Spectroscopic and Molecular Modeling Studies of Caffeine Complexes with DNA Intercalators

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ABSTRACT Recent studies have demonstrated that caffeine can act as an antimutagen and inhibit the cytoxic and/or cytostatic effects of some DNA intercalating agents. It has been suggested that this inhibitory effect may be due to complexation of the DNA intercalator with caffeine. In this study we employ optical absorption, fluorescence, and molecular modeling techniques to probe specific interactions between caffeine and various DNA intercalators. Optical absorption and steady-state fluorescence data demonstrate complexation between caffeine and the planar DNA intercalator acridine orange. The association constant of this complex is determined to be 258.4 ± 5.1 M^{-1}. In contrast, solutions containing caffeine and the nonplanar DNA intercalator ethidium bromide show optical shifts and steady-state fluorescence spectra indicative of a weaker complex with an association constant of 84.5 ± 3.5 M^{-1}. Time-resolved fluorescence data indicate that complex formation between caffeine and acridine orange or ethidium bromide results in singlet-state lifetime increases consistent with the observed increase in the steady-state fluorescence yield. In addition, dynamic polarization data indicate that these complexes form with a 1:1 stoichiometry. Molecular modeling studies are also included to examine structural factors that may influence complexation.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine) (See Fig. 1 for structures of caffeine and DNA-intercalating compounds) represent a class of molecules with conjugated planar ring systems that constitute the most widely distributed naturally occurring methylxanthines. Caffeine is regularly consumed from dietary sources including coffee, tea, cola beverages, and chocolate. The estimated average intake of caffeine for persons over the age of 18 is 2.5 mg/kg/day (Dews et al., 1984). This relatively high daily consumption, as well as the abundance of methylxanthines (primarily caffeine) in dietary substances, has stimulated extensive research into the question of methylxanthine toxicity particularly with respect to coronary heart disease and cancer (Bertrand et al., 1970; Jick et al., 1973; Mann and Thorogood, 1975; Rall, 1980; Curatolo and Robertson, 1983; Dews et al., 1984; Pozniak, 1985). Caffeine has been shown to inhibit enzymes required for DNA synthesis, cause an increase in chromatin condensation, increase the length of the G_1 phase, and exhibit antimutagenic activity against *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Borodina et al., 1979; O'Neal, 1979; Levin, 1982; Roberts, 1984; Kunicka et al., 1990; Selby and Sancar, 1990). In the presence of DNA-modifying agents, high concentrations of caffeine (>10 mM) appear to enhance cell mortality (Roberts, 1984). At lower concentrations, however, caffeine can protect against nucleotide damage caused by several DNA-intercalating agents such as 14-hydroxydaunomycin hydrochloride (DOX), novantrone (NOV), ethidium bromide (EBR), and ellipticine but has no effect on nucleotide damage caused by hydroxyurea or cisplatinum (Ross et al., 1979; Kimura and Aoyama, 1989; Traganos et al., 1991). These studies have further demonstrated that caffeine can alter the absorption spectrum of a model DNA intercalator, acridine orange (3,6-bis-(dimethylamino)acridine hydrochloride; AO) by forming a π-π complex with the dye in aqueous solution, suggesting a role for caffeine as an interceptor molecule (Traganos et al., 1991; Kapuscinski and Kimmel, 1993). Although it is evident that caffeine interacts with model intercalators in solution, the structural nature of such complexes is uncertain.

In the present study we have employed optical absorption spectroscopy, steady-state and time-resolved fluorescence, and molecular modeling techniques to obtain a better understanding of the relationship between the forces involved in formation of caffeine-intercalator complexes. Specifically, we have determined the binding constants and stoichiometry for caffeine complexes with two distinctive DNA intercalators, EBR and AO. Although caffeine complexes with AO have been previously described, this is the first determination of binding stoichiometries and the first direct evidence of caffeine complexation with EBR (Traganos et al., 1991; Kapuscinski and Kimmel, 1993). We have also examined energy-minimized structures of caffeine complexed with the DNA intercalators NOV, DOX, AO, and EBR as well as caffeine complexes with aromatic model compounds.

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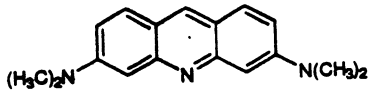
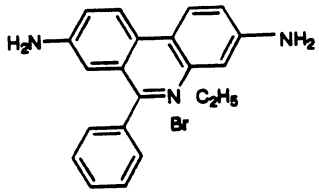


Acridine Orange



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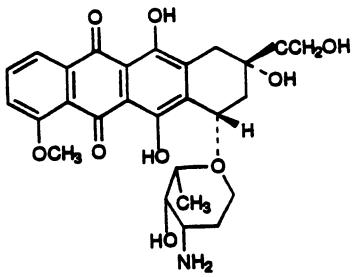
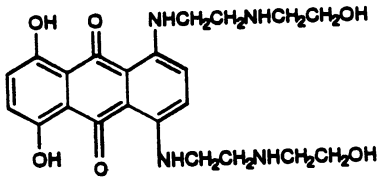
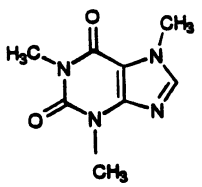


Doxorubicin



Novantrone



Caffeine

FIGURE 1 Structural diagrams of the DNA intercalators used in this study.

MATERIALS AND METHODS

Caffeine (Scientific Products, Irvine CA), HEPES (Sigma Chemical Co., St. Louis, MO), and AO (Sigma) were used without further purification. Ethidium bromide was generously donated by Dr. Clifford Morden (Department of Botany, University of Hawaii). Caffeine, EBR, and AO stock solutions (50 mM) were prepared in 5 mM HEPES, pH 7.0.

Optical titrations were performed in a 1-cm quartz optical cell housed in a thermoelectrically modulated cell holder to maintain a constant temperature. Spectra were obtained on a Milton Roy Spectronic 3000 diode array spectrophotometer. Optical titrations were carried out by diluting a dye stock solution to 10 μM in 2 ml of 5 mM HEPES, pH 7.0. Aliquots of the caffeine stock solution were then titrated and absorption spectra were recorded after each addition. Difference spectra were obtained by subtracting the uncomplexed dye spectrum from the dye spectrum in the presence of caffeine corrected for dilution. Caffeine-dye binding constants were then determined by the following equilibrium expression:

$$D + L \leftrightharpoons D\text{-}L$$

where D, L and D-L represent dye (AO or EBR), caffeine, and complex, respectively. This leads to the following equation for the association constant (K):

$$\Delta A \ (\text{or}\Delta F) = KA[L]/(1 + K[L])$$

where ΔA (or ΔF) is the change in absorbance (or fluorescence) after each addition of caffeine (ΔA or $\Delta F \propto$ [D-L]), A or (F) is the absolute absorbance (or fluorescence) of the intercalator in the absence of caffeine, [L] is the total caffeine concentration after each addition. Fits of the binding curves were performed by nonlinear least squares analysis with Enzfitter software.

Steady-state fluorescence spectra were obtained using an SLM 8000C spectrofluorometer (SLM Aminco, Champaign, IL) modified with data acquisition electronics and software from ISS (Champaign, IL). Time-resolved fluorescence was obtained using an ISS K2 multifrequency and phase modulation spectrofluorometer. Excitation of each sample was accomplished using the 514-nm line from an argon ion laser (Spectra-Physics model 2045). The resulting emission above 550 nm was observed through a Schott RG570 cutoff filter. For lifetime measurements the exciting light was polarized parallel to the vertical laboratory axis, whereas the emission was viewed through a polarizer oriented at 55° (Spencer and Weber, 1970).

The multifrequency phase and modulation approach for time-resolved fluorescence relies on intensity modulation of the excitation source, and the phase shift and relative modulation of the emitted light, with respect to the excitation, are determined (Spencer and Weber, 1969; Jameson and Hazlett, 1991). Lifetimes are then calculated according to the following equations:

$$\tan[P] = \omega\tau^{P} \qquad M = [1 + (\omega\tau^{M})^2]^{-1/2}$$

where P is the phase shift, M is the relative modulation (the AC/DC ratios of the excitation and emission waveforms), and ω is the angular modulation frequency. Two independent lifetime determinations, τ^{P} and τ^{M} are thus obtained. An emitting system characterized by a single exponential decay will yield identical phase and modulation lifetime values irrespective of the modulation frequency. In the case of heterogeneous emitting systems (multiple non-interacting fluorescent species), the phase lifetime will be less than the modulation lifetime and those values will furthermore be dependent upon the modulation frequency, namely, decreasing as the modulation frequency increases (Spencer and Weber, 1969). The measured phase and modulation values may be analyzed as a sum of exponentials by using a nonlinear least squares procedure (Jameson et al., 1984; Jameson and Gratton, 1983) wherein the goodness of fit to a particular model (for example, single or double exponential) is judged by the value of the reduced χ^2 as defined by:

$$\chi^2 = \sum \{[P_c - P_m/\sigma^{P}] + [M_c - M_m/\sigma^{M}]\}/(2n - f - 1)$$

where the sum is carried out over the measured values at n modulation frequencies and f is the number of free parameters. The symbols P and M correspond to the phase shift and relative demodulation values, respectively, and the indices c and m indicate the calculated and measured

values, respectively; σ^P and σ^M correspond to the standard deviations of each phase and modulation measurement, respectively.

The calculated values of phase and modulation are obtained by the equations:

$$P = \tan^{-1}[S(\omega)/G(\omega)] \qquad M^2 = S(\omega)^2 + G(\omega)^2$$

where the functions $S(\omega)$ and $G(\omega)$ have different expressions depending on the fitting model used (Weber, 1981).

For the fit using a sum of exponentials, the functions $S(\omega)$ and $G(\omega)$ are given by:

$$S(\omega) = \sum f_i \omega\tau_i/(1 + \omega^2\tau_i^2)$$

$$G(\omega) = \sum f_i/(1 + \omega^2\tau_i^2) \qquad \sum f_i = 1$$

where the index i depends on the number of exponentials used for the fit; f_i is the contribution to the steady-state fluorescence of the ith component; τ_i is its lifetime and ω is the angular frequency of light modulation.

In addition to fluorescence lifetime determinations, the multifrequency phase and modulation method permits the characterization of the rotational modes of fluorophores (Gratton et al., 1984; Weber, 1977; Lakowicz et al., 1985). Such information is obtained by differential polarized phase fluorimetry (also known as dynamic polarization). In this approach, the sample is illuminated by parallel polarized light, the intensity of which is modulated at high frequencies. The phase delay between the parallel and perpendicular components of the emission (Φ and $\Phi_\perp$) are determined and the differential tangent function ($\Delta\Phi$) for a single, spherical rotator may be calculated as:

$$\Delta\Phi = \tan^{-1}\left[\frac{18\omega r_0 R}{(k^2 + \omega^2)(1 + r_0 - 2r_0^2) + 6R(6R + 2k + fr_0)}\right]$$

where $\Delta\Phi$ is the phase difference, ω is the angular modulation frequency, r_0 is the fundamental anisotropy, k is the radiative rate constant ($1/\tau$), and R is the rotational diffusion coefficient. Analysis of the phase delay (or modulation ratio) versus frequency curves thus permits the characterization of a fluorophore's rotational parameters.

Energy-minimized structures of the various caffeine-dye complexes were generated using HyperChem (Autodesk) software. The force field employed for these calculations is MM2 with default parameters provided with the software. Conjugate gradient methods were used to search for geometry-optimized structures with a convergence criterion of 0.001 kcal/A. Energy-minimized complexes were obtained by first geometry optimizing each component of the complex in vacuum. These geometry-optimized components were then brought together in a face-to-face orientation and to within van der Waals radii and re-optimized. Relative binding energies were determined by subtracting the sum of the geometry-optimized energies of the isolated components from the total energy obtained for the geometry-optimized complex.

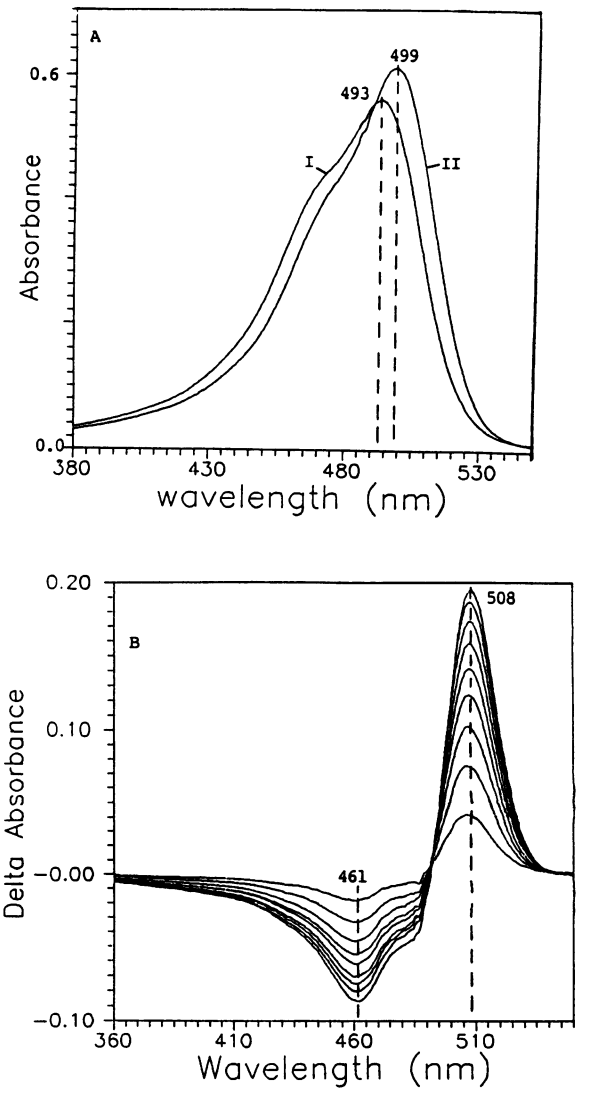


FIGURE 2 (*A*) Optical absorption spectra of the caffeine-AO complex in the absence (I) and presence (II) of caffeine. (*B*) Absorption difference spectra of AO titrated with caffeine. Intercalator concentration is 10 μM in 5 mM HEPES buffer, pH 7.0. Caffeine is titrated in increments of 1 mM from a 50 mM stock solution. Spectra of the caffeine-intercalator complexes contain 5 mM caffeine.

RESULTS

Optical titrations and steady-state fluorescence of caffeine with complexing agents

The optical spectra of AO is displayed in Fig. 2 *A*. The spectrum exhibits an absorption band in the visible region with absorption maxima at 493 nm. Upon addition of caffeine, the absorption maxima of AO shifts to 499 nm. The red-shift in the absorption maxima of AO in the presence of caffeine provides a convenient method for determining the equilibrium constants of complex formation. This method is demonstrated in Fig. 2 *B*, which displays optical difference titrations of AO with caffeine. The optical difference spectrum of the caffeine-AO complexes displays a single isosbestic point characteristic of only two absorbing species in solution. In addition, the corresponding binding curve (Fig. 3 *A*) fits well to a model in which the caffeine and intercalator form a 1:1 complex. The binding constant for the caffeine-AO complex was determined to be 258.4 ± 5.1 M^{-1}. Similar titrations involving EBR and caffeine revealed no detectable change in the absorption maxima of EBR found at 478 nm up to a final concentration of 10 mM. Upon addition of EBR to a solution containing 50 mM caffeine, however, the absorption maxima of EBR shifted to 482 nm consistent with a weakly bound complex.

Steady-state fluorescence of AO and EBR in the absence and presence of caffeine are shown in Fig. 4. The fluores-

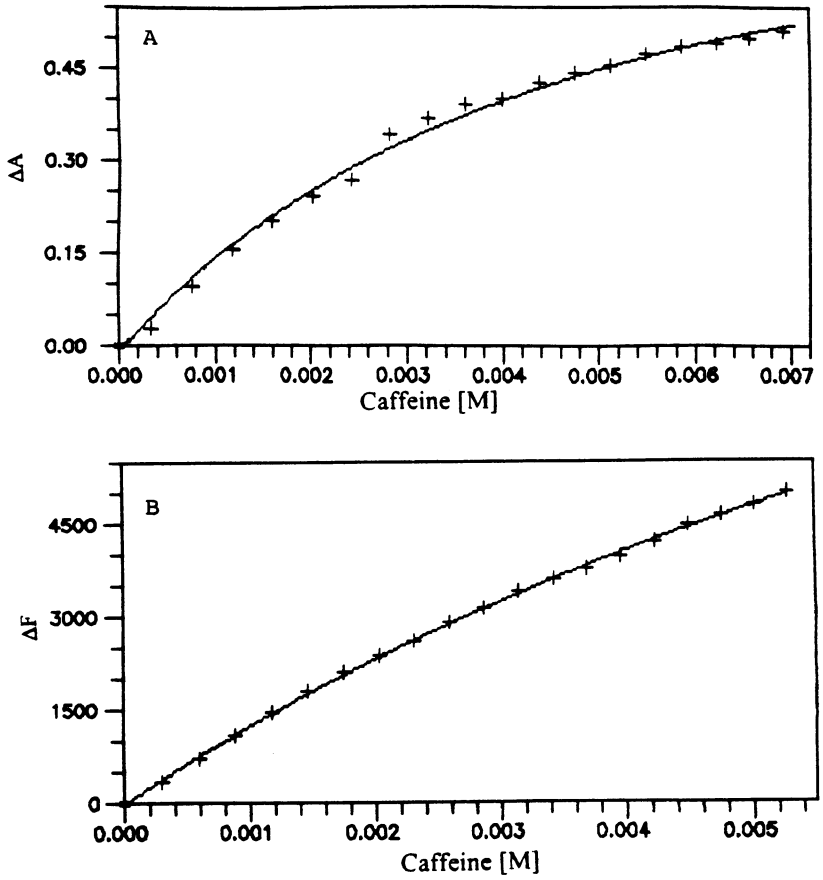


FIGURE 3 (*A*) Binding isotherm for the optical titration of AO with caffeine. The solid line corresponds to a binding constant of 200 ± 1.4 M^{-1} with a 1:1 stoichiometry. (*B*) Binding isotherm for the fluorescence titration of EBR with caffeine. The solid line corresponds to a binding constant of 84.5 ± 3.5 M^{-1} and a 1:1 stoichiometry.

cence maxima for AO and EBR, in the absence of caffeine, are observed at 526 and 609 nm, respectively. Upon addition of caffeine, changes are observed both in position of the fluorescence maximum and in the overall fluorescence intensity. Specifically, the fluorescence maximum of AO shifts to 532 nm whereas that of EBR shifts to 615 nm. In addition the integrated fluorescence intensity increases by 53% for AO and 74% for EBR in the presence of caffeine. The change in steady-state fluorescence of EBR upon addition of caffeine allows for the determination of the bind4ing constant for this complex. A least squares fit to a plot of fluorescence intensity versus caffeine concentration gives a binding constant of 84.5 ± 3.5 M^{-1} for complexation of EBR with caffeine (Fig. 3 *B*).

Time-resolved fluorescence studies

The results of intensity decay multifrequency phase and modulation measurements for AO and EBR in the absence and presence of caffeine are summarized in Table 1. Both AO and EBR can be fit to an expression displaying single exponential decays in the absence of caffeine with lifetimes of 2.0 and 1.8 ns, respectively, consistent with previously reported lifetimes for these molecules (Jameson and Hazlett, 1991; Brun and Harriman, 1994). In the presence of caffeine, the AO data can be fit to a single exponential decay with a lifetime of 3.34 ns whereas that of EBR was best fit to a double exponential decay, the major component having a lifetime of 6.6 ns. Interestingly, the analysis of the EBR/caffeine lifetime data directly yielded a shorter component of 1.8 ns; i.e., this value was not fixed in the analysis. It seems reasonable to conclude that this component corresponds to free EBR.

The fractional intensities observed for the lifetime components in the EBR/caffeine case may then be related to the relative concentrations of the free and bound EBR by assuming that the relative quantum yields parallel the relative lifetime values. Hence the relative molar ratios of the bound and free EBR are found to be 84 and 16%, respectively. Using these relative molar ratios as well as the concentrations of EBR and caffeine (30 μM and 44 mM, respectively, for the lifetime measurements), an equilibrium constant for complexation formation is calculated to be ~100 M^{-1}, which is consistent with that determined by steady-state fluorescence titrations. In the case of AO the equilibrium constant for complex formation was determined from absorption titrations to be 258.4 ± 5.4 M^{-1}. Using this value and the concentrations of AO and caffeine used for lifetime measurements (30 μM and 44 mM, respectively), the relative molar ratios of bound and free AO are calculated to be 92 and 8%, respectively. The corresponding fractional intensities expected from the lifetime data then would be on the order of 95 and 5% for bound and free AO, respectively, assuming that the relative quantum yields parallel the rela-

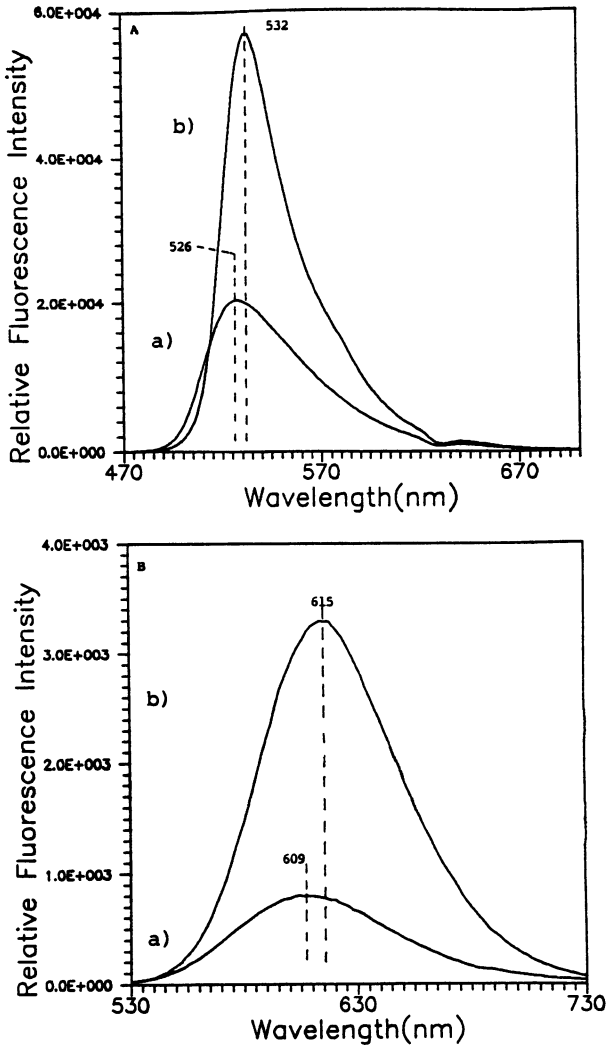


FIGURE 4 Steady-state fluorescence spectra of AO (*A*) and EBR (*B*) in the absence (*a*) and presence (*b*) of caffeine. Sample conditions are as described in Fig. 2.

tive lifetimes. The fact that the χ^2 (6.6) did not improve upon analyzing for two components is a consequence of the small difference in lifetimes between the free and bound AO (2.0 and 3.34 ns, respectively) as well as the low relative fractional intensity of free AO (5%). (The higher χ^2 for the single exponential fit may result, in part, from the presence of the two components.)

We have also examined the dynamic polarization of AO and EBR in the presence and absence of caffeine (Fig. 5). Dynamic polarization data (phase only) for EBR in the absence of caffeine fit to a single component with a rotational correlation time (ϕ) of 0.101 ns (χ^2 = 0.86). In the presence of caffeine the data could be fit to two components exhibiting rotational correlation times of 0.101 and 0.162 ns (while fixing the relative intensities according to the lifetime results, i.e., 0.043 and 0.957 for the free and bound components, respectively; χ^2 = 0.67). In the case of AO the

TABLE 1 Summary of fluorescence lifetime data and fractional populations data for AO and EBR in the absence and presence of caffeine

Species	τ_1 (ns)	f_1	τ_2 (ns)	f_2	χ^2
AO	2.0	1.0			2.37
AO-caffeine	3.34	1.0			6.69
EBR	1.8	1.0			3.61
EBR-caffeine	6.6	0.84	1.8	0.16	5.11

Concentrations used: caffeine, 44 mM; AO, 30 μM; EBR, 30 μM.

rotational correlation time of the free molecule was found to be 0.110 ns. Unlike the EBR/caffeine case, AO in the presence of caffeine fit well to a single rotational correlation time of 0.152 ns (χ^2 = 1.0). Plots of the χ^2 surfaces for the various fits are shown in Fig. 6. The fact that the rotational correlation time is related to the effective hydrodynamic molar volume allows for an unambiguous determination of molecular stoichiometries for the caffeine-dye complexes. For a spherical rotator the relationship between ϕ and molar volume is given by:

$$Y = V\eta/RT$$

where η is the solvent viscosity, R is the gas constant, and T is the temperature (Jameson and Hazlett, 1991). If the approximation is made that the molar volume scales as the molecular weight then the percent increase in correlation time provides a measure of the increase in molecular weight of the dye upon complexation. In the case of EBR the correlation time increases by 49%. For a 1:1 complex the corresponding molecular weight of the complex is increased by 51% relative to unbound EBR. For AO the percent increase in the correlation time is 41%. The corresponding increase in molecular weight for a 1:1 complex is 52%.

The suggested 1:1 stoichiometry for the caffeine-AO complex is in contrast to data reported earlier by Kapuscinski and Kimmel (1993) who reported mixed aggregate complexes between caffeine and AO. These authors suggest both $(AO)_n$-$(caffeine)_i$-$(AO)_n$ and $(caffeine)_n$-$(AO)_i$-$(caffeine)_n$ type complexes. Interestingly, statistical thermodynamic analysis of such models reveals an ionic-strength-dependent binding constant of 250 M^{-1}, which is nearly identical (within experimental error) to the value obtained assuming a 1:1 complex. This observation suggests that under low ionic strength conditions caffeine forms a 1:1 complex with AO or that the caffeine-AO complex (or mixed aggregate) is homogeneous.

Molecular modeling studies of caffeine complexes with DNA intercalators

The results of our molecular modeling calculations obtained for complexes between caffeine and the DNA intercalators DOX, NOV, AO, and EBR are summarized in Table 2. The minimized energy values for the various complexes reported in the table are obtained by subtracting the sum of the

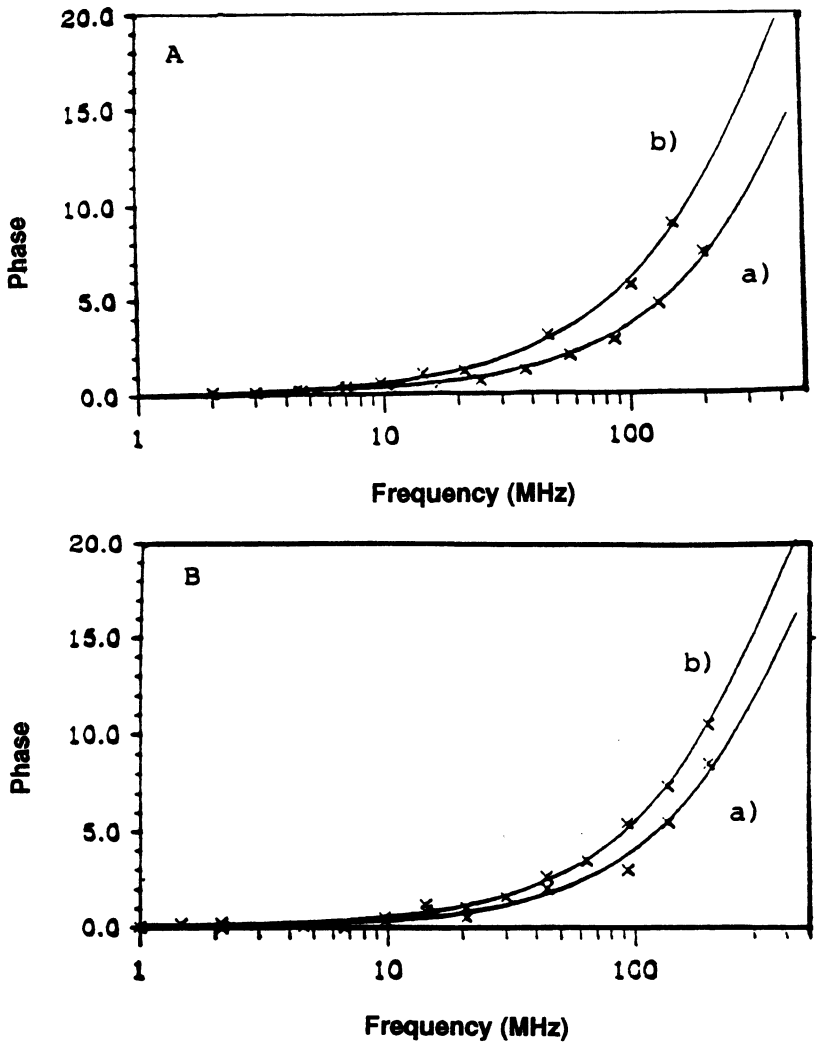


FIGURE 5 Dynamic polarization phase data. (*A*) EBR in the absence (*a*) and presence (*b*) of caffeine. (*B*) AO in the absence (*a*) and presence (*b*) of caffeine. Solid lines correspond to rotational correlation times of 0.101 ns (EBR), 0.101 ns and 0.162 ns with relative contributions of 0.043 and 0.957 (EBR/caffeine), 0.110 ns (AO), and 0.152 ns (AO/caffeine). The limiting anisotropy was found to be 0.37 in each case.

minimized energy of each molecule individually from the minimized energy obtained for the geometry-optimized complex (Tachino et al., 1994). Thus, more negative values of minimized energy predict a more stable complex. The results indicate that caffeine forms a favorable complex with all of the intercalators. The most stable of these complexes being that with caffeine and AO (relative binding energies of -21.6 kcal/mole for the caffeine-AO complex relative to ~ -10 to -15 kcal/mole for caffeine-DOX, caffeine-NOV, and caffeine-EBR complexes). For comparison, geometry optimization was also performed for a complex between caffeine and both hydroxyurea and hadicine. In the case of hydroxyurea, it has been reported that the addition of caffeine leads to an increase in cell death (Selby and Sancar, 1990). It should be pointed out that both hydroxyurea and hadicine are aliphatic and contain no conjugated ring systems. The results of molecular modeling studies of hydroxyurea and hadicine complexes with caffeine gave energies that are reduced relative to the isolated molecules. The relative stabilization of the aliphatic complexes, however, is much less than that of the caffeine complexes with the highly conjugated intercalators. Thus, it is expected that the nonconjugated molecules form considerably weaker complexes in solution.

The energy-minimized structures of the caffeine-DNA-intercalator complexes are displayed in Fig. 7. In general, the lowest energy conformation is that in which the caffeine is oriented directly over the conjugated ring of the various intercalators with an average face-to-face distance of 3.46 Å. It must be pointed out that the orientation of the caffeine relative to the DNA intercalator in the geometry-optimized complexes shown in Fig. 7 is not unique. For example, rotation of the caffeine by 180° relative to the DNA intercalator ring system does not significantly alter the relative binding energy. The relative binding energies derived from molecular mechanics methods can be further deconvoluted into the individual components that make up the total energy. These include potential energies associated with bond stretching, bond bending, dihedral bending, van der Waals forces, and electrostatic interactions. This analysis of the

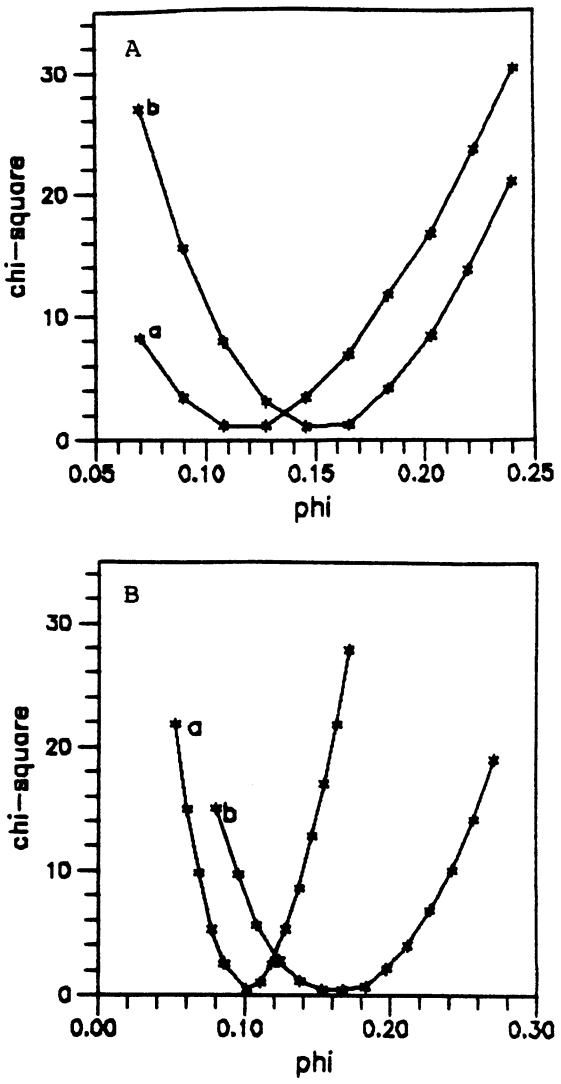


FIGURE 6 χ^2 error surface for the fluorescence lifetime analysis of AO (*A*) and EBR (*B*) in the absence (*a*) and presence (*b*) of caffeine. The χ^2 error surface obtained for AO and EBR in the absence of caffeine and AO in the presence of caffeine are for fits to discrete single component fits. The corresponding error surface for EBR in the presence of caffeine are for fits involving two discrete components.

components is also summarized in Table 2. As expected for face-to-face complexes, van der Waals contributions play a dominant role in the relative energy of the complex relative to the isolated components.

An additional point of interest is the relative differences in structure between the various intercalators. Both DOX and NOV are derived from an anthraquinone ring system whereas AO and, to some extent, EBR are pyridine based. To examine the extent to which the central ring substitutions affect complex formation, we have examined energy-minimized structures for a series of model compounds complexed with caffeine. These results are summarized in Table 3. Interestingly, these results indicate only minimal effects of central ring substitutions on energy stabilization due to complex formation. Again the dominant component to energy stabilization are van der Waals forces.

DISCUSSION

Caffeine binding to DNA intercalators

The DNA intercalators AO and EBR both exhibit optical absorption and singlet emission bands in the visible region of the spectrum that are sensitive to the solvent environment of the chromophore. The observed red-shift of the absorption and corresponding emission bands, increase in fluorescence yield, and increase in fluorescence lifetimes in the presence of caffeine are characteristic of changes in the solvent environment associated with the intercalator. In general, excited states arising from π-π^* transitions (such as the optical transitions of the DNA intercalators) are expected to decrease in energy as the solvent becomes increasingly hydrophobic (Bakhkshiev, 1961; Bayliss and McRae, 1954). Complexation between caffeine and the DNA intercalators results in the replacement of water molecules solvating the intercalator by the more hydrophobic caffeine molecule. Thus the π-π^* excited state experiences a more hydrophobic environment and a correspondingly lower energy resulting in red-shifts in the absorption and emission bands. In addition, the exclusion of water molecules surrounding the chromophore causes an increase in the fluorescence lifetime as water is an effective quencher of the excited state of the intercalators.

Molecular modeling techniques provide a useful method for examining the role of specific structural aspects of a molecule with respect to its ability to form complexes with structurally diverse partners. Molecular mechanics techniques have been successfully applied to a number of important structural problems including chlorophyllin complexes with planar mutagens (Tachino et al., 1994; Langley et al., 1991). It appears that the primary criteria for energy minimization of the caffeine-DNA-intercalator complex is the centering of the caffeine ring system over that of the DNA intercalator ring system with only a minimal contribution from the relative orientation. An exception is the caffeine-DOX complex in which the caffeine is oriented slightly off center of the intercalator ring system. Thus, the bulky substituent of the DOX influences the relative orientation of the complex but does not appear to significantly destabilize it. It is also of interest to note the structure of the complex between caffeine and EBR (Fig. 7 *C*). The energy-minimized structure of the EBR reveals a distinctive saddle shape to the ring system. Previous descriptions of complex formation between caffeine and DNA intercalators portray complexes between caffeine and intercalators with distinctly planar ring structures. The structure of the energy-minimized complex between caffeine and EBR indicate that some degree of nonplanarity does not prevent complex formation. In fact, we have observed nonplanarity or ruffling in the structure of chlorophyllin, which is also known

TABLE 2 Summary of molecular modeling calculations for caffeine-DNA-intercalating drug complexes

	Total E*	Complex energy‡	ΔE§	$\Delta V_{Stretch}$	ΔV_{Bend}	ΔV_{Dih}	ΔV_{VDW}	ΔV_{EEL}
CF-NOV	65.054	53.056	−11.997	−0.07	−0.570	0.117	−11.436	−0.037
CF-DOX	79.711	64.623	−15.089	−0.08	−0.624	0.098	−14.437	−0.044
CF-EBR	32.310	21.772	−10.679	−0.03	−0.375	−0.007	−10.135	0.0026
CF-AO	32.451	10.836	−21.615	−1.15	−3.311	4.362	−21.40	−0.120
CF-HU	26.510	21.537	−4.973	−0.013	−0.538	0.104	−4.498	−0.028
CF-HAD	24.668	18.626	−6.042	−0.01	−0.506	0.146	−6.780	−0.026

CF, caffeine; HU, hydroxyurea; HAD, hadicine. $V_{Stretch} = \Sigma K_r(r-r_o)^2$ where K_r is force constant and $(r-r_o)^2$ is the square of the bond displacement. Sum is over the number of bonds. $V_{Bend} = \Sigma K_\theta(\theta-\theta_o)^2$ where K_θ is bending force constant and $(\theta-\theta_o)^2$ is the angle displacement. Sum is over all bond angles. $V_{Dih} = \Sigma(V_n/2)(1 + \cos(n\phi-\phi_o))$ where V_n is dihedral force constant, n is the periodicity of the Fourier term, and ϕ is the dihedral angle. $V_{VDR} = \Sigma[(A_{ij}/R_{ij}^{12})-(B_{ij}/R_{ij}^{6})]$ where R_{ij} is the nonbonded distance between two atoms (R^{12} term describes repulsive interactions between two atoms and R^6 term describes attractive London dispersion interactions). A_{ij} and B_{ij} are van der Waals parameters for the interacting pair of atoms. $V_{EEL} = \Sigma(q_iq_j/\epsilon R_{ij})$ where q_i and q_j are point charges on two atoms and R_{ij} is the distance between two atoms. ε is the effective dielectric constant.

*Mathematical sum of energy (E) for each molecule in the complex in kcal/mole.

‡Total energy calculated for a 1:1 molecular complex in kcal/mole.

§Difference between mathematical sum of energies of individual molecules and calculated energies of each complex in kcal/mole.

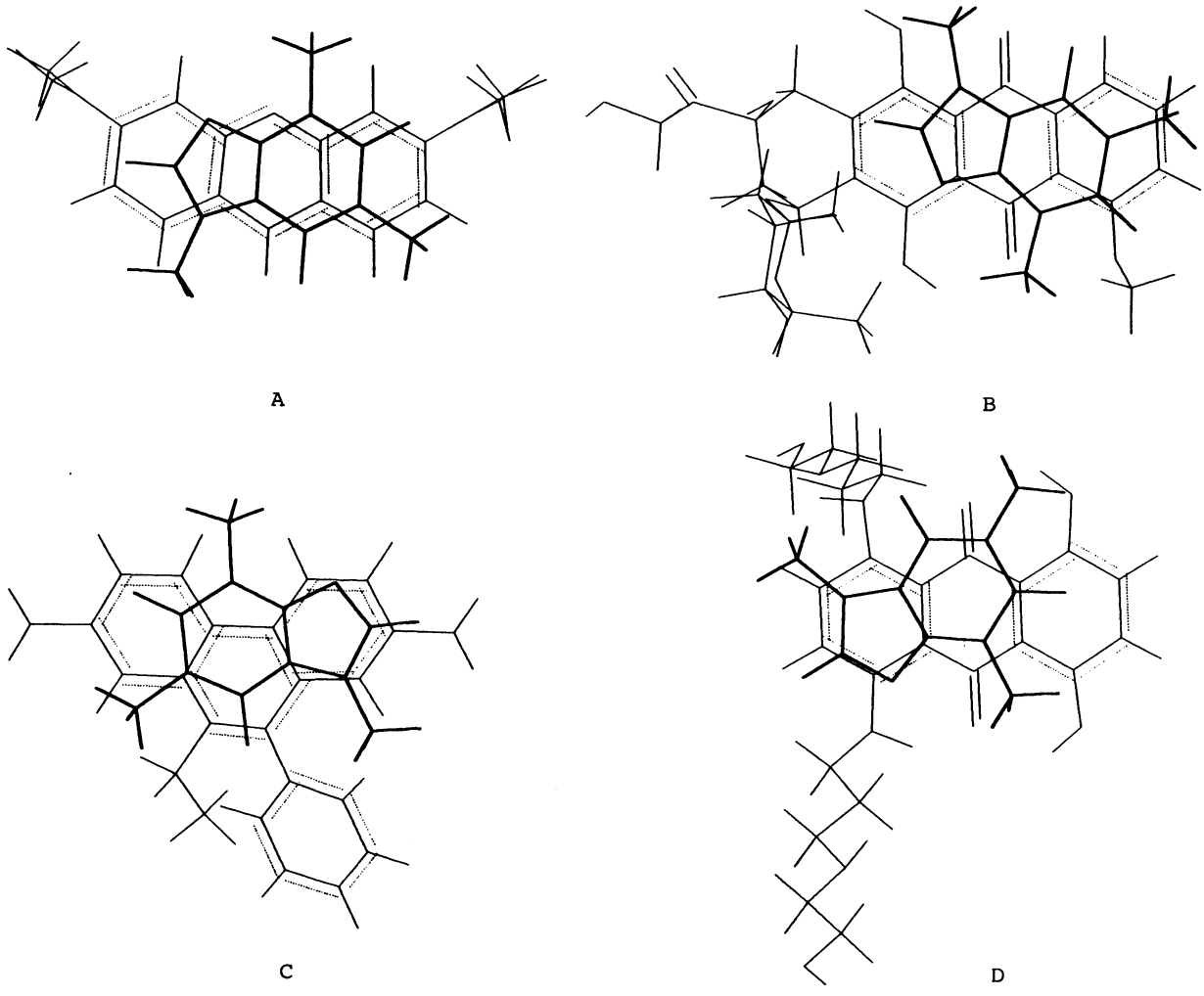


FIGURE 7 Top view of the geometry-optimized caffeine-DNA-intercalator complexes. (*A*) Caffeine-AO complex. (*B*) Caffeine-DOX complex. (*C*) Caffeine-EBR complex. (*D*) Caffeine-NOV complex. Caffeine is highlighted by heavy lines.

to form complexes with planar mutagens (Dashwood and Gao, 1993).

Caffeine as an interceptor molecule?

The results of the molecular modeling calculations and the optical titrations may provide a mechanistic model of inhibition that is consistent with in vitro studies. Previous results obtained by Traganos et al. (1991) demonstrate that caffeine can modulate the effects of DOX and NOV in L1210 cells. Specifically, the addition of DOX and NOV to L1210 cells decreases cell growth to 26 and 48%, respectively, relative to control cultures. Addition of caffeine together with either DOX or NOV results in cell growth that is 83 and 65%, respectively, relative to control cultures. In addition, it was discovered that the sequence in which caffeine was added affects the degree of cell growth with the mutagens. When caffeine is added either before or after the mutagens, caffeine afforded no protection against the mutagens. When caffeine was added along with the mutagens, cell-damaging effects are reversed. Taken together, these findings imply that co-treatment facilitates complex formation between caffeine and the mutagens and that this results in a lower effective concentration of the intercalator in solution. This assumes, of course, that the combined caffeine-intercalator complex is unable to bind to DNA or otherwise affect cellular replication.

Additional evidence for this mechanism can be found in the energy-minimized complexes of caffeine with hadicine and hydroxyurea. In the case of hydroxyurea, caffeine displays no protective properties against cellular damage caused by this compound. The molecular modeling studies show that the energetics for complexation between hydroxyurea and caffeine are much less favorable resulting in a weaker complex with a lower association constant. This phenomenon is presumably due to the aliphatic nature of hydroxyurea, which is incapable of π-π complex formation with caffeine. The result is an effective concentration of hydroxyurea that is unaffected by the presence of caffeine in solution.

Comparison of the complexing ability of caffeine relative to other proposed interceptor molecules is also of interest. Optical titrations and molecular modeling calculations of mutagen-porphyrin and mutagen-chlorophyll complexes reveal significant binding constants and relative binding energies for these complexes. Binding constants for chlorophyllin complexes with heterocyclic amines are at least one order of magnitude larger than that of caffeine with model intercalators (Hartman and Shankel, 1990; Arimoto et al., 1993; Dashwood and Guo, 1993). This trend is also reflected in the relative binding energies determined from molecular modeling calculations. These binding energies are a factor of two larger for porphyrin-mutagen complexes relative to caffeine-mutagen complexes. Thus, although caffeine may play a role as an interceptor molecule, its ability to prevent cell damage is somewhat less than porphyrin-type interceptors.

In summary, the results of molecular modeling and optical titration studies of caffeine with DNA-intercalating drugs demonstrates that caffeine can complex with the intercalators via a π-π type of interaction. The dominant force in the formation of such complexes appears to be van der Waals interactions resulting in maximal ring overlap between the two molecules of the complex. Complexation between caffeine and aliphatic mutagens show much lower binding energies relative to caffeine complexes with aromatic intercalators. The corresponding binding constants for caffeine-DNA-intercalator complexes are on the order of 250 M^{-1} (AO) and 85 M^{-1} (EBR). These results suggest a possible role for caffeine as an interceptor molecule although the inhibitory effect is expected to be less than for interceptor molecules with more extended conjugated ring systems.

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TABLE 3 Summary of molecular modeling calculations for caffeine model complexes

	Total E*	Complex energy‡	ΔE§	$\Delta V_{Stretch}$	ΔV_{Bend}	ΔV_{Dih}	ΔV_{VDW}	ΔV_{EEL}
CF-BEN	22.344	15.062	−7.2825	−0.019	−2.897	0.117	−3.707	−0.0174
CF-ANT	9.427	−1.061	−10.488	0.233	−0.455	0.098	−10.07	0.00
CF-NAP	15.876	6.566	−9.310	−0.027	−0.465	0.067	−8.861	−0.0174
CF-Q	46.075	38.432	−7.642	−0.0223	0.922	4.949	−7.216	−0.029
CF-AQ	45.147	35.559	−9.592	−0.213	−1.896	6.967	−14.013	−0.123
CF-NQ	44.830	34.094	−10.736	0.147	0.919	−6.767	−5.401	0.041
CF-PYR	29.110	22.167	−6.943	−0.016	−0.500	0.137	−6.537	−0.019
CF-QL	24.634	15.366	−9.268	−0.043	−0.491	0.122	−8.826	−0.0329
CF-AC	20.030	9.853	−10.177	−0.040	−0.493	−0.149	−9.764	−0.0274

CF, caffeine; BEN, benzene; ANT, anthracine; NAP, napthelene; Q, benzoquinone; NQ, napthoquinone; PYR, pyridine; QL, quinoline; AC, anthroquinoline. V terms are described in Table 2.

*Mathematical sum of energy (E) for each molecule in the complex in kcal/mole.

‡Total energy calculated for a 1:1 molecular complex in kcal/mole.

§Difference between mathematical sum of energies of individual molecules and calculated energies of each complex in kcal/mole.

REFERENCES

Arimoto, S., S, Fukuoka, C. Itome, H. Nakano, H. Rai, and H. Hayatsu. 1993. Binding of polycyclic planar mutagens to chlorophyllin resulting in inhibition of the mutagenic activity. *Mutation Res.* 287:293–305.

Bakhkshiev, N. G. 1961. Universal intermolecular interactions and their effects on the position and electronic spectra of molecules in two-component solutions. *Optic. Spectrosc.* 10:379–384.

Bayliss, N. S., and E. G. McRae. 1954. Solvent effects in organic spectra: dipole forces and the Franck-Condon principle. *J. Chem. Phys.* 58: 1002–1006.

Bertrand, C. A., I. Pomper, G. Hillman, J. C. Duffy, and I. Mitchell. 1970. No relation between coffee and blood pressure. *N. Engl. J. Med.* 299:315–316.

Borodina, V. M., E. E. Kirianova, O. V. Federova, and A. V. Zelenin. 1979. Cytochemical properties of interphase chromatin condensed as a result of treatment with caffeine. *Exp. Cell Res.* 122:391–394.

Brun, A. M., and A. Harriman. 1994. Energy- and electron-transfer processes involving palladium porphyrins bound to DNA. *J. Am. Chem. Soc.* 116:10383–10393.

Brunet, J. E., V. Vargas, E. Gratton, and D. M. Jameson. 1993. Hydrodynamics of horseradish peroxidase revealed by global analysis of multiple fluorescence probes. *Biophys. J.* 66:446–453.

Curatolo, P. W., and D. Robertson. 1983. The health consequences of caffeine. *Ann. Int. Med.* 98:641–653.

Damewood, J. R., W. P. Anderson, and J. J. Urban. 1988. A molecular mechanics study of neutral molecule complexation with crown ethers. *J. Comput. Chem.* 9:111–124.

Dashwood, R., and D. Guo. 1993. Antimutagenic potency of chlorophyllin in the *Salmonella* assay and its correlation with binding constants of mutagen-inhibitor complexes. *Env. Mol. Mut.* 22:164–171.

Dews, P., H. C. Grice, A. Neims, J. Wilson, and R. Wurtman. 1984. Report of the fourth international caffeine workshop, Athens. *Fd. Chem. Toxicol.* 22:163–169.

Gratton, E., D. M. Jameson, and R. D. Hall. 1984. Multifrequency phase and modulation fluorometry. *Annu. Rev. Bioenerg.* 13:105–124.

Hartman, P. E., and D. M. Shankel. 1990. Antimutagens and anticarcinogens: a survey of putative interceptor molecules. *Env. Mol. Mut.* 15:145–182.

Jameson, D. M., and E. Gratton. 1983. New Directions in Molecular Luminescence. D. Eastwood and L. Cline-Love, editors. Philadelphia, American Society for Testing Materials. 67–81.

Jameson, D. M., E. Gratton, and J. D. Hall. 1984. The measurement and analysis of heterogeneous emissions by multifrequency phase and modulation fluorimetry. *Appl. Spectrosc. Rev.* 20:55–105.

Jameson, D. M., and T. L. Hazlett. 1991. Time-resolved fluorescence in biology and biochemistry. *In* Biophysical and Biochemical Aspects of Fluorescence Spectroscopy. T. G. Dewey, editor. Plenum Press, New York. 105–133.

Jick, H., O. S. Miettinen, R. K. Neff, S. Shapiro, O. P. Heinonen, and D. Slone. 1973. Coffee and myocardial infarction. *N. Engl. J. Med.* 289:63

Kapuscinski, J., and M. Kimmel. 1993. Thermodynamic model of mixed aggregation of intercalators with caffeine in aqueous solution. *Biophys. Chem.* 46:153–163

Kimura, H., and T. Aoyama. 1989. Decrease in sensitivity to ethidium bromide by caffeine, dimethylsulfoxide or 3-aminobenzamide due to reduced permeability. *J. Pharmacobiodyn.* 12:589–595.

Kunicka, J. E., M. R. Melamed, and Z. Darzynkiewicz. 1990. Caffeine increases sensitivity of DNA to denaturation and chromatin of L1210 cells. *Cell Tissue Kinetics.* 23:31–39.

Lakowicz, J. R., H. Cherek, E. Gratton and B. P. Maliwal. 1985. Time-resolved fluorescence anisotropy of diphenyl hexatriene and perylene in solvent and lipid bilayers obtained from multifrequency phase-modulation fluorometry. *Biochemistry.* 24:376–383.

Langley, D. R., T. W. Doyle, and D. L. Beveridge. 1991. The dynemicin-DNA intercalation complex: a model based on DNA affinity cleavage and molecular dynamics simulation. *J. Am. Chem. Soc.* 113:4395–4403.

Levin, R. B. 1982. Influence of caffeine on mutations induced by nitrosoguanidine in *Salmonella typhimurium* tester strains. *Env. Mutagen.* 4:689–694.

Mann, J. I., and M. Thorogood. 1975. Coffee drinking and myocardial infraction. *Lancet.* ii:1215.

O'Neal, F. J. 1979. Differential effects of cytochalasin B and caffeine on control of DNA synthesis in normal and transformed cells. *J. Cell Physiol.* 101:201–218.

Pozniak, P. C. 1985. The carcinogenicity of caffeine and coffee: a review. *J. Am. Diet. Assoc.* 85:1127.

Rall, T. W. 1980. Central nervous system stimulants: the xanthines. *In* The Pharmacological Basis of Therapeutics, 6th ed. L. S. Goodman and A. Gilman, editors. Toronto, Macmillan. 592.

Roberts, J. J. 1984. Mechanism of potentiation by caffeine of genotoxic damage induced by physical and chemical agents. *In* DNA Repair and Its Inhibitors. A. Collins, C. S. Downes, and R. T. Johnson, editors. Oxford, IRL Press. 193–216.

Ross, W. E., L. A. Zwelling, and K. W. Kohn. 1979. Relationship between cytoxicity and DNA strand breakage by adriamycin and other intercalating agents. *Int. J. Radiat. Biol. Phys.* 5:1221–1224.

Selby, C. P., and A. Sancar. 1990. Molecular mechanism of DNA repair inhibition by caffeine. *Proc. Natl. Acad. Sci. USA.* 87:3522–3525.

Spencer, R. D., and G. Weber. 1969. Measurements of subnanosecond fluorescence lifetimes with a cross-correlation phase fluorometer. *Ann. NY Acad. Sci.* 158:361–376.

Spencer, R. D., and G. Weber. 1970. Influence of Brownian rotations and energy transfer upon the measurement of fluorescence lifetimes. *J. Chem. Phys.* 52:1654–1663.

Tachino, N., D. Guo, W. M. Dashwood, S. Yamane, R. W. Larsen, and R. Dashwood. 1994. Mechanisms of the in vitro antimutagenic action of chlorophyllin against benzo(A)pyrene: studies of enzyme inhibition, molecular complex formation, and degradation of the ultimate carcinogen. *Mutation Res.* 308:191–203.

Traganos, F., J. Kapuscinski, and Z. Darzynkiewicz. 1991. Caffeine modulates the effects of DNA-intercalating drugs in vivo: a flow cytometric and spectrophotometric analysis of caffeine interaction with novantrone, doxorubicin, ellipticine, and the doxorubicin analogue AD198. *Cancer Res.* 51:3682–3689.

Weber, G. 1977. Theory of differential phase fluorimetry: detection of anisotropic molecular rotations. *J. Chem. Phys.* 66:4081–4091.

Weber, G., 1981. Resolution of the fluorescence lifetimes in a heterogeneous system by phase and modulation measurements. *J. Phys. Chem.* 85:949–953.