0 (310° K), −3.6 kcal·mole^{-1}. The binding forces of the molecular associations were probably hydrophobic (short-range force), sometimes supported by electrostatic interaction (long-range force).

INTRODUCTION

Doxorubicin (adriamycin) is one of the most widely used and potent drugs against human cancer. It belongs to the anthracycline group of chemotherapeutic agents. Doxorubicin is a glycosidic antibiotic constituted by a pigmented aromatic aglycone linked to an amino sugar. Doxorubicin is partially ionized under physiological conditions by protonation of the amino sugar. The study of the physicochemical properties of the anthracyclines has become more interesting to membrane physiologists and oncologists after the demonstration of an apparent correlation between the ability of the anthracyclines to self-associate in aqueous solution to higher molecular weight complexes (1, 2) and the characteristics of anthracycline transport across cell membranes, e.g., the saturation kinetic (3). The self-association appears to be a function of hydrophobic forces through π electron interaction between the planar and aromatic portion of the anthracyclines. On this background it is thought that anthracyclines form complexes in aqueous solution with an extensive range of various compounds which are planar aromatics. The ability of the anthracyclines to intercalate and to form complexes with nucleic acids which contain heterocyclic aromatic bases is well known. The present paper describes the ability of doxorubicin to form complexes in aqueous solution at 37° with a broad range of low and high molecular weight compounds which contain planar aromatic ring systems. The complex formation between doxorubicin and the various compounds is demonstrated in a nonbiological system by determining the partition of doxorubicin between a lipid phase and a water phase. A shift of doxorubicin from the lipid phase toward the water phase following the addition of various water-soluble compounds indicated complex formation in the water phase, i.e., the compounds increased the water solubility of the sparsely water-soluble electroneutral doxorubicin molecule by complex formation. Furthermore, the effect of complex formation on doxorubicin transport and equilibrium distribution across human red blood cell membranes is shown. The present data are in accordance with previously published data (3) indicating that doxorubicin transport across cell membranes is predictable from the distribution of doxorubicin between a lipid and a water phase. Preliminary results of this study have appeared elsewhere (4).

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EXPERIMENTAL PROCEDURES

Materials and Methods. The partition of doxorubicin between a lipid (low-dielectric) phase and a water (high-dielectric) phase was determined by measuring the partition of doxorubicin between 1-octanol and a 0.05M Tris-distilled water solution (3). The doxorubicin and the various additional compounds were dissolved in 18 ml of Tris buffer solution and equilibrated at the appropriate temperature and pH. Three initial samples of 2 ml each were collected, and 12 ml of 1-octanol were added to the remaining 12 ml of Tris buffer solution under vigorous stirring by a magnetic bar. After 10, 20, and 40 min, respectively, a 2-ml sample was drawn from the 1-octanol and buffer phases after spontaneous separation of the two phases. The doxorubicin equilibration between the two phases was obtained within the first 10 min. The doxorubicin was extracted from the 1-octanol phase by shaking 1 ml of 1-octanol with 1 ml of 1 N HCl solution at room temperature. The doxorubicin concentration was measured in the 1 N HCl solution and in the Tris buffer solution after acidification with 5 N HCl by spectrophotofluorometry. The results were expressed by the apparent partition coefficient [(moles per liter of 1-octanol) (moles per liter of Tris buffer)$^{-1}$], which was defined as the ratio of the total amount of doxorubicin (protonated or deprotonated) per unit volume of 1-octanol and Tris buffer, respectively. The apparent partition coefficient was calculated from (a) the decrease of the doxorubicin concentration in the Tris buffer phase as well as from (b) the doxorubicin concentration in the 1 N HCl extraction solution of the 1-octanol phase. Both concentrations were expressed as fractions of the average concentration of the doxorubicin in the Tris buffer solution at equilibrium in order to obtain the apparent partition coefficient. The partition coefficient of the deprotonated and electroneutral doxorubicin species between 1-octanol and the Tris buffer was calculated from the apparent doxorubicin partition coefficient under certain assumptions (see Calculations).

The apparent partition coefficient was measured as (a) a function of doxorubicin concentration in the Tris buffer at equilibrium in the presence of various compounds added to the Tris buffer, or as (b) a function of the concentration of the various added compounds in the Tris buffer at equilibrium in the presence of a constant quantity of doxorubicin in the total system consisting of both the Tris buffer phase and the 1-octanol phase. The measured apparent doxorubicin partition coefficient was normalized by expressing the coefficient as a fraction of the apparent partition coefficient in the absence of added compounds (e.g., Fig. 2).

The effect of the various compounds on doxorubicin transport was determined by measuring the initial cellular doxorubicin net uptake in human red blood cells. The doxorubicin uptake was determined in red cells suspended in either 165 mM NaCl or autologous plasma from fasting human subjects at a hematocrit of 0.1 and at pH 7.3 (37°). The red cells were obtained from freshly drawn, heparinized blood after removal of the buffy coat and from the lower portion of the column of packed red cells obtained by centrifugation of the blood. The cells were washed three times in 165 mM NaCl, titated to pH 7.3 (37°), and washed once more to remove signs of hemolysis. The pH of the cell suspension was buffered mainly by the intracellular hemoglobin content (5). The suspension of red cells in their own plasma was equilibrated with atmospheric air until the pink color of oxyhemoglobin was obtained. The pH of the cell suspension was titrated from 7.8 to 7.3 (37°) with 1 N HCl. The number of red cells in the suspension (approximately 10^{12} cells/liter of suspension) was counted in a Bürger-Türk counting chamber (Carl Zeiss, Jena, East Germany) after the appropriate dilution of the cell suspension.

The doxorubicin net influx experiments were carried out under atmospheric air with 30 ml of the cell suspension incubated at 37° (pH 7.3) under vigorous stirring of the suspension by a Teflon-coated magnetic bar. The pH and temperature were recorded continuously and remained constant during the experiments. The test compounds were added to the cell suspension approximately 3 min before the addition of doxorubicin. Samples of cell suspension (1.5 ml) were injected into 10 ml of stopping solution at 0° at various periods after the addition of doxorubicin to the cell suspension. The cooling procedure stopped the doxorubicin transport across the red cell membrane (3). However, the pH dependence of doxorubicin transport in red cells (6) was also taken into account in the composition of the stopping solution: 140 mM NaCl, 10 mM sodium succinate, and 10 mM KH_2PO_4 (0°, pH 6.1). The cells were washed twice with 165 mM NaCl at 0° in order to remove doxorubicin from the extracellular fluid and the cell surfaces. The plots of the cellular doxorubicin uptake versus time intersected with the point of the origin when this stopping procedure was employed.

The cellular doxorubicin content was determined by measuring the total drug fluorescence extracted from the drained cell pellet (approximately 150 μl of red cells) after the two washes. The doxorubicin was extracted from the cell pellet with 10 ml of extraction solution [75% acetone (v/v) and 25% *N,N*-dimethylacetamide (v/v)] after hypotonic hemolysis of the cells with 0.5 ml of water (6). The extraction solution with cells was shaken for 10 min at room temperature in the dark, and a water-clear supernatant was obtained after centrifugation at room temperature (4×10^3 rpm for 5 min). The fluorescence of the supernatant was measured by spectrophotofluorometry (Aminco Bowman spectrophotofluorometer; American Instrument Company, Silver Spring, Md.) with excitation and emission wavelengths of 470 nm and 590 nm, respectively, at room temperature. The fluorescence of the two blanks obtained at the start of the experiments before the addition of doxorubicin to the cell suspension was measured after the washing and extracting procedures in parallel with the doxorubicin-containing samples. The latter samples were corrected accordingly. Furthermore, blanks were used in each experiment to check absence of a doxorubicin fluorescence quench. Appropriate standards were used and the linearity of fluorescence versus drug concentration was tested. The determination of doxorubicin concentration in the stock solution was carried out by spectrophotometry (SP 1800 spectrophotometer; Pye Unicam Ltd., Cambridge, England) of an

acidic methanolic solution [more than 97% methanol (v/v)] of the doxorubicin stock solution at 478 nm at room temperature. The doxorubicin concentration before reading was adjusted to give an absorbance of 0.1–0.3 unit in order to obtain a linear relationship between absorbance and drug concentration. An extinction coefficient of $E_{1\,cm}^{1\%} = 225$ was employed.

Calculations. The apparent association constants between doxorubicin and the various compounds in aqueous solution were calculated from partition studies of doxorubicin between 1-octanol and the Tris buffer solution. The studies were carried out under conditions where doxorubicin and the various compounds did not self-associate, i.e., at doxorubicin concentrations below 5 μM (cf. Fig. 1), and at concentrations of DNA-derived bases, nucleosides, and nucleotides below 10 mM (7, 8). Furthermore, the concentrations of the various compounds were more than 300 times the doxorubicin concentration at equilibrium. The apparent doxorubicin partition coefficient was measured at a constant doxorubicin ionization, i.e., (a) at pH 7.3 (37°) or (b) at various pH values depending on the influence of temperature on the pK of the Tris buffer, which equals the temperature effect on the pK of the amino group on doxorubicin (3).

The apparent doxorubicin partition coefficient in the absence of other compounds is described by the equation

$$K^0 = [C]_0/[C]_b \quad (1)$$

where $[C]_0$ and $[C]_b$ are the concentrations of the total amount (protonated and deprotonated) of doxorubicin in the 1-octanol phase and the Tris buffer solution, respectively. Hetero-association consisting of a 1:1 complex in the Tris buffer solution is described by

$$[C]_b + [N]_b \overset{K_a}{\rightleftharpoons} [CN]_b \quad (2)$$

where $[N]_b$ and $[CN]_b$ are the concentrations (molar) in the Tris buffer solution of free and hetero-associated compound after complex formation with doxorubicin. The complex formation in the aqueous solution is described by the mass law

$$[CN]_b = K_a\,[C]_b[N]_b \quad (3)$$

where $K_a(M^{-1})$ is the apparent molar association constant. The apparent doxorubicin partition coefficient in the presence of the various compounds at equilibrium is described by using Eq. 3:

$$K^N = [C]_0/([C]_b + [CN]_b) = [C]_0/([C]_b(1 + K_a[N]_b)) \quad (4)$$

The apparent doxorubicin partition coefficients at various concentrations of added compounds were normalized (e.g., Fig. 2A) by expressing the coefficients as fractions of the current doxorubicin partition coefficient in the absence of added compounds (Eq. 1)

$$K^N/K^0 = (1 + K_a(N)_b)^{-1} \quad (5)$$

The values of K_a were determined from the plots of the reciprocal value of the normalized partition coefficients versus the concentrations of the added compounds in the Tris buffer solution at equilibrium (e.g., Figs. 2B and 4A). The graphs were fitted to the data by eye and passed through unity on the ordinate. The values of $K_a(M^{-1})$ were calculated from the slopes of the graphs.

The partition coefficient of the unprotonated and electroneutral doxorubicin species between the 1-octanol and the Tris buffer phase in the absence or presence of electroneutral compounds can be estimated from the apparent partition coefficient by multiplication with the factor, $Q = (1 + 10^{pK-pH})$, where pK is the pK of the amino group on doxorubicin and pH is the pH of the Tris buffer. This calculation presupposes that (a) the pK of doxorubicin is unaffected by complex formation, and (b) the affinity of the protonated doxorubicin for the added compounds equals that of the unprotonated doxorubicin. The factor Q equals 3 at pH 7.3 (37°), assuming a pK of 7.6 at 37° (6).

The thermodynamic quantities of the complex formation between doxorubicin and ATP was determined from the partition studies. The apparent standard enthalpy, ΔH^0 (cal·mole^{-1}), was calculated from the slope of a logarithmic plot (Fig. 4B) of the apparent association constant $K_a(M^{-1})$, versus the reciprocal value of the absolute temperature, T(°K), by use of the indefinite integral of the van't Hoff equation

$$\ln K_a = (-\Delta H^0/R)\,(1/T) + q \quad (6)$$

where R is the universal gas constant (cal·mole^{-1}·°K^{-1}) and q is the constant of the integration. The apparent standard free energy change of the reaction at temperature T, $\Delta G^0(T)$ (cal·mole^{-1}) was calculated from the equation

$$\Delta G^0(T) = -RT \ln K_a(T) \quad (7)$$

where $K_a(T)$ is the association constant of the complex formation at temperature T(°K). The apparent standard entropy, ΔS^0, was calculated from the equation

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (8)$$

and expressed in entropy units (Clausius) per mole (cal·°K^{-1}·mole^{-1}).

The apparent doxorubicin permeability coefficient, P (cm·sec^{-1}), was calculated from the initial cellular doxorubicin net uptake (moles/10^{20} red cells·min) and the assumptions of a surface area of 1.42×10^{-6} cm^2 per single cell and an initial doxorubicin concentration difference across the rate-limiting barrier of the red cell membrane equal to the calculated initial doxorubicin concentration in the cell suspending medium, $(D)_m$ (mol·cm^{-3}). The following equation was used

$$P\ (\text{cm}\cdot\text{sec}^{-1}) = M(D)_m^{-1} \quad (9)$$

where M is the initial doxorubicin net influx (mole·cm^{-2}·sec^{-1}). The apparent doxorubicin activity coefficient in plasma, $a_D(p1)$, was calculated from the equation

$$a_D(p1) = P_{p1}/P_s \quad (10)$$

where P_{p1} or P_s is the apparent doxorubicin permeability coefficient in red cells when they are suspended in autologous plasma or a salt solution, respectively. The calculation was carried out under the assumptions that (a) the activity coefficient of doxorubicin in a salt solution was equal to unity at doxorubicin concentrations below 10

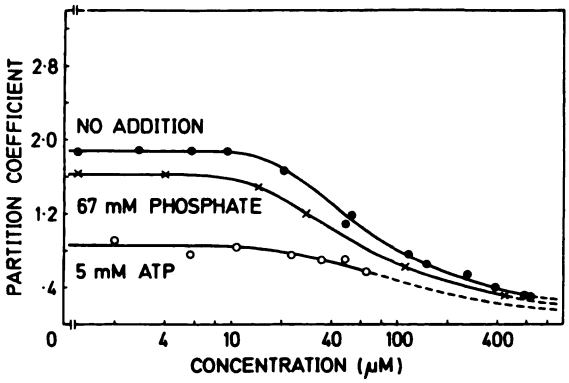

FIG. 1. *Doxorubicin partition coefficient between a l-octanol and a 0.05 M Tris buffer solution as a function of the doxorubicin concentration in the Tris buffer at equilibrium (37°, pH 7.3)*

●, In the absence of any additional compounds; ×, in the presence of 67 mM sodium phosphate; ○, in the presence of 5 mM sodium ATP.

μM, and (b) the effect of plasma on the apparent doxorubicin permeability coefficient was due only to doxorubicin interaction with plasma components.

Drugs. Doxorubicin was obtained commercially as doxorubicin·HCl in lactose (adriamycin; Carlo Erba Farmitalia, Milan, Italy). Adenine, adenosine, and inosine were obtained from E. Merck (Darmstadt, West Germany); cytidine, uridine, guanosine, caffeine, L-tryptophan, L-histidine, and theophylline from British Drug House Chemicals Ltd. (Poole, England); 2′-deoxyadenosine, AMP, ADP, ATP, NAD, propantheline bromide, and imipramine·HCl from Sigma Chemical Company (St. Louis, Mo.); chloroquine diphosphate from Serva Feinbiochemica (Heidelberg, West Germany; and thiamazolum from Gea Ltd. (Copenhagen, Denmark). Propranolol·HCl was obtained from Ferrosan Ltd. (Malmoe, Sweden). The octanol was obtained as 1-octanol reinstituted (Lot 991) from E. Merck.

RESULTS

Figure 1 shows the apparent partition coefficient of doxorubicin between 1-octanol and the Tris buffer phase as a function of the doxorubicin in the Tris buffer at equilibrium (37°, pH 7.3). The partition coefficient was approximately 2 in the absence of added compounds at low doxorubicin concentration. The coefficient decreased with concentration above 20 μM. A decrease in the partition coefficient demonstrated that doxorubicin had become more hydrophilic; i.e., the activity coefficient of doxorubicin was decreased in the aqueous phase. Furthermore, the addition of inorganic phosphate or ATP to the aqueous phase decreased the partition coefficient at all doxorubicin concentrations. However, the greatest effect on the partition coefficient was observed at low doxorubicin concentration. The influence of only 5 mM ATP on the coefficient was much more pronounced than the effect of 67 mM phosphate at a given doxorubicin in concentration.

Figure 2A shows the normalized apparent doxorubicin partition coefficient as a function of the concentration of various DNA-derived bases, nucleosides, and nucleotides at equilibrium and at a constant amount of doxorubicin in the system (37°, pH 7.3). All added compounds decreased the doxorubicin partition coefficient with increasing concentration of the compounds. However, the effect of the purine derivatives was more distinct than the effect of the pyrimidine derivatives. Furthermore, the decrease of the partition coefficient of doxorubicin was more pronounced in the presence of the electronegative ATP than in the presence of adenosine. The partition coefficients of purine and pyrimidine bases, nucleosides, and nucleotides themselves between the 1-octanol and the Tris buffer phase were measured in separate experiments, and the concentrations in the aqueous phase were corrected accordingly. The ATP, guanosine,

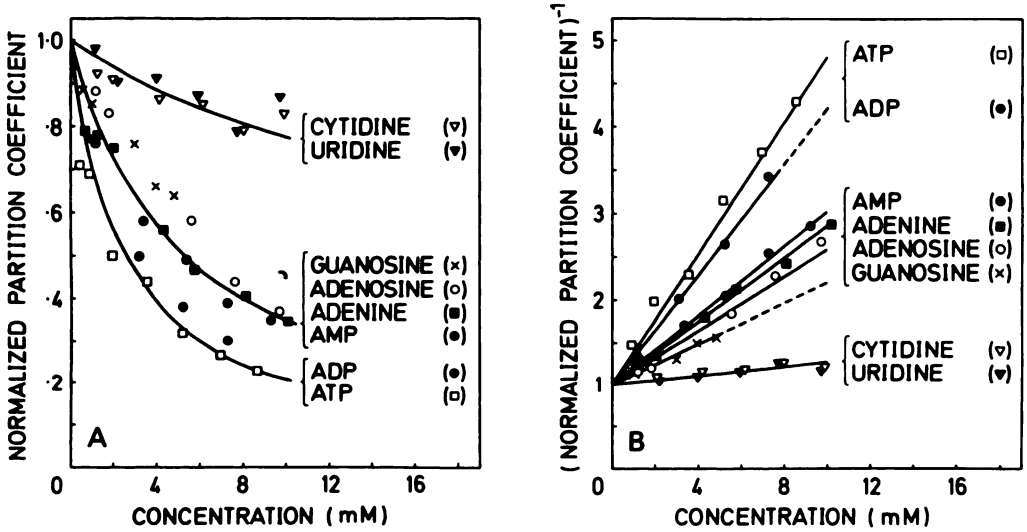

FIG. 2. *Effect of heterocyclic compounds on the doxorubicin partition coefficient between 1-octanol and a 0.05 M Tris buffer solution as a function of the concentration of the various compounds in the Tris buffer at equilibrium (37°, pH 7.3)*

The measured partition coefficients were normalized to the measured partition coefficients in the absence of added compounds (A). The apparent doxorubicin molar association constants were calculated from the plots of the reciprocal normalized partition coefficients versus the concentration of the various compounds (B). The doxorubicin concentration in the Tris buffer was 4 μM at equilibrium in the absence of added compounds.

TABLE 1

Apparent molar association constant, K_a, of doxorubicin to various heterocyclic compounds at 37° (pH 7.3) calculated from 1-octanol/0.05 M Tris buffer solution partition studies

Compound	k_a
	M^{-1}
Adenine	185
Cytidine	35
Uridine	25
Inosine	100
Guanosine	120
Adenosine	160
2'-Deoxyadenosine	160
AMP	205
ADP	320
ATP	380
NAD	190

cytidine, and uridine remained in the Tris buffer solution. The partition coefficients of adenosine and adenine were 0.07 and 0.84, respectively, at an initial concentration in the Tris buffer of 5 mM (37°, pH 7.3).

Figure 2B shows the reciprocal normalized and apparent doxorubicin partition coefficient as a function of the concentration of the various compounds in the Tris buffer at equilibrium (37°, pH 7.3). The apparent molar association constants between doxorubicin and the compounds were calculated from the slopes of the linear graphs (see Calculations) and are shown in Table 1. Table 2 shows the normalized apparent doxorubicin partition coefficients in the presence of various compounds with an initial concentration of the compounds of 5 mM in the Tris buffer at 37° (pH 7.3). No corrections were made in the results of Table 2 for a possible partition coefficient of the added compounds different from zero.

The effect of temperature on the complex formation between doxorubicin and the various compounds was investigated at a constant doxorubicin ionization (see Materials and Methods). Figure 3 shows that the partition coefficient was not affected by temperature (21–60°) at low doxorubicin concentration (4 μM, 37°, pH 7.3) in the absence of additional compounds. In the presence of ATP, the effect of temperature on the doxorubicin partition coefficient increased with ATP concentration. Figure 4A shows the reciprocal normalized partition coefficient of doxorubicin at various temperatures as a function of the ATP concentration in the Tris buffer. Figure 4B demonstrates the effect of temperature on the apparent doxorubicin association constant to ATP between 21° and 60° (a Van't Hoff plot). The apparent standard enthalpy, ΔH^0, and the apparent standard entropy, ΔS^0, were −9.5 kcal·mole^{-1} (−40 kJ·mole^{-1}) and −19 eu·mole^{-1}, respectively. The ΔH^0 was constant between 21° and 60°. The standard free energy change of the complex formation, ΔG^0 (310° K, pH 7.3), was −3.6 kcal·mole^{-1} at 37° (pH 7.3). The ΔG^0 (298° K, pH 7.7) was −3.8 kcal·mole^{-1}.

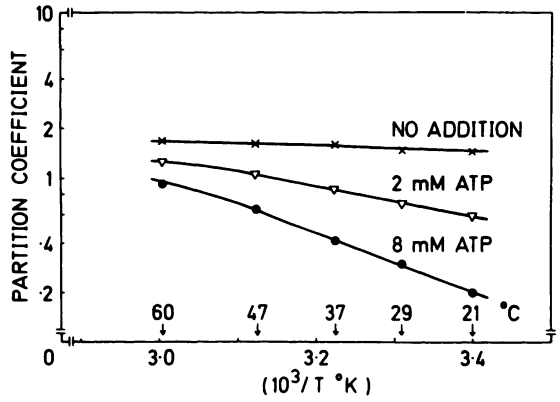

FIG. 3. *Effect of temperature on the doxorubicin partition coefficient between 1-octanol and a 0.05 M Tris-buffer solution at three different concentrations of ATP*

The doxorubicin concentration in the Tris buffer was 4 μM (37°, pH 7.3) in the absence of added ATP.

The various compounds affected the initial cellular doxorubicin net uptake and equilibrium distribution in human red blood cells (37°, pH 7.3). Figure 5 shows the effect of ATP on the net influx into red cells suspended in (a) a salt solution or (b) their own plasma at an initial doxorubicin concentration of 5 μM in the cell suspending medium. In both experimental situations ATP decreased the initial doxorubicin influx and the doxorubicin equilibrium distribution between cells and medium relative to control experiments. Furthermore, Fig. 5 shows that the initial doxorubicin influx and equilibrium distribution decreased in the presence of autologous plasma relative to the values observed with red cells suspended in a salt solution. The apparent doxorubicin permeability coefficient of human red blood cells suspended in a salt solution was 4×10^{-6} cm·sec^{-1} (37°, pH 7.3) when the coefficient was calculated from the initial influx. The permeability coefficient decreased to 1.5×10^{-6} cm·sec^{-1} in the presence of 5 mM ATP. The permeability coefficient of red cells suspended in their own plasma was calculated to be 6×10^{-7} cm·sec^{-1} and decreased to 4×10^{-7} cm·sec^{-1} in the presence of 5 mM ATP (37°, pH 7.3).

The doxorubicin equilibrium distribution ratio [(moles per liter of cells) (moles per medium)$^{-1}$] between cells and the cell-suspending medium at 37° (pH 7.3) was determined to be 7.7 (SD 0.7, $n = 12$) when the cells were suspended in a salt solution. The distribution ratio decreased to 1.6 (SD 0.07, $n = 8$) when the cells were suspended in autologous plasma at doxorubicin concen-

TABLE 2

Doxorubicin partition coefficient, K^N/K^O, at equilibrium between 1-octanol and a 0.05 M Tris buffer solution in the presence of various compounds expressed as a fraction of the partition coefficient in the absence of added compound (37°, pH 7.3)

The various compounds were added to the Tris buffer to obtain an initial concentration of 5 mM with the exception of tryptophan and histidine (10 mM).

Compound	K^N/K^O
Theophylline	0.64
Caffeine	0.29
Propantheline bromide	0.39
Thiamazolum	0.45
Chloroquine diphosphate	0.25
Imipramine·HCl	0.16
Propranolol·HCl	0.08
Tryptophan	0.5
Histidine	1.0

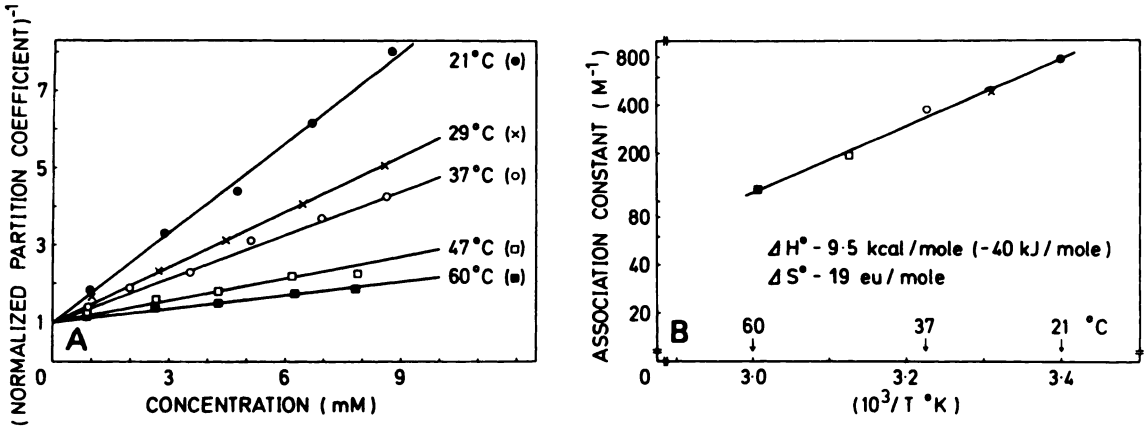

FIG. 4. *Effect of temperature on the interaction between doxorubicin and ATP at constant doxorubicin ionization*

A, The reciprocal-normalized doxorubicin partition coefficient between 1-octanol and 0.05 M Tris buffer solution as a function of the ATP concentration in the Tris buffer at various temperatures. The doxorubicin concentration in the Tris buffer was 4 μM at equilibrium (37°, pH 7.3) in the absence of added ATP. B, The apparent molar doxorubicin association constant, $K_a(Y)$, to ATP as a function of the reciprocal absolute temperature, $T(X)$. The relationship was described by the equation $\ln Y = 4.78\ (10^3/X) - 9.60$; $r^2 = 0.99$.

trations in the suspending medium of less than 100 μM (3–100 μM).

DISCUSSION

The present data add various low molecular weight molecules to the long list of compounds which interact with doxorubicin through complex formation in aqueous solution. Anthracyclines interacted with high molecular weight structures composed of negatively charged phospholipids (9–11), sulfated mucopolysaccharides (12), nonhistone protein from the rat liver (13), and tubulin (14). The present data demonstrate that rather simple and low molecular weight molecules interacted with doxorubicin in aqueous solution. The complex formation between doxorubicin and the low molecular weight compounds in aqueous solution was demonstrated by a decrease in the l-octanol/Tris buffer partition coefficient, meaning that the low molecular weight molecules increased the water solubility of doxorubicin by decreasing the apparent activity coefficient of doxorubicin in the water phase. The ability of small hetero-cyclic compounds to increase the water solubility of the sparsely water-soluble electroneutral doxorubicin molecule was in accordance with the solubilization of 3,4-benzpyrene in aqueous solutions of caffeine and other nitrogenous bases first described in 1938 by Brock *et al.* (15).

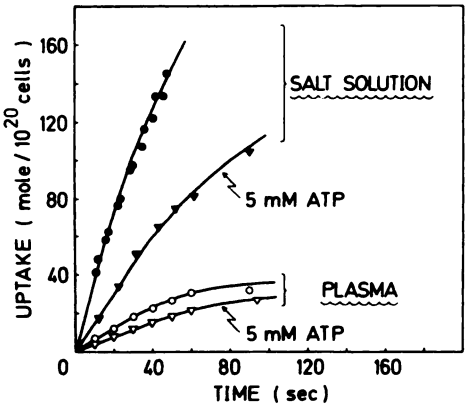

FIG. 5. *Effect of ATP on the cellular doxorubicin net uptake as a function of time after the addition of doxorubicin to human red blood cells suspended in a salt solution or their own plasma in the absence or presence of 5 mM ATP*

The initial doxorubicin concentration in the suspending medium was 5 μM (37°, pH 7.3).

The present paper calls attention to the apparent doxorubicin partition coefficient for two reasons: (a) the apparent partition coefficient is defined by the actually measured doxorubicin concentrations in the lipid and water phases, and (b) many biological phenomena correlate to the apparent partition coefficient (e.g., membrane transport of lipophilic compounds). An increased water solubility of doxorubicin in the presence of ATP and a decreased apparent doxorubicin permeability coefficient in human red blood cells was in accordance with the observation that doxorubicin transport in red cells showed saturation kinetics. The saturation kinetics correlated to a decreasing doxorubicin apparent partition coefficient, i.e., an increased water solubility, with increasing doxorubicin concentration (3) because the doxorubicin molecules self-associated in aqueous solution (1, 2).

The linear relationship between the reciprocal normalized partition coefficient of doxorubicin versus the concentration of the added compounds in the Tris buffer solution might indicate that the complex formation mainly took place as a 1:1 molecular association reaction (see Calculations, Eq. 2). The interaction of daunomycin with dinucleoside phosphates has also been suggested to take place as a bimolecular reaction (16).

The partition coefficient of the electroneutral and deprotonated doxorubicin is calculable from the apparent doxorubicin partition coefficient under certain assumptions by us of the multiplication factor Q (see Calculations). The partition coefficient of deprotonated doxorubicin is approximately 6 (37°, pH 7.3) in the absence of added compounds, a value which indicates a lipid solubility of deprotonated doxorubicin of the same magnitude as l-propanol and l-butanol.

The effect of temperature on the complex formation

between doxorubicin and ATP was investigated by using a special technique. It has been shown that the apparent doxorubicin partition coefficient is a sensitive function of pH following protonation of the doxorubicin amino group (2). Furthermore, the ionization enthalpy of the amino group on doxorubicin is as high as that for other amino groups such as tris(hydroxymethyl)aminomethane (3). Consequently, it is necessary to measure the doxorubicin partition coefficient at a constant fractional doxorubicin ionization at all temperatures in order to determine the influence of temperature on the complex formation between doxorubicin and added compounds. The latter condition is obtainable only by shifting the pH of the reaction solution with temperature. The necessary pH shift with temperature was achieved by use of an amino group containing buffer such as Tris. Figure 3 demonstrates that the necessary condition was fulfilled because the apparent partition coefficient of doxorubicin was temperature-independent in the absence of added compounds with the technique employed, although the pH of the Tris buffer solution shifted approximately 1 pH unit between 20° and 60°.

The thermodynamic quantities of the reaction of doxorubicin with ATP were similar to those of the reactions of other aromatic compounds with nucleotides which were characterized by large negative values of ΔH^0 and ΔS^0 and rather small negative values of standard free energy changes. The thermodynamic quantities of the complex formation between another intercalative compound, actinomycin, and nucleotides have been estimated to have a ΔH^0 of approximately -9 kcal·mole^{-1} and a ΔS^0 of -17 eu·mole^{-1} with a standard free energy change of -4 kcal·mole^{-1} at room temperature (17–19). The thermodynamic quantities of the complex formation reaction between various indole derivatives such as tryptophan and nucleotides have been estimated to have a ΔH^0 of -3 kcal·mole^{-1} and a ΔS^0 of -8 eu·mole^{-1} with a free energy change of -1 kcal·mole^{-1} at room temperature (20). A number of other reactions very likely promoted by hydrophobic interactions also show large negative values of ΔH^0 and ΔS^0, such as the self-association of actinomycin (21), the self-association of DNA-derived bases and nucleosides (22), and the stacking of bases in nucleic acids (23, 24). The most obvious interpretation of the present data is that, in complex, the doxorubicin chromophore and the aromatic portion of the various compounds were oriented parallel to each other, with interaction of the π electrons of the two aromatic ring systems. An additional long-range interaction force was apparently the electrostatic interaction between the doxorubicin cation and ADP and ATP. The equilibrium of the complex formation reaction was shifted to the left (see Calculations, Eq. 2) with temperature (destacking) as for other exothermic processes following the Le Châtelier-Braun principle. However, the thermodynamic quantities indicate that the complex formation first becomes energetically unfavorable at temperatures above 225° under the assumption that ΔH^0 and ΔS^0 are independent of temperature.

It has been suggested that there exist three types of binding forces between daunomycin and the high molecular weight structure, DNA: i.e., (a) hydrophobic interaction due to the intercalated aglycone ring system, (b) electrostatic interaction between the protonated 3′-amino group of the amino sugar and the phosphate groups of the nucleotides, and (c) hydrogen bonds of unspecific character (e.g., ref. 16). The effect of electrically uncharged compounds at neutral pH, such as DNA-derived bases and nucleosides on the doxorubicin partition coefficient, demonstrated the importance of hydrophobic forces on the complex formation in aqueous solutions. However, the stronger effect of ATP on the doxorubicin partition coefficient demonstrated the importance of electrostatic forces. Our data on doxorubicin interaction with nucleosides and nucleotides might support the suggestions of the binding forces between daunomycin and DNA.

Two types of molecular association might be of importance *in vivo* with regard to the therapeutic and toxicological action of doxorubicin: (a) the various compounds might compete with doxorubicin for the binding sites in the cells, and (b) the various compounds decrease the activity coefficient of doxorubicin in the cellular and extracellular aqueous solutions by complex formation. The former situation might account for the decreased ventricular content of doxorubicin after addition of propranolol to the perfusion fluid of isolated cat hearts (25). The meaning of complex formation in aqueous solution with regard to transport and equilibrium distribution of doxorubicin was demonstrated in human red blood cells. In the presence of the impermeant, ATP, both the uptake and distribution were decreased in accordance with the partition experiments. Furthermore, the effect of intracellular ATP on the activity coefficient of doxorubicin might be significant because of the considerable variation of intracellular ATP concentration from 2 mM in red cells, 5 mM in the liver cells, to 13 mM in heart cells. In the latter case the activity coefficient of doxorubicin in the cytosol is expected to be 0.15 as a result of the effect of ATP alone (37°).

The ability of plasma to decrease the activity coefficient of doxorubicin was considerable. The activity coefficient in plasma was approximately 0.15 relative to the value in a salt solution when the activity coefficient was calculated from the apparent permeability coefficients. The smaller activity coefficient observed in plasma might explain the smaller apparent association constant of doxorubicin with DNA when the measurements were made in serum instead of a salt solution (26).

The smaller cellular doxorubicin equilibrium uptake in human red blood cells suspended in plasma has also been demonstrated in nucleated cells (27, 28). The results indicated that the effect of complex-forming compounds on the doxorubicin activity coefficient was additive to the effect of plasma itself, probably by competition between all of the doxorubicin binding compounds for the binding of doxorubicin. This means that the effect of a doxorubicin-binding compound on the activity of doxorubicin in the presence of plasma at a given doxorubicin concentration was less in absolute values than in the absence of plasma because the doxorubicin activity—which determined the magnitude of complex formation—had already been diminished by binding to the other doxorubicin complex-forming compounds present in

plasma. However, the observed percentage decrease of the doxorubicin permeability coefficient in red cells at 5 mM ATP was considerable both in the absence (60%) and presence (30%) of autologous plasma at a given doxorubicin concentration (Fig. 5).

The doxorubicin distribution ratio between red cells and a salt solution was 7.7 at equilibirum, indicating that doxorubicin was bound to cellular components. The data indicated that doxorubicin did not quantitatively adsorb to any major extent to the cell surface of human red cells. However, doxorubicin has been shown to interact with membrane proteins, spectrin and hemoglobin (29, 30). The doxorubicin binding to cellular components appeared largely to be reversible because the doxorubicin distribution ratio decreased to 1.6 in the presence of plasma in short-term experiments.

The present work demonstrates the usefulness of human red blood cells in the determination of the effect of other compounds on doxorubicin activity in aqueous solution containing labile macromolecules (e.g., proteins) which denature in contact with hydrophobic molecules such as l-octanol. In the present paper, complex formation has been demonstrated both in transport experiments and in equilibrium distribution experiments.

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