dose curves in the *Results*. Where other values were used, they are noted in the appropriate figure legends. In the *Results*, values of important drug and system parameters were varied systematically to ascertain their effects on the dose dependency of absorption.

REFERENCES

(1) P. A. Shore, B. B. Brodie, and C. A. M. Hogben, *J. Pharmacol. Exp. Ther.*, **119**, 361 (1957).

(2) H. Stricker, *Drugs Made Ger.*, **13**, 35 (1970).

(3) H. Stricker, *Drugs Made Ger.*, **14**, 93 (1971).

(4) J. A. Clements, R. C. Heading, W. S. Nimmo, and L. F. Prescott, *Clin. Pharmacol. Ther.*, **24**, 420 (1978).

(5) M. A. Osman, R. B. Patel, D. S. Irwin, W. A. Craig, and P. G. Welling, *Biopharm. Drug. Dispos.*, **3**, 89 (1982).

(6) P. G. Welling, R. H. Barbhaiya, R. B. Patel, T. S. Foster, V. P. Shah, J. P. Hunt, and V. K. Prasad, *Curr. Ther. Res.*, **31**, 379 (1982).

(7) J. E. Baer, H. L. Leidy, A. V. Brooks, and K. H. Beyer, *J. Pharmacol. Exp. Ther.*, **125**, 295 (1959).

(8) H. Davenport, in "Physiology of the Digestive Tract," 4th ed., Yearbook Medical Publishers, Chicago, Ill., 1980, p. 63.

(9) L. Bueno, J. Fioramonti, and Y. Ruckebusch, *J. Physiol.*, **249**, 69 (1975).

(10) W. G. Crouthemal, G. H. Tan, L. W. Dittert, and J. T. Doluisio, *J. Pharm. Sci.*, **60**, 1160 (1971).

(11) M. C. Meyer and A. B. Straughn, *Curr. Ther. Res.* **22**, 573 (1977).

(12) P. G. Welling and R. H. Barbhaiya, *J. Pharm. Sci.*, **71**, 32 (1982).

(13) H. P. Deppeler, in "Analytical Profiles of Drug Substances," Vol. 10, K. Florey, Ed., Academic, New York, N.Y., 1981, p. 405.

(14) B. Beerman and M. Groschinsky-Grind, *Eur. J. Clin. Pharmacol.*, **12**, 297 (1977).

(15) "Martindale: The Extra Pharmacopoeia," 27th ed., A. Wade, Ed., The Pharmaceutical Press, London, 1978, p. xxvii.

(16) "The Merck Index" 9th ed., M. Windholz, Ed., Merck and Co., Rahway, N.J., 1976, p. 277.

Microcalorimetric Investigation of the Binding of Some Chemotherapeutic Agents and Related Molecules to Calf Thymus DNA

WILLIAM R. VINCENT *, LEONARD S. ROSENBERG, and STEPHEN G. SCHULMAN ˣ

Received May 24, 1982, from the *College of Pharmacy, University of Florida, Gainesville, FL 32601.* Accepted for publication January 10, 1983.

*Present address: Arnold & Marie Schwartz College of Pharmacy and Health Science, Long Island University, Brooklyn, NY 11201.

Abstract □ Batch microcalorimetry was used to estimate directly the standard enthalpies of the binding of small molecules to DNA. These values were compared with those obtained from spectrophotometric binding constants and van't Hoff plots. The close agreement between the independently obtained enthalpies indicates that the appropriate (best) binding model has four phosphates per binding site. Thermodynamic binding constants were obtained from apparent binding constants measured at different ionic strengths. From these and the measured standard enthalpies, standard free energies and standard entropies of binding were calculated. The weak, presumably external, binding alleged to occur at high formal molar concentration ratios of ligand to DNA bases could not be detected by a measurable heat of binding.

Keyphrases □ Microcalorimetry—binding of chemotherapeutic agents and related molecules to calf thymus DNA □ Binding—chemotherapeutic agents and related molecules to calf thymus DNA, microcalorimetry □ DNA—microcalorimetry, binding of some chemotherapeutic agents and related molecules to calf thymus DNA

The binding of certain aromatic cations to nucleic acids for some time has been implicated as a primary process in antibacterial, antiprotozoal, antiviral, mutagenic, and antineoplastic activity (1–7). The changes in the thermal stability and the hydrodynamic and spectroscopic properties of DNA to which aromatic cations are bound have been suggested to indicate that at high ratios of formal DNA base to cation concentrations, intercalation of the flat aromatic portion of the ligand between adjacent base pairs on DNA occurs (8). Intercalation (also called type I binding) entails insertion of the flat aromatic portion of the ligand between base pairs (8–10) or bases (11–12) on DNA, accompanied by a partial unwinding of the double helix. In crystalline samples, X-ray diffraction spectrometry has provided direct evidence for the intercalation of the fungal antibiotic, dactinomycin, between the adjacent base pairs of DNA (13, 14).

The intercalative mode of binding does not appear to extend to all ligand molecules bound to a DNA helix (4). Rather, it is believed that only one molecule of ligand can be accommodated in an intercalative fashion for every four or five phosphate groups of the DNA helix. When nearly all intercalative binding sites of a DNA double helix are occupied, further interaction of the DNA with excess ligand is still able to occur. However, this binding is weaker than the intercalative binding, is not accompanied by hydrodynamic changes in the DNA solutions, and is believed to entail the electrostatic association of the cationic ligands with the anionic phosphodiester linkages at the surface of the helix. This is referred to as external (or type II) binding and occurs in solutions of low DNA base to ligand formal concentration ratios. At high ligand concentrations, some of the larger polycyclic aromatic molecules appear to be bound to the surface of DNA as dimers, because complexes can be spectroscopically distinguished in which there are two ligand molecules bound for each DNA phosphate group (15). The apparent relationship of the reversible reactions of aromatic cations with nucleic acids to the antimicrobial, antineoplastic, and mutagenic activities of the former has resulted in considerable interest in the qualitative and quantitative investigation of these interactions. These studies have usually been concerned with the stoichiometries and formation constants of the complexes formed between the ligands and the nucleic acids (1–22), although more recent work has been centered about the kinetics of binding (16, 21). Techniques such as electronic absorption spectrophotometry, fluorometry, differential spectrophotometry, and circular dichroic spectrophotometry have been employed to determine the amounts of bound and free ligand at each point in the titration of a so-

0022-3549/84/0900-1279$01.00/0

lution of the free ligand with a concentrated solution of nucleic acid.

THEORETICAL SECTION

Methods for evaluating equilibrium association constants for the binding of small molecules to DNA are generally derived from the independent-site model, developed by Scatchard (23), to deal with the binding of small molecules to proteins and the extension of this model by McGhee and von Hippel (24) to include ligand cooperativity. Peacocke and Skerrett (25) redefined the parameters of the Scatchard equations to allow their application to nucleic acids. In this approach, the ligand is titrated with a solution of DNA of known phosphate concentration, and the concentration of free ligand [B] is determined at each point in the titration either by direct spectrophotometry or by spectrophotometry subsequent to separation of free and bound ligand by equilibrium dialysis or some other suitable method. From the formal ligand concentration C_B, the former DNA phosphate concentration C_P, and the equilibrium free ligand concentration, the average number of bound ligand molecules (occupied binding sites) per phosphate (r) is calculated at each point in the titration:

$$r = \frac{C_B - [B]}{C_P} \qquad \text{(Eq. 1)}$$

For two classes of independent, noninteracting binding sites I (intercalative) and II (external):

$$r = \frac{n_I K_I [B]}{1 + K_I [B]} + \frac{n_{II} K_{II} [B]}{1 + K_{II} [B]} \qquad \text{(Eq. 2)}$$

where n_I and n_{II} are the reciprocals of the numbers of phosphates in each type of binding site and K_I and K_{II} are the equilibrium association constants, belonging to class I and class II binding, respectively. The reactions characterized by K_I and K_{II} may be written:

$$B + S_I \rightleftarrows BS_I \qquad \text{(Eq. 3)}$$

and:

$$B + S_{II} \rightleftarrows BS_{II} \qquad \text{(Eq. 4)}$$

where S_I and S_{II} are the type I and type II binding sites on DNA. The equilibria may be written:

$$K_I = \frac{[BS_I]}{[B][S_I]} \qquad \text{(Eq. 5)}$$

and:

$$K_{II} = \frac{[BS_{II}]}{[B][S_{II}]} \qquad \text{(Eq. 6)}$$

A plot of $r/[B]$ *versus* r is then constructed. This plot is generally hyperbolic in appearance, and if the components of the system are sufficiently dilute so that no ligand aggregation occurs on the polymer, the plot is usually considered to represent a superposition of the type I and type II processes. If K_I and K_{II} differ by at least an order of magnitude, the type I and type II binding may be resolved into straight lines at the high and low $r/[B]$ extrema, respectively, of the $r/[B]$ *versus* r plot. In this case, the extrema of the hyperbola are linearly extrapolated to the $r/[B]$ and r axes. The line of steep slope (at low values of r) is associated with the type I (intercalative) binding and has a slope $-K_I$ and an intercept on the $r/[B]$ axis of $K_I n_I + K_{II} n_{II}$. The line of shallow slope (at high values of r) associated with the type II (external) binding has a slope of $-K_{II}$ and an intercept on the r-axis of $n_I + n_{II}$.

However, the extrapolation of the legs of the hyperbolic plot to obtain slopes and intercepts places an inordinately high degree of reliance on the data at the extrema of the titrations. It is at the extrema that the data, regardless of how they were obtained, are the least precise.

During the course of investigations in this laboratory (26) of the binding of the singly charged cation of 3-aminoacridine, at pH 5.9 ($pK_a = 8.04$) and ionic strength 5.0×10^{-3}, to double-stranded calf thymus DNA, it was found that at high values of $r/[B]$, there was sufficient curvature in the extremum of the plot of $r/[B]$ *versus* r that it was very difficult to make reasonable linear extrapolation to the $r/[B]$-axis. This led us to hypothesize that the reaction represented by Eqs. 3 and 5 might be inappropriate to represent, even qualitatively, the type I binding. An attempt was made to rewrite the type I binding reaction in a way that might fit the experimental data. A reaction of the form:

$$B + qS \rightleftarrows B(S)q \qquad \text{(Eq. 7)}$$

was chosen, in which an integral number of binding sites, q, would react with BH^+ to form the type I complex $B(S)q$. The equilibrium expression corresponding to Eq. 7 would then be:

$$K = \frac{[B(S)q]}{[B][S]^q} \qquad \text{(Eq. 8)}$$

Since n phosphates are associated with each type I complex:

$$m \cdot q = n \qquad \text{(Eq. 9)}$$

where m is the number of phosphates associated with one type I site, S. In most binding studies, it has been demonstrated that the type I binding is characterized by $n = 4$ or 5. However, in our empirical approach, values of $q = 1$-4 and of $m = 1$-6 were tried in Eqs. 8 and 9. It was found that the best straight lines could be obtained when $m = 2$ and $q = 2$ (*i.e.*, two sites per complex, each site having two phosphates). Hence, for the type I binding of the 3-aminoacridinium monocation:

$$K = \frac{[B(S)_2]}{[B][S]^2} \qquad \text{(Eq. 10)}$$

corresponding to the essentially termolecular overall reaction:

$$B + 2S \rightleftarrows B(S)_2 \qquad \text{(Eq. 11)}$$

The reaction shown in Eq. 11 is difficult to rationalize if both sites which react with B lie on the same DNA molecule, as indeed they must, unless intercalation involves the bridging of ligand between two distinct macromolecules. To test the thermodynamic feasibility of the variously proposed reaction models ($q = 1$, $m = 4$; $q = 1$, $m = 5$; and $q = 2$, $m = 2$) for the intercalative binding of small molecules to DNA, it was decided to attempt to determine the standard enthalpy of binding ($\Delta H°$) for the reactions of several cationic drugs and dyes with calf thymus DNA. These values of $\Delta H°$ could be determined from van't Hoff plots of the appropriately calculated apparent binding constants as a function of temperature. It should be mentioned that the occurrence of a reasonably linear van't Hoff plot alone might be an indication of the chemical significance of the way in which the equilibrium expression is written. The values of $\Delta H°$ taken from the van't Hoff plots could then be compared with enthalpies of reaction (ΔH) determined directly by microcalorimetry. Since the standard state for a solute in dilute solution is taken to be the infinitely dilute solution at standard conditions, the calorimetrically determined values of ΔH may be taken to approximate $\Delta H°$ very well in each case because the concentrations of the reactants will be kept very low ($\sim 10^{-4}$-10^{-3} M). Agreement between the graphically and calorimetrically determined values of $\Delta H°$ then should lend support to the validity of the model used in the calculation of the equilibrium constants giving the best values of $\Delta H°$. Additionally, once the appropriate intercalative binding model is known and the best values of $\Delta H°$ are in hand, it should be possible to determine thermodynamic binding constants by extrapolation to infinite dilution and then to determine the standard entropy of binding ($\Delta S°$). Assessments of the relative importance of DNA-ligand interactions and ligand-water interactions in the binding process could then be attempted. With these objectives in mind, the following study of the binding of four compounds to calf thymus DNA was undertaken.

EXPERIMENTAL SECTION

The pH was maintained throughout this study at 5.50 with an acetic acid-sodium acetate buffer to ensure that all ligands utilized were in only one state of protonation and thereby obviate the necessity to correct for competitive prototropic equilibria.

Ethidium bromide[1] and 9-aminoacridine hydrochloride[2] were recrystallized from 95% ethanol. Doxorubicin hydrochloride[3] was used without further purification. 3-Aminoacridine was prepared by the procedure of Martin and Tong (27). The sodium salt of calf thymus DNA[4] was used without further purification.

The molar absorptivity of each ligand was determined by accurately weighing and dissolving each compound in a 10-mL volumetric flask and then delivering successive 50-μL aliquots of this stock solution into 9.00 mL of pH 5.50 buffer and recording the long-wavelength absorption spectra. A Beer's law plot was constructed for each ligand, with the final concentration of ligand equal to or greater than the final concentration employed in the determination of the DNA-ligand binding constants. Linearity was observed in all cases, indicating that aggregation of the free ligand in solution was negligible within the concentration range employed.

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Pfaltz and Bauer, Stanford, Conn.
[3] Gift from Adria Laboratories, Columbus, Ohio.
[4] Calbiochem, La Jolla, Calif.

The molar absorptivity of the intercalated form of each ligand was determined analogously by adding the stock solution to a concentrated solution of DNA and recording the absorption spectra after each addition. A Beer's law plot was then constructed and found to be linear. This indicates that successively bound ligands have identical molar absorptivities, irrespective of the degree of saturation of polymer. The final concentration of bound ligand was equal to or greater than the concentration used in the determination of the intrinsic association constant. Variation of temperature and/or ionic strength had no effect on the measured molar absorptivities. The exact molar absorptivity of native calf thymus DNA is disputed in the literature (28–30). Values range from 6300 $L \cdot mol^{-1} \cdot cm^{-1}$ (29) to 6600 $L \cdot mol^{-1} \cdot cm^{-1}$ (31), with the wavelength maximum at either 259 (31) or 260 nm (29). Throughout this study, the concentrations of DNA solutions were determined absorptiometrically at 259 nm with a corresponding molar absorptivity of 6500 $L \cdot mol^{-1} \cdot cm^{-1}$. All absorptiometric measurements were carried out on a recording UV-visible spectrophotometer[5], using cylindrical 5-cm quartz cells.

The intrinsic association constants of all ligands with native double-stranded DNA were determined as a function of temperature and ionic strength. A measured volume and concentration of ligand was added to a known volume and concentration of DNA prior to the titration. Absorption spectra of the preequilibrated DNA-ligand solution were then recorded at temperatures and ionic strengths corresponding to those from which intrinsic association constants were calculated. The titration was then carried out differentially by placing an identical volume of the preequilibrated DNA solution in both the sample and reference compartments. The preequilibration of the DNA solution with ligand ensures that the concentration of unbound ligand is measurable from the outset and allows the differential spectral technique to be optimized for that region of the titration. Aliquots of the known concentration of ligand solution were then added with a micropipet[6] to the sample cell, and the resulting rise in the absorption spectra was recorded. The transfer of ligand solutions was found to be more reproducible than the more commonly used method of transferring the DNA solution, owing to the relatively high viscosity of the latter. Absorption spectra of all ligands during the titration were recorded starting at wavelengths at least 20 nm greater than the first observable absorbance and ending ~20 nm below the analytical wavelength of choice. Analytical wavelengths were chosen according to the ligand and were at the long-wavelength maxima of the free drug. The titrations were performed in duplicate for at least three temperatures between 15°C and 40°C for each ligand and at ionic strengths of 0.1, 0.05, and 0.01. The temperature in the absorbance cell was monitored by a submersible temperature probe and maintained with a constant-temperature bath[7] and a jacketed cell compartment. At least 10 points, and usually 20 points, were acquired in each titration.

The conservation of mass of the ligand requires that:

$$C_B = [B] + [BS] \qquad \text{(Eq. 12)}$$

where B and BS are the free and bound ligand, respectively, and C_B is the total concentration of ligand. At any point in the titration, assuming no aggregative binding, the absorbance is:

$$A_T = \epsilon_B[B]l + \epsilon_{BS}[BS]l \qquad \text{(Eq. 13)}$$

where A_T is the total absorbance at the analytical wavelength of choice, and l is the optical path length of the sample cell. Combination of Eqs. 12 and 13 gives:

$$[B] = \frac{A_T - \epsilon_{BS}C_B l}{(\epsilon_B - \epsilon_{BS})l} \qquad \text{(Eq. 14)}$$

and:

$$[BS] = \frac{\epsilon_B C_B l - A_T}{(\epsilon_B - \epsilon_{BS})l} \qquad \text{(Eq. 15)}$$

for the concentration of free and bound ligand at any point in the titration.

The heat generated by the binding of the ligands by DNA was measured with a batch microcalorimeter[8]. The reaction cells employed in the batch microcalorimeter are of the mixing type, each having two compartments into which liquid reactant can be introduced. The maximum volume that can be contained within each divided cell is 4.5 and 2.5 mL to minimize the heat of dilution and maximize concentration. DNA was introduced into the 4.5-mL compartment, and the ligand was introduced into the 2.5-mL chamber. The temperature within the microcalorimeter was maintained at 25.00 ± 0.01°C with a thermostatically controlled air bath[9] with an external adjunct water bath[10]. The temperature inside the sealed microcalorimeter was checked with a calibrated thermostat embedded in a heat sink. The microcalorimeter at thermal equilibrium was rotated, allowing the reactants to mix, and the heat of reaction was recorded. The output from the microcalorimeter was recorded on a linear strip chart recorder with a disk-driven integrator[11]. The microcalorimeter was calibrated with an electrical calibration current which passed through the reactant cell for a measured period of time[12]. A comparison of the signal from the calibration run with that measured for the heat of reaction of ligand with DNA yields an exact molar enthalpy of reaction. The heat of dilution of both reactants was measured and subtracted from the measured heat of reaction. The apparent enthalpy of binding can be calculated from:

$$\Delta H = \frac{Q}{C_B V_i f_{BS}} \qquad \text{(Eq. 16)}$$

where Q is the measured heat, V_i is the initial volume of ligand, C_B is the initial concentration of ligand, and f_{BS} is the fraction of the total ligand bound by the DNA at the end of the reaction. An *a priori* knowledge of the intrinsic association constant at the same temperature and ionic strength is necessary to calculate f_{BS}. However, to a first approximation, the value of the binding is not necessary since an excess of DNA was used, such that f_{BS} was >0.95. The error incurred in this assumption is <5% of the final value. The heat of binding for each compound was measured at ionic strengths of 0.1, 0.05, and 0.01. Data were analyzed using the facilities of the Northeast Regional Data Center[13].

RESULTS AND DISCUSSION

Intercalative binding of a cationic ligand with native DNA entails the slipping of the aromatic drug in between adjacent base pairs on the double helix. Concomitantly, the molar absorptivity of the ligand decreases dramatically. The concentration of free and bound ligand can be evaluated at each point in the titrations by Eqs. 14 and 15. These values were subsequently used for the evaluation of apparent binding constants from Eqs. 8 and 9 with values of $m = 1$–6 and $q = 1$–4, as described previously (26). Particular attention was paid to the values of $m = 4$, $q = 1$ and $m = 5$, $q = 1$ (corresponding to the modified Scatchard treatment of type I binding), $m = 3$, $q = 1$ (corresponding to the suggested stoichiometry for type II binding), and $m = 2$, $q = 2$ [for the termolecular treatment of type I binding (26)]. Of these, only values of $m = 4$, $q = 1$ and $m = 2$, $q = 2$ yielded binding constants which did not exhibit systematic variance and were positive over the entire titration interval. In addition, the values of $m = 4$, $q = 1$ gave estimated errors in the binding constants which were consistently less than those calculated by using values of $m = 2$, $q = 1$. However, both models gave feasible results, and each must be considered further.

Binding data for each compound at a different absolute temperature (T) were treated in the manner described above, with values of $m = 4$, $q = 1$ (bimolecular overall reaction) and $m = 2$, $q = 2$ (termolecular overall reaction). The logarithms of the apparent binding constants calculated therefrom are plotted against reciprocal absolute temperature (van't Hoff plots) in Figs. 1 and 2, respectively. The best first-order least-squares lines for these data are also shown, the slopes of which should be $-\Delta H°/2.303R$, where R is the universal gas constant. The calculated values of $\Delta H°$ are listed in Table I. The van't Hoff plots are apparently linear, with the deviations from linearity being very similar for both models. If the linearity of the plots is taken as a criterion for the validity of a binding model, then both models appear to be equally correct. However, a major difference between them is the magnitudes of the resultant values of $\Delta H°$ which are significantly larger for the termolecular model than those from the bimolecular model. Thus, to establish the appropriate model, the standard enthalpies of binding of each compound to DNA must be measured by an independent experimental procedure.

The calorimetric measurement of the binding enthalpies was therefore performed by batch microcalorimetry. The major advantage of batch microcalorimetry over the more commonly used flow calorimetry is the relatively small amounts of reagents required for the analysis. The heats of binding were found to be virtually identical at three ionic strengths and were subsequently averaged, and the resulting standard enthalpies for each compound were calculated (Table I). The calorimetrically determined enthalpies are in agreement with the values described above for the bimolecular model and significantly different from the termolecular values. This agreement of the standard enthalpies of binding, which were determined independently by

[5] Model 219; Varian-Cary.
[6] Finnpipette.
[7] Lauda K-2/R; Brinkmann.
[8] Model 2107-111; LKB.
[9] Model 2107-210; LKB.
[10] Lauda K-2/R; Brinkmann.
[11] *Omniscribe*; Houston Instruments, Houston, Tex.
[12] Control Unit 2107-310; LKB.
[13] 470 v/6-11 computer; Amdahl.

Table I—Thermodynamic Parameters of the Binding of Ligands to DNA

Ligand	$\Delta H°$, kcal·mol^{-1} [a,b]	$\Delta H°$, kcal·mol^{-1} [a,c]	ΔH, kcal·mol^{-1} [d]	$\Delta G°$, kcal·mol^{-1}	$\Delta S°$, cal·deg^{-1}mol^{-1}
Ethidium bromide	6.2 ± 0.5	10 ± 1	−7.5 ± 0.2	−9.30 ± 0.04	6.0 ± 0.7
9-Aminoacridine	6.3 ± 0.5	13 ± 2	−6.1 ± 0.2	−8.70 ± 0.07	8.7 ± 0.7
3-Aminoacridine	7 ± 1	11 ± 2	−6.9 ± 0.2	−7.69 ± 0.05	2.6 ± 0.7
Doxorubicin	5.3 ± 0.5	6.6 ± 0.7	−5.4 ± 0.3	−9.9 ± 0.1	15 ± 1

[a] From the least-squares slope of the van't Hoff plot. [b] Bimolecular model ($m = 4, q = 1$). [c] Termolecular model ($m = 2, q = 2$). [d] From calorimetry.

spectrophotometry and calorimetry, is strong evidence for the bimolecular model with four bases per binding site ($m = 4, q = 1$).

The determination of enthalpic contributions to the binding process inevitably leads to questions about the corresponding entropic contributions and to the standard free energy of the system. To calculate the standard free energy of binding from the association constant for the DNA-ligand interaction, the thermodynamic association constant must be known. Only from the thermodynamic constant can the standard free energy of binding ($\Delta G°$) be calculated. The standard entropy of binding ($\Delta S°$) can then be calculated from $\Delta G°$ and the standard enthalpy of binding, $\Delta H°$, using:

$$\Delta S° = \frac{\Delta G° - \Delta H°}{T} \qquad \text{(Eq. 17)}$$

The majority of reported association constants in the literature for DNA-ligand interactions are apparent equilibrium association constants. The use of an apparent association constant in the calculation of the free energy of binding does not yield $\Delta G°$ but rather an entity which varies with the ionic strength. The calculated entropy of binding will then also appear to fluctuate with ionic strength. However, the determination of a thermodynamic affinity constant extrapolated to zero ionic strength allows the direct and unambiguous determination of $\Delta G°$ and, subsequently, $\Delta S°$, which are thermodynamic constants of the reaction.

The Debye-Hückel relationship (Eq. 18) can serve as a starting point from which to correct the apparent association constants for ionic screening of the various reactants. The activity coefficient of the ith species can be calculated from:

$$-\log \gamma_i = \frac{AZ_i^2\mu^{1/2}}{1 + \mu^{1/2}} \qquad \text{(Eq. 18)}$$

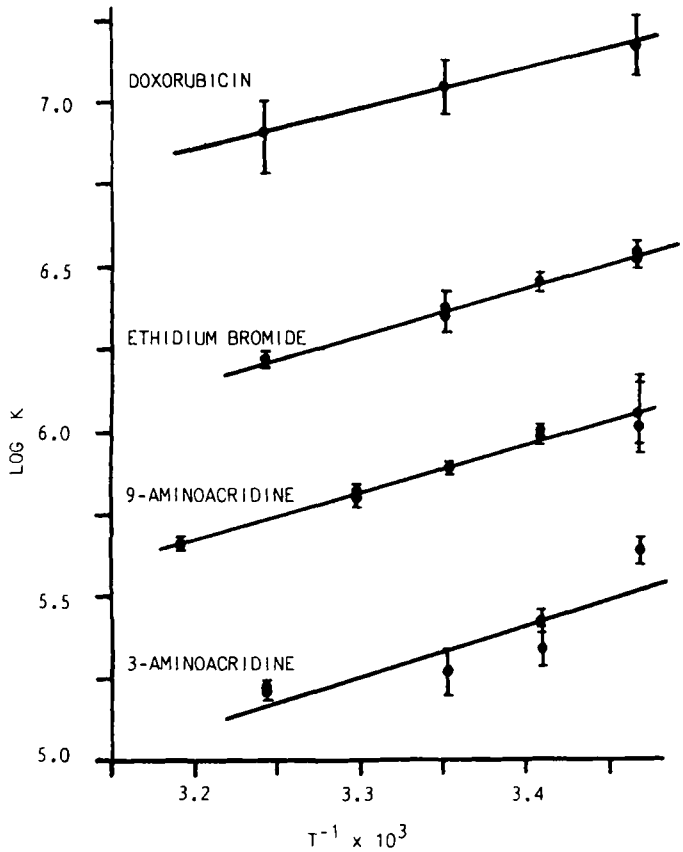


Figure 1—*Van't Hoff plots for the indicated compounds, assuming a bimolecular model* (m = 4, q = 1).

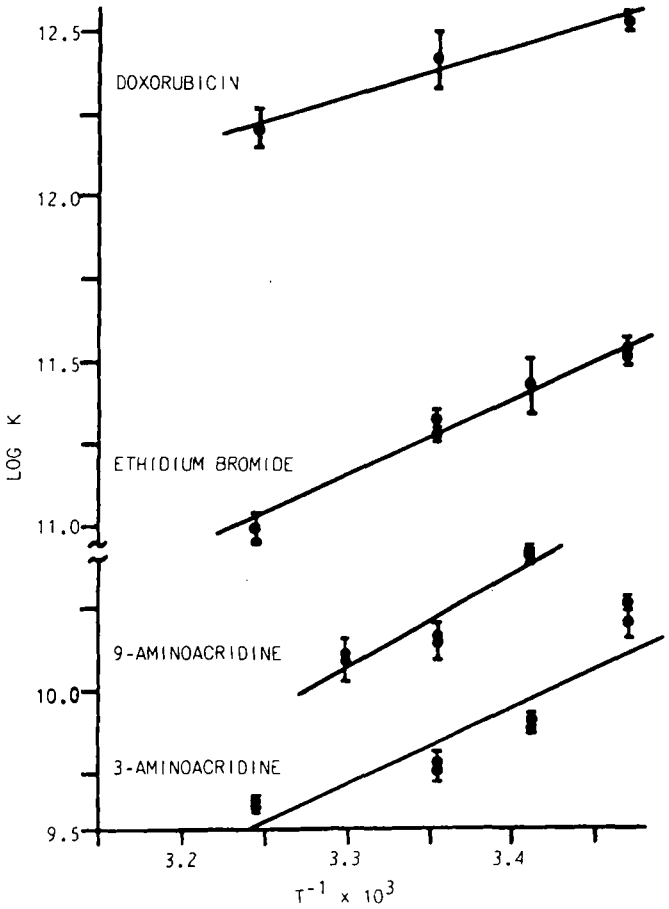


Figure 2—*Van't Hoff plots for the indicated compounds, assuming a termolecular model* (m = 2, q = 2).

where A is a constant dependent on the temperature and dielectric constant of the solute, Z_i is the charge on the ith species in solution, and μ is the ionic strength of the solution.

The thermodynamic equilibrium constant $K°$ can be related to the apparent constant K by:

$$K° = K\frac{\gamma_{BS}}{\gamma_B\gamma_S} \qquad \text{(Eq. 19)}$$

where the subscripts of the activity coefficients refer to the reactant species. Combination of Eqs. 18 and 19 yields, in logarithmic form:

$$\log K = \log K° + C\frac{\mu^{1/2}}{1 + \mu^{1/2}} \qquad \text{(Eq. 20)}$$

It is then possible to extract a thermodynamic association constant. Even if the Debye-Hückel equation is not quite correct for DNA solutions, the fact that log $K°$ is an intercept and not a slope minimizes the dependence on how γ_i is calculated. The apparent association constants at three ionic strengths and the thermodynamic association constant taken from the corresponding least-squares intercept are given for each ligand in Table II. The association constants agree well with the results of other workers (32–36). Values for all thermodynamic parameters are given in Table I. Note that the precision of the calorimetrically obtained enthalpies is better than that which could be obtained from spectrophotometric data.

Examination of the thermodynamic parameters gives some insight into the molecular forces which control the binding process. A favorable enthalpy of binding should be evident with large, planar, aromatic molecules. Their pla-

Table II—Parameters For the Binding of Ligands to DNA

Ligand	Wavelength, nm	Molar Absorptivity × 10^{-3} L·mol^{-1}·cm^{-1}		Log K^a			Log K° [b]
		Free	Bound	μ = 0.100	μ = 0.050	μ = 0.010	
Ethidium bromide	480	5.60 ± 0.06	2.46 ± 0.04	5.61 ± 0.02	5.93 ± 0.02	6.36 ± 0.04	6.82 ± 0.03
9-Aminoacridine	422	7.48 ± 0.16	2.84 ● 0.02	5.08 ± 0.07	5.54 ± 0.02	5.87 ± 0.01	6.38 ± 0.05
3-Aminoacridine	452	12.60 ± 0.11	8.50 ± 0.06	4.70 ± 0.03	4.95 ± 0.01	5.28 ± 0.05	5.64 ± 0.04
Doxorubicin	480	10.00 ± 0.15	5.96 ± 0.02	6.60 ± 0.07	6.80 ± 0.03	7.00 ± 0.09	7.25 ± 0.07

[a] Apparent equilibrium association constants. [b] Equilibrium association constants extrapolated to μ = 0.

Table III—Variation in the Measured Heat of Binding Due to the Fraction of Ligand Bound [a]

Percent Bound	Concentration, ×10^{-5} M				Molar Enthalpy of Binding, kcal/mol
	Total Drug	Bound Drug	Free Drug	Total DNA	
99	3.36	3.33	0.03	6.74	−6.2
95	3.50	3.33	0.170	2.39	−6.2
90	3.70	3.33	0.370	1.82	−6.1
85	3.92	3.33	0.590	1.64	−6.3
80	4.16	3.33	0.803	1.55	−6.2
70	4.76	3.33	1.43	1.46	−6.2
60	5.55	3.33	2.22	1.41	−6.3
50	6.66	3.33	3.33	1.39	−6.2

[a] T = 25°C; μ = 0.010 for 9-aminoacridine.

narity allows their insertion into the DNA helix with minimal disturbance of the DNA structure. Larger compounds enhance the opportunity for the van der Waals binding interaction between the ligand and the DNA bases. The largest binding enthalpy for ligands studied herein is that of ethidium bromide. In contrast to 9-aminoacridine and 3-aminoacridine, which also contain tricyclic aromatic ring systems, ethidium bromide has an exocyclic phenyl substituent which might at first be expected to sterically inhibit its intercalation. However, the observed large enthalpy of binding relative to those of the aminoacridines suggests that the phenyl ring of ethidium bromide reinforces binding. This would seem to indicate that the phenyl substituent serves to extend the area of the ligand which is bound to the DNA base pairs, perhaps by becoming coplanar with the aromatic nucleus of the ligand. In contrast, doxorubicin, which exhibits the least-favorable binding enthalpy, has a nonplanar ring linearly annulated with the aromatic system and an exocyclic amino sugar group. The weakness of the enthalpic binding contribution may be attributed to either the steric inhibition of the insertion of the planar portion of the molecule into the DNA helix or to the disruption of the DNA structure by the intercalation of the buckled ring segment or the amino sugar side chain.

The entropy of binding is probably influenced mainly by changes in the translational freedom due to the binding or release of solvent molecules by the ligands and/or the DNA. This is evidenced by the entropy of binding for doxorubicin which is much larger than that observed for the other ligands. The binding of the doxorubicin can be expected to displace water from its amino sugar side chain. In addition, the structure of the DNA helix and its accompanying waters of hydration may be disrupted significantly if the buckled ring or the amino sugar group makes contact with the double helix. Because of the limited number of compounds investigated in this study and the substantial structural differences between them, it would probably be premature and excessively speculative to attempt to account for the differences between their enthalpies and entropies of binding to DNA in any but those most obvious examples just cited.

Batch microcalorimetry can be used to investigate type II binding as well. The only condition necessary for the separation of type I and type II binding is that their heats of complexation be different and measurable. In that type II binding becomes prevalent in the presence of an excess concentration of free ligand, type II binding should demonstrate a heat of interaction in this region of titration, and an enthalpy of binding should be measurable by microcalorimetry. By using excess DNA, the heat for intercalative binding can be accurately calculated at any known concentration of DNA and ligand. By using these calculated values as a reference and by repeating the measurement with excess free ligand, any deviation from the reference value will be taken as being due to external binding. Table III shows the results of this experiment. Even in the presence of an excess free ligand concentration, which is equal to the total concentration of bound ligand, the measured enthalpy of binding is invariant from the reference value for intercalative binding. The heats of binding were checked at a number of bound ligand–free ligand concentrations, and in all cases the resulting enthalpy was equal to the reference value within experimental error.

The lack of a measurable heat of binding for type II interactions can be explained in two ways. First, type II binding may have a small, unmeasurable heat of complexation, such that the batch microcalorimeter cannot detect this heat above the reference value. Second, type II binding may be an artifact of the Scatchard plot. As such, external binding may not occur for nonaggregating ligands. For ligands which aggregate, if the concentration is maintained at a low value, external binding again may not occur.

The inaccuracy in measuring the exact concentration of free and bound ligand, when either is in a large excess, is great. Such is the case for external binding, in which it is an excess of free ligand that creates the difficulties. The curvature in the modified Scatchard plots is probably due to this inability to accurately measure free and bound ligand concentrations.

REFERENCES

(1) R. I. DeMars, *Nature (London)*, **172**, 964 (1953).

(2) J. Hurwitz, J. J. Furth, M. Malamy, and M. Alexander, *Proc. Natl. Acad. Sci. U.S.A.*, **48**, 1222 (1962).

(3) C. S. Woese, R. Naono, R. Soffer, and F. Gros, *Biochem. Biophys. Res. Commun.*, **11**, 435 (1963).

(4) A. Blake and A. R. Peacocke, *Biopolymers*, **6**, 1225 (1968).

(5) A. R. Peacocke in "Heterocyclic Compounds: Acridines," vol. 9, R. M. Acheson, Ed. Interscience, New York, N.Y., 1973, pp. 723–757.

(6) E. R. Lochmann and A. Micheler in "Physico-Chemical Properties of Nucleic Acids," vol. 1, J. Duchesne, Ed., Academic, New York, N.Y., 1973, pp. 223–267.

(7) S. Georghiou, *Photochem. Photobiol.*, **26**, 59 (1977).

(8) L. S. Lerman, *J. Mol. Biol.*, **3**, 18 (1961).

(9) L. S. Lerman, *Proc. Natl. Acad. Sci. U.S.A.*, **49**, 94 (1963).

(10) L. S. Lerman, *J. Cell. Comp. Physiol.*, Suppl. 1, **64**, 1 (1964).

(11) N. J. Pritchard, A. Blake, and A. R. Peacocke, *Nature (London)*, **212**, 1360 (1966).

(12) D. S. Drummond, V. F. W. Simpson-Gildemeister, and A. R. Peacocke, *Biopolymers*, **3**, 135 (1965).

(13) H. M. Sobell and S. C. Jain, *J. Mol. Biol.*, **68**, 21 (1972).

(14) H. M. Sobell, *Prog. Nucleic Acid Res. Mol. Biol.*, **13**, 153 (1973).

(15) E. Frederica and M. Houssier, *Biopolymers*, **10**, 2281 (1972).

(16) J. H. Li and D. M. Crothers, *J. Mol. Biol.*, **39**, 461 (1968).

(17) M. Sakoda, K. Hiromi, and K. Akasaka, *Biopolymers*, **10**, 1003 (1971).

(18) K. Akasaka, M. Sakoda, and K. Hiromi, *Biochem. Biophys. Res. Commun.*, **40**, 1239 (1970).

(19) M. Sakoda, K. Hiromi, and K. Akasaka, *J. Biochem.*, **71**, 891 (1972).

(20) J. L. Bresloff and D. M. Crothers, *J. Mol. Biol.*, **95**, 103 (1975).

(21) P. Davanloo and D. M. Crothers, *Biochemistry*, **15**, 5299 (1976).

(22) G. Duportail, Y. Mauss, and J. Chambron, *Biopolymers*, **16**, 1397 (1977).

(23) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1948).

(24) J. D. McGhee and P. H. von Hippel, *J. Mol. Biol.*, **86**, 469 (1974).

(25) A. R. Peacocke and J. N. H. Skerrett, *Trans. Faraday Soc.*, **52**, 261 (1956).

(26) A. C. Capomacchia and S. G. Schulman, *J. Pharm. Sci.*, **67**, 89 (1978).
(27) R. F. Martin and J. H. Y. Tong, *Aust. J. Chem.*, **22**, 487 (1969).
(28) E. Frederica and C. Houssier, *Biopolymers*, **11**, 2281 (1972).
(29) G. Felsenfeld and S. Z. Hirschman, *J. Mol. Biol.*, **13**, 407 (1965).
(30) N. N. Zhadin and V. L. Rapoport, *Mol. Biol.*, **9**, 187 (1975).
(31) H. R. Mahler and D. D. Mehrotra, *J. Mol. Biol.*, **9**, 801 (1964).
(32) M. Waring, *J. Mol. Biol.*, **13**, 269 (1965).
(33) C. M. Lee, C. T. Chang, and J. G. Wetmur, *Biopolymers*, **12**, 1099 (1973).
(34) S. Aktipis and A. Kindelis, *Biochemistry*, **12**, 1213 (1973).
(35) P. S. Drummond, N. J. Pritchard, V. F. W. Simpson-Gildemeister, and A. R. Peacocke, *Biopolymers*, **4**, 1091 (1966).
(36) S. R. Byrn and G. D. Dolch, *J. Pharm. Sci.*, **67**, 688 (1978).

Antiradiation Compounds XIX: Metal-Binding Abilities of Thioureas

WILLIAM O. FOYE ˣ and CHING-CHIU CHAO

Received July 13, 1983, from the *Samuel M. Best Research Laboratory, Massachusetts College of Pharmacy and Allied Health Sciences, Boston, MA 02115.* Accepted for publication August 5, 1983.

Abstract □ Metal-binding stability constants for a series of *N*- and *N,N'*-substituted thioureas with Cu(II), Ni(II), Al(III), and Fe(III) ions were determined by potentiometric titration. The sequence of constants for thiourea, *N*-methylthiourea, and *N,N'*-dimethylthiourea indicated steric effects of the methyl groups and that both nitrogen and sulfur were involved in the complexation. The magnitude of the constants was somewhat lower than those of the simple peptides. The mechanism of protection against ionizing radiation by thioureas is probably due to hydrogen-atom transfer rather than binding of metal ions that catalyze cellular oxidations.

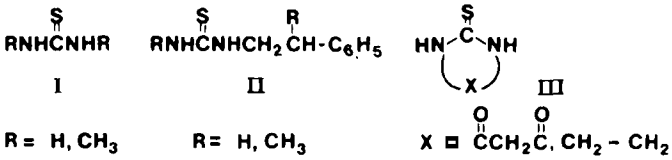
Keyphrases □ Thioureas—metal-ion complexes, stability constants, antiradiation protection □ Antiradiation protection—thioureas, metal-ion complexes, stability constants □ Stability constants—thioureas, metal-ion complexes, antiradiation protection

Metal-ion complexation has been shown or postulated to be involved in a number of biological activities. The effect of complexing agents such as dithiocarbamates (1) and some thioureas (2) on the inhibition of dopamine-β-oxidase, a copper-containing enzyme, is well known. Metal-ion complexation has also been postulated for the antithyroid activity of thiouracil (3), the inhibitory effects of dithiocarbamates against *Mycobacterium tuberculosis* (4), and the fungistatic effects of dithiocarbamates and other complexing agents (5). The nitrate reductase complex of *Neurospora crassa* is also sensitive to inhibition by metal-binding agents, including thiourea (6), and polyphenol oxidase is also inhibited by thiosinamine (allylthiourea) (7).

Several postulations (8–10) regarding the protective effects of a number of metal-binding radiation-protective agents have also been made. Although thiourea and its simple derivatives have some protective ability for whole-body irradiation of animals, these effects have not been appreciable. Thioureas have, however, shown substantial radiation-protective effects in other systems, such as mouse lung (11), human erythrocytes (12), T_2 phage (13), Ehrlich ascites cells (14), and macromolecules (15) (Table I). The radiation-protective effects of thiourea have been attributed to metal binding (11).

With such a significant number of postulations involving the metal-binding aspects of thioureas, it is surprising to find that, other than for thiourea itself (16), metal-binding abilities of substituted thioureas have not been determined. Stability constants for a series of cyclic thioureas, including mercaptoimidazoles and mercaptouracils, were determined with copper, iron, and aluminum ions (17), and the constants were surprisingly high. It was considered important, therefore, to measure the metal-binding stability constants for a series of alkyl, aralkyl, and cyclic thioureas. Knowledge of the magnitude of the constants would indicate whether thiourea complexes were capable of existence in the presence of cellular complexing agents, such as peptides, or whether thiourea might function as a transient metal complexer.

Compounds of the general structures I–III were measured for binding abilities to Cu(II), Ni(II), Al(III) and Fe(III) ions using the method of potentiometric titration.



EXPERIMENTAL SECTION

Materials—Analytical reagent-grade[1] aluminum chloride hexahydrate, cupric chloride dihydrate, ferric nitrate nonahydrate, and nickel(II) chloride hexahydrate were used for the metal complexation determination. Carbonate-free 0.01 M KOH was prepared according to the method of Armstrong (18). Solutions of the metal salts were prepared in boiled distilled water and stored in polyethylene bottles under nitrogen; they were diluted quantitatively with carbon dioxide-free water just prior to use. Normalities were checked against potassium biphthalate.

The organic ligands were obtained commercially[2]. *N*-Methyl-*N'*-phenethylthiourea was reported previously (19), and *N*-methyl-*N'*-phenyl-2-propylthiourea was described earlier (20). Purity was ascertained by TLC using chromagram sheets[3]. Solutions (0.1%) of the compounds in ethanol were spotted, and the sheets were developed with benzene–methanol (8:2); spots were detected with iodine vapor.

Ionization Constants—The method of Albert and Serjeant (21) was used, which consisted of titrations of 0.001 M solutions of the compounds in 95% ethanol with 0.01 M KOH in 0.5-mL portions. The pH was recorded with a pH meter[4] with glass and calomel electrodes after each addition. Each titration thus yielded 10 pH values, giving 10 values for the pK_a, which were averaged (Table II). Since most of the pH values fell outside the 5–9 range, corrections were made for hydrogen-ion concentrations.

Stability Constants—Potentiometric titrations were carried out under nitrogen in 95% ethanol at 25°C with the described pH meter. Volumes of 50 mL of the 0.001 M solutions of the organic ligands were titrated with 0.01 M KOH in 0.5-mL portions, first in the absence of metal ions, and then in the

[1] J. T. Baker Analyzed Reagents.
[2] Aldrich Chemical Co., Eastman Organic Chemicals, or Fisher Scientific Co.
[3] Eastman Organic Chemicals.
[4] Beckman Instruments.

0022-3549/84/0900-1284$01.00/0