Dimerization of Coralyne and Its Propyl Analogue and Their Association with DNA

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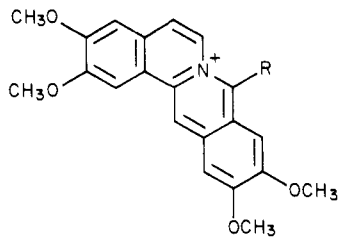
Dimerization of coralyne, Ia, and its propyl analogue Ic has been analyzed at neutral pH as a function of ionic strength. Even at low ionic strength (I = 0.02) dimerization constants (molar units) for both compounds are 10^5 or greater, which is considerably larger than values obtained for similar intercalating molecules. Coralyne seems to undergo association to higher aggregates somewhat easier than Ic, which could be due to the fact that the propyl group on Ic provides some steric hinderance in forming higher aggregates. Both compounds readily associate with DNA. At high ratios of ligand to DNA, the spectra for Ia or Ic are similar to that of a highly aggregated complex. At low ratios of ligand to DNA, the spectra for both compounds approach a limit, which is relatively independent of ionic strength and concentration, and can be identified with the intercalated species. The slight differences between Ia and Ic in dimerization or in association with DNA do not seem capable of explaining the significant differences in antileukemic activity of these compounds.

We have analyzed the interaction of several antiparasitic and antineoplastic drugs with deoxyribonucleic acid (DNA) as their potential bioreceptor.[1-5] The goal of these studies with drugs of quite varied structure is to develop some general principles relating activity to structure. As part of this effort, we have reported that the antineoplastic drug coralyne (Ia), synthesized by C. C. Cheng and co-workers,[6,7]



Ia, R = CH_3
Ib, R = CH_2CH_3
Ic, R = $CH_2CH_2CH_3$

binds to DNA by intercalation.[5] Coralyne has relatively low activity (P388 and L1210 leukemia in mice) but it also has quite low toxicity, making this general lead an attractive one for future synthetic efforts. There are also some interesting molecular features relating the activity of coralyne and its derivatives. For example, the activity of the ethyl-substituted analogue (Ib) of coralyne has significantly higher activity than coralyne, but the similarly substituted propyl analogue (Ic) has essentially no activity.[6]

Cheng and co-workers have postulated that this activity loss with Ic could be due to unfavorable steric interactions with the bioreceptor for this class of compounds.[6] Since DNA is a potential receptor for intercalating compounds of this type and is known to bind coralyne in an intercalation complex,[5] we have initiated an analysis of the interaction of coralyne and its derivatives with DNA to see if changes in binding can account for their activity differences.

Unfortunately, even in dilute solution at low ionic strength, coralyne and its derivatives do not obey Beer's law and do not give isosbestic points when titrated with a DNA solution. This suggests that these molecules undergo aggregation or self-association. Before the interaction of these compounds with DNA can be quantitatively understood, the aggregation phenomena must be analyzed, and in this paper we report dimerization constants[8,9] for coralyne and its inactive propyl derivative. We also present qualitative spectral results on the relative interaction of these two compounds with DNA. More quantitative studies on these and other coralyne derivatives are made extremely difficult by the self-association and DNA-induced aggregation and precipitation of these compounds.

Experimental Section

Materials. Coralyne (Ia) and propylcoralyne (Ic) samples were synthesized by Dr. C. C. Cheng and co-workers and were generously supplied by Dr. Harry B. Wood, Jr., of the National Cancer Institute. Fresh stock solutions of these compounds were prepared for all experiments, and all manipulations were done under filtered light to prevent decomposition of the drugs.[7] Calf thymus DNA samples were prepared as previously described.[2,4] All solutions, unless otherwise indicated, were prepared in the following buffer: 7.5×10^{-3} M NaH_2PO_4, 10^{-3} M EDTA adjusted to pH 7.0 with NaOH. The ionic strength of this buffer was adjusted to any desired value by the addition of NaCl before final adjustment of pH.

Methods. Spectra were recorded on either a Cary 17D (calibrated in wavelength) or Beckman Acta V (calibrated in wavenumbers) spectrophotometer. All titrations were done in 28-mm o.d., 10-cm quartz spectrophotometer cells. The holder for these cells was immobilized in the spectrophotometer, and the cell could be removed and replaced without any detectable change in absorbance. The holder was thermostated with a Haake refrigerated water bath at 25 °C. Titrations (adding salt, drug, or DNA solutions) were then done by adding desired amounts of the titrant, with a calibrated microliter syringe, mixing, and scanning or reading absorbance at a fixed wavelength. For determination of dimerization constants, this process was repeated for at least three titrations, and the results of all experiments were averaged.

Results

Effect of Ionic Strength on Aggregation. A qualitative comparison of the aggregation of Ia and Ic can be seen in Figure 1, where visible spectra of the two compounds are shown at different salt concentrations. Normalized plots of these data in Figure 2 illustrate that coralyne has a much greater tendency to aggregate than the propyl derivative. The spectrum of coralyne monomer above 300 nm, where DNA does not absorb, has resolved peaks at 421 and 403 nm, in addition to a smaller peak at 357 nm. The highly aggregated complex at 0.5 M NaCl, however, has only a broad peak at approximately 422–424 nm, no peak near 357 nm, and a generally reduced extinction coefficient in this wavelength range. This qualitative change in spectra will be of interest when analyzing the DNA complex of coralyne shown below.

Effect of Drug Concentration on Aggregation. If coralyne and its derivatives yield only dimers and no higher aggregate on self-association, isosbestic points should be obtained in absorbance vs. wavelength plots at different drug concentrations, provided the effect of increased concentration can be eliminated by changing cell size or instrument expansion. Because of the several instrument scale expansions available on the Cary 17D, we adjusted concentrations in a linear inverse manner to these scales.

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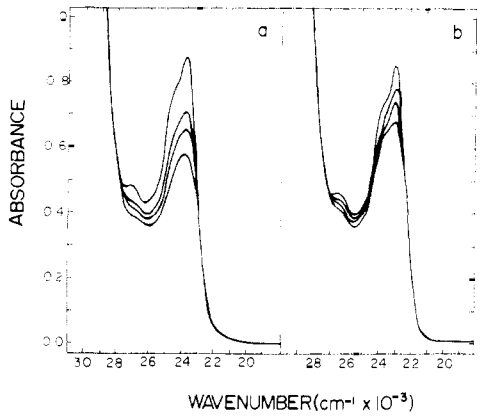

Figure 1. The effect of ionic strength on the spectrum of (a) coralyne and (b) propylcoralyne. The ionic strengths for curves from top to bottom in both a and b are 0.02, 0.12, 0.22, and 0.52, respectively. Experiments were done in 10-cm quartz cuvettes with a Beckman Acta V spectrophotometer.

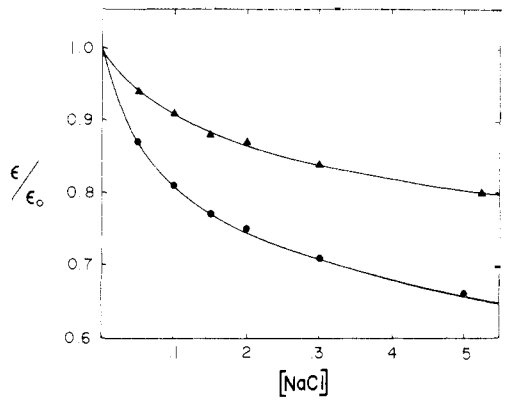

Figure 2. Results from Figure 1a,b and similar experiments at other salt concentrations were converted to extinction coefficients (ϵ) and plotted as ϵ/ϵ_0, where ϵ_0 is the extinction coefficient with no added salt, as a function of the molarity of added NaCl.

The results, as shown in Figure 3, indicate that coralyne even in 0.1 M NaCl can give the two peaks at 421 and 403 nm characteristic of the monomer compound, but as the concentration is increased the spectrum shifts to an aggregated complex. There is no isosbestic point for these spectra, indicating that coralyne can form multiple aggregates which differ in spectra. Similar experiments with coralyne at other ionic strengths indicated that isosbestic behavior could be obtained for this concentration region (Figure 3) at ionic strengths only below 0.05. Similar spectral experiments with other intercalating drugs, such as acridine orange,[8-11] actinomycin,[12,13] daunorubicin,[14,15] proflavin,[16,17] ellipticine,[18] quinacrine,[19] and ethidium bromide,[20,21] have illustrated that the initial equilibrium of these dyes, characterized by isosbestic behavior, is a dimerization. At higher concentration, most of these planar aromatic compounds continue to associate to higher aggregates. Dimerization for the ring systems discussed above results in characteristic absorption spectral changes, such as those seen in Figure 3.[8-21]

Over the same concentration range used in Figure 3, propylcoralyne gives isosbestic behavior to nearly 0.1 ionic strength. A typical series of plots, analogous to Figure 3, are shown for propylcoralyne in Figure 4. Coralyne gives a very similar plot in the same buffer as Figure 4. Both give isosbestic points at 433 nm (Figure 4) as part of their initial equilibrium.

As concentration is increased at constant ionic strengths or as ionic strength is increased at constant drug concentration, the spectra shift to the associated form, but for both drugs a point is reached where the isosbestic point is lost and the overlap of the absorption curves shifts to longer wavelengths (cf. Figure 3). Coralyne loses the isosbestic behavior at lower ionic strengths than propylcoralyne. A quantitative analysis of the equilibrium in regions of aggregation where there is no isosbestic point involves many assumptions, and the following discussion will be restricted to the initial equilibrium.

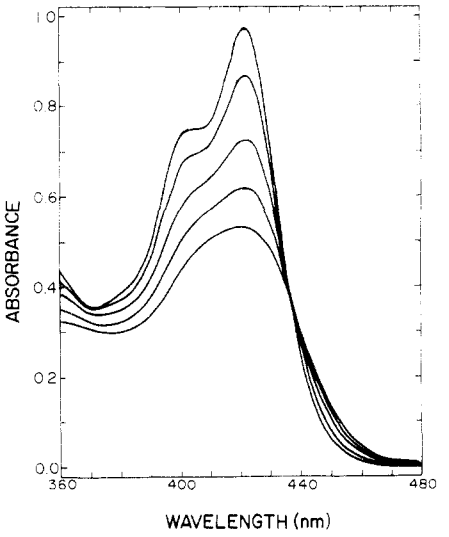

Figure 3. The effect of coralyne (Ia) concentration on its self-association in 0.10 M NaCl (the total ionic strength is 0.12). Coralyne concentrations and absorbance scales from top to bottom in the figure are: 3.52×10^{-7} M, 0.1; 7.04×10^{-7} M, 0.2; 3.95×10^{-6} M, 0.5; 7.89×10^{-6} M, 1.0; 1.57×10^{-5} M, 2.0; respectively. Experiments were conducted in 10-cm quartz cells at 25 °C in a Cary 17D spectrophotometer.

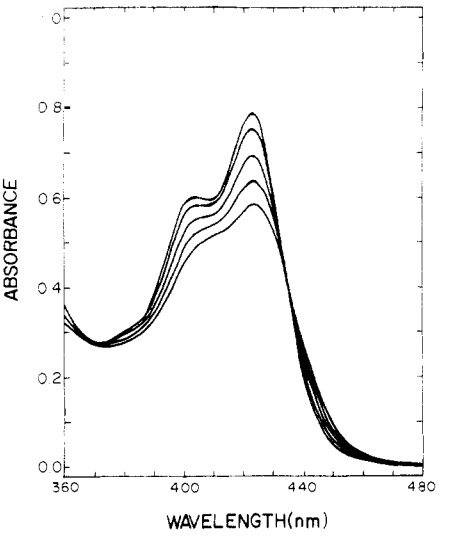

Figure 4. The effect of propylcoralyne (Ic) concentration on its self-association at an ionic strength of 0.02. Propylcoralyne concentrations and absorbance scales from top to bottom are: 5.01×10^{-7} M, 0.1; 1.00×10^{-6} M, 0.2; 2.51×10^{-6} M, 0.5; 4.99×10^{-6} M, 1.0; 9.99×10^{-6} M, 2.0; respectively. There is an isosbectic point at 433 nm. Experiments were conducted as described with Figure 3.

Dimerization Constants. Numerous experiments have been conducted on the self-association of planar aromatic drug molecules (cf. ref 8–21). In all cases where structural information was obtained, the initial equilibrium, characterized by isosbestic behavior, is a dimerization by stacking aromatic rings as might intuitively be expected. Essentially all of these compounds can form even higher aggregates

by continued stacking of aromatic molecular areas as concentration is increased. Proof of dimerization as vs. some more complex equilibria in solution has generally rested on analysis of equilibrium data for consistency with various aggregation models.[8-10,12,14-15,17] Absorption spectral methods show rather characteristic changes for most dimerization reactions, and, in addition, these techniques are used to collect equilibrium data.[8-10,16-17,19] Because of its relatively high molecular weight, low tendency to form higher aggregates, and small spectral shifts on dimerization, actinomycin self-association could be analyzed by equilibrium centrifugation and also fits a dimerization model.[12] Because of the low concentrations at which coralyne loses isosbestic behavior and its pronounced spectral changes on aggregation, it could not be analyzed by molecular-weight methods. We have used spectral experiments such as the one shown in Figure 4 (but with more data points) to test dimerization models for aggregation. For example, using the symbolism of Lamm and Neville[9] for the monomer (M) – dimer (D) equilibrium (eq 1) the

$$2M \rightarrow D \quad (1)$$

total concentration (C) is defined as shown in eq 2 and the

$$C = C_M + 2C_D \quad (2)$$

apparent extinction coefficient is as shown in eq 3, when

$$\epsilon = \alpha \times \epsilon_M + (1 - \alpha)\epsilon_D/2 \quad (3)$$

ϵ_M and ϵ_D are extinction coefficients of the monomer and dimer, respectively, and α is the fraction of the total drug present as monomer. The equilibrium for dimerization (eq 1) will then be represented by eq 4. From experimental

$$K = \frac{(1 - \alpha)}{2\alpha^2 C} \quad (4)$$

values of ϵ and C, K is evaluated using an iterative computer program which first picks an arbitrary K value, calculates α by eq 4, and then determines the root mean square deviation of experimental ϵ values from those predicted by the best straight-line fit to eq 3. The K value is changed and the above process repeated until a minimum in the root mean square deviation is found.[8,9] The K value at this minimum is, then, the experimental dimerization constant. Correlation coefficients calculated from the best linear fit to the dimerization equilibrium were in general greater than 0.99, indicating that this model is consistent with the experimental data in the isosbestic region and that the initial equilibrium is a dimerization.[8,9] Equilibrium dimerization constants for coralyne determined in this manner are 1.1×10^5 in water, 1.5×10^5 in buffer ($I = 0.02$), and 1.6×10^5 in buffer with 0.02 M NaCl ($I = 0.04$). For propylcoralyne, the dimerization constant is 0.9×10^5 in buffer ($I = 0.02$) and 0.5×10^5 in buffer and 0.04 M NaCl ($I = 0.06$). These dimerization constants with coralyne are especially difficult to measure because the compound shows such a strong tendency to associate to higher aggregates (cf. Figure 3) and this compound could not be accurately analyzed above an ionic strength of 0.04.

Once dimerization has been established, spectral methods such as NMR spectroscopy can be used to gain some information about the solution structure of the dimer.[11,13,18] With coralyne and its derivatives, however, the dimerization equilibrium constants are once again too high to allow accurate NMR experiments at the very low concentrations where the monomer–dimer equilibrium predominates.

Association with DNA. The spectral changes which coralyne undergoes when titrated with DNA are shown in Figure 5. The initial spectrum (no DNA) is characteristic of monomer–dimer equilibrium. After adding a small amount of DNA (large coralyne to DNA ratio), the spectrum changes dramatically to resemble that of a higher aggregate of coralyne (cf. Figure 1). As DNA is added, the spectra progressively change again to a limit, at high DNA to coralyne ratios which is the spectrum of coralyne intercalated into DNA.[5] The initial spectrum changes dramatically with changes in salt and drug concentrations (cf. Figures 1 and 3), but the final spectrum is essentially independent of these solution changes as would be expected for the intercalated complex (not shown). The spectrum at the lowest concentration in Figure 1 is approximately 70% monomer (using the dimerization constants given above), and it can be seen comparing this spectrum to Figure 5 that the intercalated spectrum is red shifted relative to the monomer in solution as expected for intercalating molecules.[1,3,4]

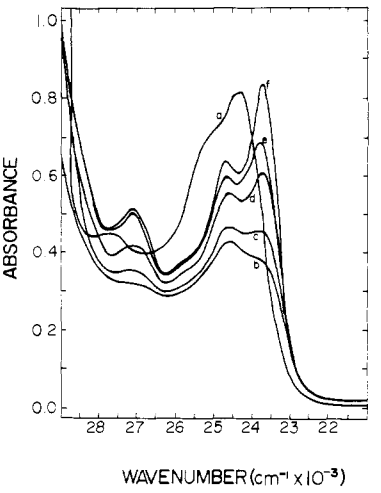

Figure 5. The effect of DNA on the coralyne spectrum. Curve a is coralyne at 8.32×10^{-6} M with no added DNA. Curves b–f are at the same conditions but with increasing DNA concentrations. The ratios of coralyne to DNA nucleotides are as follows: (b) 0.995, (c) 0.332, (d) 0.199, (e) 0.166, and (f) 0.033. Experiments were conducted in 10-cm quartz cells in a Beckman Acta V spectrophotometer at an ionic strength of 0.02.

As would be expected for this complex behavior, no isosbestic points are obtained in titrating either coralyne or propylcoralyne with DNA. Titration of propylcoralyne under identical conditions to Figure 5 (not shown) gives very similar effects for the limit of high DNA concentration (intercalation), but the aggregated spectrum at low DNA concentrations is more similar to a less highly aggregated species, with higher absorbance near 423 nm and a slight peak remaining at 357 nm.

Discussion

Many drugs which bind to DNA are known to dimerize.[8-12] Acridine orange has one of the highest reported dimerization constants, approximately 1×10^4 in water and 2×10^4 in 0.2 M KCl (concentrations measured in molarity).[8,9] Other intercalating compounds, for example, quinacrine ($K \approx 500$ at $I = 0.02$),[19] ellipiticine[18] ($K \approx 10^3$ at $I = 0.05$), ethidium bromide ($K \approx 70$ at $I = 0.1$),[20] proflavin ($K \approx 500$ at $I = 0.01$),[16] and actinomycin ($K \approx 900$ at $I = 0.2$)[12] have much lower dimerization constants. It is striking then to compare the value for the related nitrogen heterocycle coralyne ($K > 10^5$ at $I = 0.02$) to these compounds. The loss of isosbestic behavior for coralyne also indicates that this compound must be present as a higher aggregate at physiological ionic strength even in solutions which are quite dilute (10^{-5} M and above). If coralyne

interacts with its bioreceptor only significantly as a monomer, this extensive aggregation must lower its effective concentration and activity. This could account in part for the relatively high dose level required for coralyne to reach significant activity.[6]

Propylcoralyne dimerizes slightly less than coralyne ($K \approx 10^5$ at $I = 0.02$) and does not seem to have the marked tendency that coralyne has to form higher aggregates. Analysis of Corey–Pauling–Kolthum molecular models reveals that coralyne can exist as an essentially flat molecule with the methoxy groups rotated into the plane of the aromatic ring system. The propyl group of Ic, because of steric hinderance, cannot be rotated into the plane of the aromatic ring system. This bulky group apparently does not greatly hamper dimerization but does inhibit higher aggregation. This could not occur if the propyl groups (and positive charges) point in opposite direction in the dimer. In higher aggregates, however, at least two propyl groups must point in the same direction, giving the potential for unfavorable steric interactions if the hydrophobic interactions are to be maximized, as the large dimerization constant would suggest. Propylcoralyne will form higher aggregates but only at higher concentration and/or higher ionic strength than coralyne.

NMR experiments on acridine orange,[11] actinomycin,[13] and ellipticine[18] have indicated that the "inverted stack" type of association is favored by these molecules and may be a general feature of self-association of planar aromatic compounds. This type complex with acridine orange, ellipticine, and coralyne would be favored because it allows maximum overlap of the aromatic ring systems with minimum charge repulsion.

It is difficult to see how these results relate to the dramatically different activity of coralyne and propylcoralyne. The propyl compound does undergo higher aggregation less well than coralyne, but the ethyl derivative Ib is closer to Ic than Ia in this respect (not shown). In the same manner, Ia and Ic do not show any striking differences in their interaction with DNA. The aggregation of these compounds can lead to their precipitation under physiological conditions, and this has been reported to occur after interperitoneal injection of coralyne in mice.[14] Even here, however, we have qualitatively not observed any dramatic precipitation differences among Ia, Ib, and Ic. The dramatic differences, between the closely related Ib and Ic, in in vivo activity would seem to require a bioreceptor with higher selectivity than apparently exists in DNA for these two compounds.

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Potential Antiinflammatory Compounds. 2.[1a] Acidic Antiinflammatory 1,2-Benzisoxazoles

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A number of 1,2-benzisoxazoles, substituted in the 3 position with 4-substituted phenyl groups and in the 5–7 positions with acetic and propionic acid residues, have been synthesized and tested in the rat carrageenan foot edema assay. Activity has been found in the 6- and 7-substituted acids.

The leads provided by indomethacin and ibufenac in the nonsteroidal antiinflammatory field have stimulated workers to synthesize considerable numbers of substituted acetic and propionic acids.[1b] These compounds have had nuclei chosen from a wide range of substituted aromatic and heterocyclic systems. Among the more significant systems which have been exploited in the last few years are 1-phenylnaphthalene[2] and 2-phenylbenzoxazole.[3]

In seeking a molecule which combined, as nearly as possible, the structural features of the 1-phenylnaphthalenes and the 2-phenylbenzoxazoles, we decided to investigate the effect on biological activity of placing

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