Structure of Mutagen Nucleic Acid Complexes in Solution. Proton Chemical Shifts in 9-Aminoacridine Complexes with dG-dC, dC-dG, and dA-dT-dG-dC-dA-dT†

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ABSTRACT: The influence of self-complementary oligodeoxynucleotides on the chemical shifts of protons of the mutagenic acridine dye 9-aminoacridine has been measured. Upfield shifts indicative of intercalative binding are found in the cases of dG-dC, dC-dG, and dA-dT-dG-dC-dA-dT but not in dA-dT. Geometries for the complexes that are compatible with the chemical-shift data and the X-ray structure of the complex between ri^{5}C-rG and 9-aminoacridine determined by Sakore et al. [Sakore, T. D., Jain, S. C., Tsai, C., and Sobell, H. M. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 188–192] can be identified using recent theoretical estimates of shifts induced by nucleotide bases.

The dye 9-aminoacridine (9AA) is one of a large class of acridines that are mutagenic (Crick et al., 1961) and that interact with DNA in vitro (see Bloomfield et al., 1974, for a review). In general, physical studies have shown that the binding of acridines to DNA can occur through several distinct modes. The tightest binding mode has been interpreted structurally in terms of an intercalation complex (Lerman, 1961, 1963), in which the planar ring system is sandwiched between adjacent parallel base pairs of the DNA double helix. This model rationalizes the biological activity of certain acridines which function as potent frameshift mutagens (Crick et al., 1961; Ames et al., 1973) with the ability of these molecules to alter the apparent length of helical DNA (Cohen and Eisenberg, 1969) and the superhelix density of closed covalent circular DNA (Bauer and Vinograd, 1970; Waring, 1970).

The most direct evidence for intercalation stems from X-ray crystallographic structure determinations on complexes between ribodinucleoside monophosphates and acridines (Tsai et al., 1975; Seeman et al., 1975; Sakore et al., 1977). These reveal some molecular details of the process. For example, the structure of the 2:2 9AA:i^{5}CpG complex is found to exhibit two different kinds of interaction between the acridine and the complementary i^{5}C·G base-paired minihelix.[1] One involves a pseudosymmetric stacking between 9AA within two i^{5}C·G pairs, the other being an asymmetric association predominantly involving the G rings (see Figure 5A,B). The structure of the 9AA–ApU complex indicates only a single mode of stacking between the acridine and bases from different ApU molecules in the crystal.

On the basis of absorbance, CD, and magnetic resonance data (see Reinhardt and Krugh, 1977, and the references therein) it has been suggested that complementary dinucleoside monophosphates and dinucleotides can bind certain molecules to form miniature analogues of intercalation complexes in solution also. Detailed studies on complexation of actinomycin D (Patel, 1974; Kurgh et al., 1977, and the references therein) and ethidium bromide (Reinhardt and Krugh, 1977) have been reported.

In order to elucidate the structures of intercalation complexes between 9AA and various nucleic acid sequences in solution, we have measured the chemical shifts induced in the proton NMR spectrum of 9AA as a function of the concentration of the self-complementary deoxyribosyl dinucleotides d-GpC, d-CpG, and d-ApT, as well as the hexanucleotide d-ATGCAT. We present here a comparison of the experimental complex formation shifts with values estimated according to recent theoretical calculations of the shifts induced by nucleobases (Giessner-Prettre and Pullman, 1976) in conjunction with structural models based on that of the 9AA:i^{5}CpG crystalline complex (Sakore et al., 1977). In this way, a limited class of structures compatible with the shift data can be discriminated.

Materials and Methods

The deoxydinucleotides and deoxyhexanucleotide were obtained from Collaborative Research, Inc., and were used without further treatment. 9-Aminoacridine hydrochloride, a product of Sigma Chemical Co., was lyophilized from D_2O. Concentrations of stock solutions were determined spectrophotometrically and then diluted volumetrically. The pH was adjusted to a meter reading of 7.5 using NaOD or DCl. The methyl signal of a small amount (ca. 0.3 mM) of *tert*-butyl alcohol served as an internal standard for chemical-shift referencing.

Proton magnetic resonance spectra were recorded with a Varian 220-MHz spectrometer operating in the Fourier transform mode. Depending on the concentration of the compound of interest, between 10 and 100 transients were accumulated. The peak separation from the reference signal was measured on a precalibrated chart supplied by the instrument manufacturer with a precision of ±2 Hz.

Models of intercalation complexes were constructed by superposing scale drawings of the relevant molecules. Purines and pyrimidines were drawn along with the isoshift lines that include both ring current and anisotropy contributions at a perpendicular distance of 3.4 Å to the base planes (Giessner-Prettre and Pullman, 1976). The acridine C–H bond length was taken to be 1.084 Å. Theoretical proton shifts for 9-ami-

† From the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received August 25, 1977; revised manuscript received February 27, 1978.* This investigation was supported in part by Grant NP-187 from the American Cancer Society.

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[1] Note that this is a ribosyl derivative.

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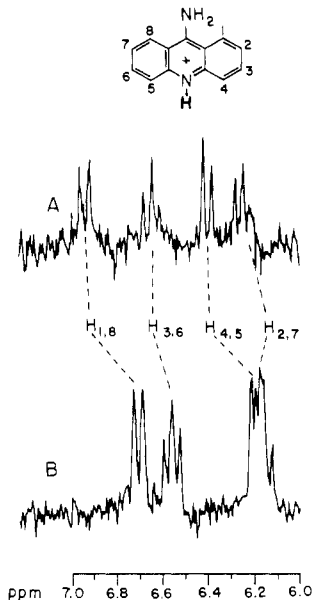


FIGURE 1: Structure and proton NMR spectrum of 9-aminoacridine at two different concentrations in D_2O, pD 7.5, T = 18 °C: (A) 0.3 mM; (B) 1.5 mM. Shifts are measured downfield from tert-butyl alcohol.

TABLE I: Chemical Shifts of 9-Aminoacridine Extrapolated to Zero Concentration.

Proton	δ, ppm[a]
1, 8	6.95
3, 6	6.67
4, 5	6.42
2, 7	6.25

[a] Downfield from *tert*-butyl alcohol.

noacridine were estimated for a fixed orientation of the dye molecule relative to the bases by linear interpolation along a radius vector drawn from the point corresponding to the shift maximum in the given plane, neglecting contributions beyond the line of zero shift. Systematic and random errors associated with this procedure are estimated to be less than 15% of a given shift value.

Results

Self-Association of 9-Aminoacridine. Proton spectra of 9-aminoacridine along with the numbering scheme are shown in Figure 1. The resonance assignments are according to Acheson and Harvey (1976). As seen, there is a strong concentration dependence of the chemical shifts resulting from self-association. A linear concentration dependence of the shifts was observed for concentrations below 2 mM. The chemical-shift values extrapolated to zero concentration are summarized in Table I. These values are assumed to represent the unassociated 9-aminoacridine molecule in aqeuous solution and will be used as a reference point for comparison with the shifts observed upon interaction with the di- and hexanucleotides.

Interaction of 9-Aminoacridine with d-CpG and d-GpC. The effects of increasing concentrations of d-GpC or d-CpG on the shifts of 9-aminoacridine in a 2 mM solution of the dye are shown in Figure 2A,B. Interaction with the dinucleotide results in upfield shifts in the limit of fast exchange. The formation of a strong 1:2 9-aminoacridine–dinucleotide complex is indicated by the sharp breaks in the titration curves at a dinucleotide concentration corresponding to this stoichiometry. The shifts remain roughly constant at higher dinucleotide

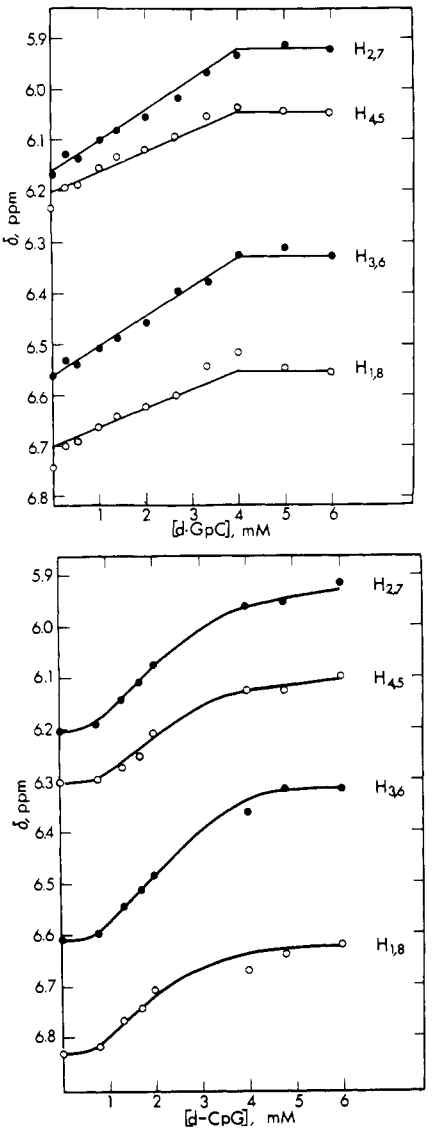


FIGURE 2: Titration of 2.0 mM 9-aminoacridine with dinucleotide in D_2O, pD 7.5, T = 18 °C: (A, top) dGpC; (B, bottom) dCpG. Shifts are measured downfield from *tert*-butyl alcohol.

concentrations. These results suggest the formation of an intercalation complex with a dye molecule inserted between the base pairs of a miniature helix formed between two self-complementary dinucleotide molecules (Patel 1974; Krugh et al., 1977).

The shifts of the dinucleotide protons were also examined. The reciprocal titration was carried out, varying the 9AA concentration with fixed dinucleotide. In this case, it can be seen from Figure 3 that upfield shifts continue to increase beyond the stoichiometry observed in Figure 2. This probably represents other binding modes of the dye, or higher complex formation involving additional dye molecules, free or associated. The shifts of the 9-aminoacridine protons at low dinucleotide concentrations are likely to contain contributions from such stacked complexes as well as from self-association of dye molecules. In view of the complexity of this system, attempts to analyze the results in terms of the equilibria involved were abandoned.

The limiting shift values observed for the 9-aminoacridine protons at high dinucleotide concentrations are taken as representative of the intercalation complex. The net shifts due to intercalation, i.e., the difference between the shifts at infinite dilution and those of the 1:2 complex with d-GpC, are sum-

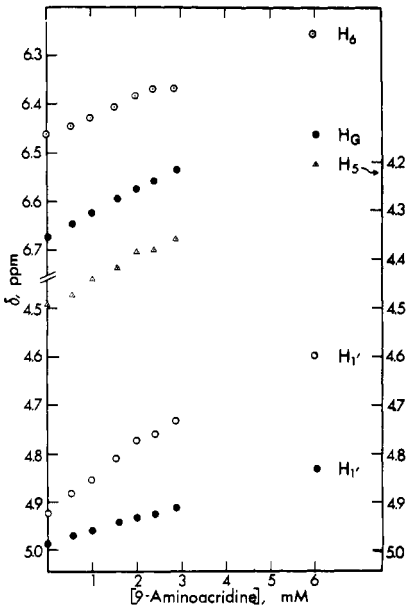


FIGURE 3: Titration of 2.0 mM dGpC with 9-aminoacridine in D_2O, pD 7.5, T = 18 °C.

TABLE II: Upfield Shifts[a] of 9-Aminoacridine Induced by Intercalation with Oligonucleotides.

Oligonucleotide	Temp, °C	Δδ1,8	Δδ3,6	Δδ4,5	Δδ2,7
d-CpG	18	0.33	0.35	0.32	0.34
d-GpC	18	0.40	0.34	0.37	0.34
d-GpC	3.5	0.64	0.56	0.61	0.56
d-ApTpGpCpApT	18	0.71	0.79	0.69	0.84

[a] In ppm.

marized in Table II. Also given in Table II are the results obtained with d-GpC at 3.5 °C and with d-CpG at 18 °C. The latter produced concentration effects similar to those observed with d-GpC (cf. Figure 2). Note that larger complex formation shifts are observed at the lower temperature. Finally, a similar titration of d-ApT with 9AA led to results indicative of no strong interaction or intercalation.

Interaction of 9-Aminoacridine with d-ApTpGpCApT. The results of the titration of a 1.7 mM 9-aminoacridine solution with the self-complementary hexanucleotide are shown in Figure 4. The break in the curves is observed at a concentration approximately corresponding to a 3:2 dye–hexanucleotide stoichiometry. Data points at lower hexanucleotide concentrations could not be collected due to precipitation. Complexes of higher dye–hexanucleotide stoichiometry are apparently closer to electroneutrality and thus likely to be insoluble. The net complex formation shifts are given in the last row of Table II. Note that these values exceed the low-temperature dinucleotide shifts.

Structural Models and Calculated Shifts. Theoretical shift values were computed (see Materials and Methods) for the four representative configurations illustrated in Figure 5. Structures A and B are respectively the pseudosymmetric and asymmetric intercalative modes found in crystals of 9-aminoacridine complexes with a related ribodinucleotide by Sakore et al. (1977). Structures C and D represent classes of trial configurations. The results are summarized in Table III. One entry in Table III was computed with the isoshift lines from ring currents only (Giessner-Prettre and Pullman, 1970). Note

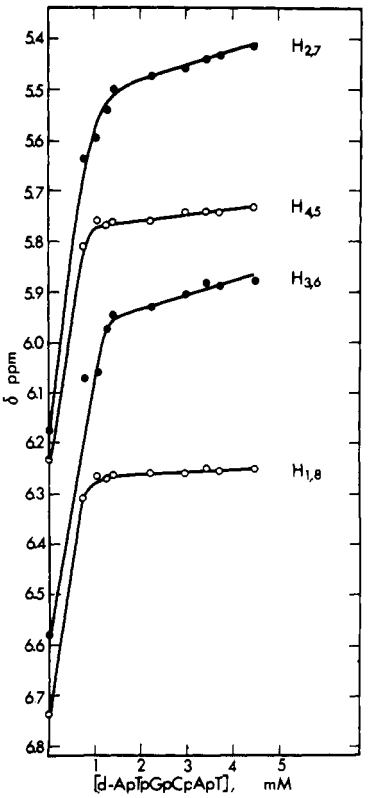


FIGURE 4: Titration of 1.7 mM 9-aminoacridine with the hexanucleotide d-ATGCAT in D_2O, pD 7.5, T = 18 °C.

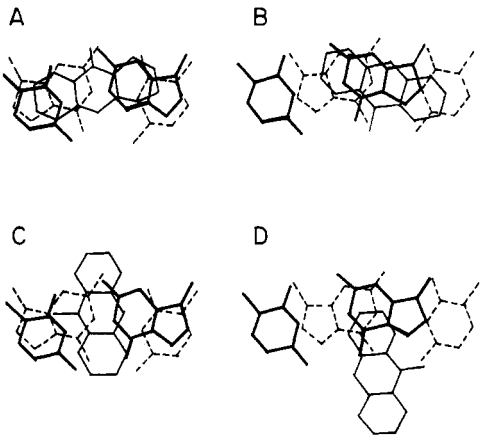


FIGURE 5: Scale drawings of four possible complexes of a dinucleotide with 9-aminoacridine, based on the crystal structures of Sakore et al. (1977).

these latter values are lower than the others. The following implicit assumptions in the shift calculations should be emphasized: (1) the computed values correspond to a static structure in solution; (2) molecular planes were taken to be parallel with interplanar distances of 3.4 Å; (3) for the hexanucleotide T was replaced with U and next nearest contributions were neglected. Table III includes entries for specific 3-site and random 3-site shift averages in accordance with the observed 3:2 dye–hexanucleotide stoichiometry.

Discussion

The pattern of chemical-shift changes induced in proton resonances of 9AA upon adding d-GpC or the self-complementary hexanucleotide d-ATGCAT suggest formation of complexes with defined stoichiometry. There is no such indi-

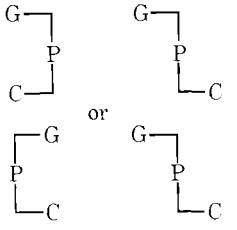
TABLE III: Theoretical Upfield Shifts of 9AA Induced by Intercalation with Oligonucleotides.[a]

Structure[b]	Comments	Δδ1,8	Δδ3,6	Δδ4,5	Δδ2,7
GpC					
A		0.58	1.24	0.78	0.81
A	Ring current only	0.28	0.65	0.31	0.37
B		0.67	0.60	0.56	0.84
C		0.75	0.21	0.75	0.23
D	Good fit	0.66	0.68	0.64	0.62
d-ApTpGpCpApT					
A					
1	AT·TA site	0.79	1.23	0.85	0.93
2	TA·GC site*	0.65	1.27	0.86	0.87
3	GC·CG site	0.60	1.30	0.80	0.79
4	CG·AT site*	0.74	1.30	0.79	0.87
5	AT·TA site		same as **1**		
(**2** + **3** + **4**)/3	3 sites	0.66	1.27	0.82	0.84
Random site selection	3 sites				
B					
1		0.87	0.63	0.70	0.90
2		0.76	0.65	0.71	0.83
3		0.67	0.60	0.56	0.84
4		0.80	0.62	0.62	0.88
5			same as **1**		
(**2** + **3** + **4**)/3	Good fit	0.74	0.63	0.63	0.85
Random site selection		0.80	0.63	0.66	0.87
D					
1		0.83	1.16	0.86	1.06
2		0.79	0.87	0.62	0.92
3		0.66	0.68	0.64	0.62
4		0.70	0.97	0.89	0.76
5			same as **1**		
(**2** + **3** + **4**)/3	Good fit	0.72	0.84	0.72	0.77

[a] Entries with an asterisk correspond to two different directions of the $HN-NH_2$ vector in 9AA in the site. By symmetry **2** and **3** should be equivalent. [b] See Figure 5.

cation in the case of d-CpG, on the other hand. In the former cases, we cannot determine the structure of any complex uniquely on the basis of these data. All the shifts observed in this study correspond to a fast-exchange process between free and bound dye in the first place. Hence, we detect a time average that includes contributions from several different complexes if these are present. We can nevertheless rule out a number of possible structures and show that fully or partially intercalated models are consistent with both our data and available information on base pairing in complementary oligonucleotides.

Consider first the case of the 1:2 complex between 9AA and d-GpC. A number of structures can be invoked to explain the upfield chemical shifts of protons both in 9AA and the bases (Figure 2A). However, the critical nucleation size for G·C base pairs is close to two, so that nucleation of GpC molecules by 9AA should make structures in which there is no provision for base pairing, for example,



less likely than those such as are illustrated in Figure 5, which do. The relative concentratons of noncomplementing forms should be below 10^{-4} of the paired forms if each GC nucleated pair has $\Delta G^\circ = -3$ kcal/mol at 25 °C, for example (see Kallenbach and Berman, 1977). For this reason, we confine our attention to the models in Figure 5. It should be noted that either kind of structure would be consistent with the increased chemical shifts seen at lower temperature, since both stacking and pairing of bases exhibit large negative enthalpies of formation. One would then expect the population of bound molecules to increase as T decreases, yielding larger values of the shifts observed in the rapid-exchange limit seen here. More detailed investigation of the chemical shifts of the 9AA–2d-GpC complexes with temperature will be presented elsewhere. At present we cannot distinguish between the alternative explanations of (1) an increased population of complexed form(s) at low temperature or (2) change in structure of the complex(es) with changing temperature. In the case of an all-or-none process (Davanloo and Crothers, 1976) one would expect the ratios of induced shifts for different 9AA or dinucleotide protons to remain constant. This seems to be the case for the 9AA–d-GpC interaction. However, this is not necessarily true generally, because a mixture of states could be present favoring different structures at different temperatures, for example, or there could be more complicated kinetic mechanisms involved in forming the complex. Again, the fast-exchange process observed fails to provide a basis for discrimination.

Comparison of the experimentally observed shifts for d-GpC with those estimated using theoretically calculated values for the shifts due to bases in conjunction with the set of trial structures (Table III) reveals a number of interesting features. First, only one model (Figure 5B) corresponding to a structure observed in a related crystalline system (albeit with i^5C substituted for C) and in a ribonucleotide gives reasonable agreement with the observed shifts. Second, a trial structure

involving only partial insertion of the 9AA (Figure 5D) also yields reasonable consistency with the experiment, so that there is no basis to claim that the proton chemical-shift data alone favor any particular model. Nor, in fact, can it be stated that the observed results do not represent an average of both types, or possibly more, appropriately weighted. Third, of the various theoretical chemical-shift values that have been assigned to the nucleobases (Giessner-Prettre and Pulman, 1970) only values that include "local" contributions to the diamagnetic anisotropies (Giessner-Prettre and Pullman, 1976) seem capable of providing agreement with experiment. In the absence of the latter contributions, the predicted results including only ring-current effects (Giessner-Prettre and Pullman, 1970) are invariably too low.

The case of the hexanucleotide interaction with 9AA is qualitatively similar. In this case, however, there are a total of five potential intercalation sites available, and the self-complementarity of the hexanucleotide can lead to formation (at 25 °C) of a helical duplex in the absence of 9AA even in conditions of low ionic strength (Patel, 1975; Patel and Tonelli, 1975; Patel and Hilbers, 1975; Hilbers and Patel, 1975). In this oligonucleotide, duplex formation is strongly concentration dependent as well as sensitive to available counterions. Interaction with 9AA thus favors the duplex because of electrostatic effects. Binding is clearly tighter in this case (Figure 4). The limiting stoichiometry observed is three molecules of 9AA per duplex. This may be due to next-neighbor exclusion (Armstrong et al., 1970) of the sites adjacent to the central GpC/CpG one; on the other hand, d-ApT alone represents a poor interaction site for 9AA because of the lack of strong interaction observed. As in the case of 9AA + $(d\text{-}GpC)_2$, calculated values of the models corresponding to the structures B and D in Figure 5 are consistent with the observed shifts. Yet it can be seen in Table II that the shifts measured in the hexanucleotide case differ from those in d-GpC by 0.08 ppm to 0.28 ppm. Such effects could arise if there are substantial next-nearest-neighbor contributions to the chemical shift of nucleobases (Arter and Schmidt, 1976).

We have presented an investigation of the chemical shifts of dye protons in the case of interactions between 9AA and several self-complementary nucleotide sequences. It is evident from our data that there is a striking selectivity in this process, as shown by the different behavior of d-GpC and d-CpG. Such selectivity is well known from the example of actinomycin D (Jain and Sobell, 1972; Sobell and Jain, 1972). However, 9AA lacks the peptide rings of the latter molecule, which enable it to form specific hydrogen bonds to the bases (Jain and Sobell, 1972; Sobell et al., 1972). Frameshift reversion data (Ames et al., 1973) suggest that 9AA appears capable of a high degree of selectivity in vivo as well. The nature of this specificity and the forces responsible for it are of considerable interest. So far we have been unable to define precisely the structure of the strongly bound states of 9AA with d-GpC and d-ATGCAT, although we find that intercalated or partially intercalated models can yield shifts consistent with those observed on the basis of a recent theoretical estimate of the shielding due to nucleobases. Whether these models are actually present, or whether the theory is adequate, can be tested using this system. An important element in any such test is to attempt to establish conditions of slow exchange between complexed and free dye. This work is in progress.

Acknowledgment

The authors thank Rong-Ine Ma for her expert technical assistance.

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