Several synthetic DNAs were prepared containing the unusual bases 7-deazaadenine (c'A) and 7-deazaguanine (c'G). As judged from changes in melting temperatures these modified DNAs bound ethidium to a similar extent as the parent polymers. However, duplexes such as poly[d(7dG)]·poly[d(CA)] and poly[d(TC)]·poly[dc'GA)] gave no enhancement of ethidium fluorescence in a standard ethidium fluorescence assay. Fluorescence spectra in the range 400–650 nm showed that ethidium bound to poly[d(TC)]·poly[d(Gc7A)] gave 70% of the fluorescence of the parent polymer poly[d(TC)]·poly[d(GA)], whereas the fluorescence of poly[d(TC)]·poly[dc'GA)] was essentially 0%. Even the intrinsic fluorescence of ethidium in solution was quenched in the presence of poly[d(TC)]·poly[d(c'GA)]. Binding constants were estimated from Scatchard analysis and were 4.8, 3.4, and 2.0 × 10^6 M^{-1} for poly[d(TC)]·poly[d(GA)], poly[d(TC)]·poly[d(Gc7A)], and poly[d(TC)]·poly[d(c'GA)], respectively. This reduction in binding constant cannot account for the loss of fluorescence. The UV spectrum of ethidium was measured in the presence of these DNAs, and some significant differences were noted. Presumably the presence of 7-deazaguanine alters the electronic structure of bound ethidium so that it can no longer fluoresce.

The molecular basis for the fluorescence enhancement has been postulated to be due to an increase in the energy separation between a triplet and excited singlet states (13). There is also evidence that when ethidium is intercalated there is a reduction in the rate of transfer of protons from the excited state to solvent molecules (14). Thus the lifetime of the excited state is increased giving rise to enhanced fluorescence. This model is attractive since proton acceptors quench the fluorescence, whereas in the presence of D2O it is enhanced (14). Presumably the ethidium is less accessible to solvent in the intercalated state; similar reasoning may also explain why the triplex poly(dT)·poly(dA)-poly(dT) gives greater fluorescence enhancement than the duplex poly(dA)-poly(dT) (11).

Recently we have prepared several synthetic DNAs containing the unusual bases 7-deazaadenine (c'A)1 and 7-deazaguanine (c'G), the idea being that these polymers should be unable to form triplexes because Hoogsteen hydrogen bonds would be blocked (15–17). In light of the extensive literature on the properties of ethidium, it came as somewhat of a shock to discover that duplexes containing c'G no longer enhanced ethidium fluorescence.

**Materials and Methods**

**RESULTS AND DISCUSSION**

Synthetic DNAs containing c'A and c'G were originally prepared because these modified bases should prevent triplex formation. This prediction has indeed been confirmed.2 The Tm values of these polymers are listed in Table I, and in general the presence of either c'A or c'G lowers the Tm compared with the parent polymer. One exception is poly[d(7dG)]·poly[d(CA)], whose Tm is indistinguishable from poly[d(TG)]·poly[d(CA)], which tends to confirm that stacking interactions are sequence-dependent. Previous reports (21) also showed that duplexes containing all c'G were destabilized, but contrary to the data in Table I it was reported that the oligomers (d(c'AT))n and (d(c'AT))n had increased Tm values compared with the parent duplexes (22). A most surprising observation was that incorporation of c'A and c'G led to large changes in the fluorescence intensity. Indeed for most polymers containing c'G no fluorescence could be detected at all (Table I). Fluorescence spectra of ethidium bound to the three polymers related to poly[d(TC)]·poly[d(GA)] are shown in Fig. 1. For poly[d(TC)]·poly[dc'GA)] no fluorescence could be observed over the range of 400–650 nm with any

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1 The abbreviations used are: c'A, 7-deazaadenine; c'G, 7-deazaguanine.

2 Portions of this paper (including “Materials and Methods,” part of “Results,” and Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

3 L. J. P. Latimer and J. S. Lee, unpublished results.
triplet intersystem crossing is enhanced and the fluorescence of poly[d(Gc7A)]. It is tempting to suggest that this change in the duplexes containing deazapurines, but simple calculations show that the level of ethidium binding under the conditions of 10 μl of the DNA melting at 260 nm.

For the poly[d(TC)].poly[d(c7GA)] family, accurate binding parameters were measured from Scatchard plots as shown in Fig. 3. The binding constant, K, is significantly lower for both the duplexes containing deazapurines, but simple calculations show that the level of ethidium binding under the conditions of 10 μl of the DNA melting at 260 nm.

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combination of excitation or emission wavelengths. In other words the fluorescence of ethidium is actually being quenched. It was considered possible that these polymers had altered conformations which no longer bound ethidium. This was most easily tested by measuring Tm values in the presence of the drug. As shown in Fig. 2 ethidium increases the Tm of poly[d(TC)].poly[d(GA)] and its analogues to a similar extent. This was also found to be the case with the other deazapurine-containing DNAs (data not shown). Thus ethidium certainly binds to these DNAs, although it is difficult to estimate binding constants from Tm data. There is also no evidence that these polymers readily adopt non-B conformations; for example there is no dramatic change in Tm upon changing the pH and the UV spectra have 260:285 absorption maxima for ethidium bound to poly[d(TC)].poly[d(c7GA)]. It seemed possible that the electronic structure of the drug might be altered, in which case the UV spectrum would also show changes. As shown in Fig. 4 there is some evidence to support this idea, since there is a significant change in the absorption maximum for ethidium bound to poly[d(TC)].poly[d(Gc7A)]. It is tempting to suggest that this change in the absorption maximum of bound ethidium leads to a reduction in the singlet-triplet energy separation so that singlet to triplet intersystem crossing is enhanced and the fluorescence is quenched (13). The possibility that c7G somehow accelerates proton transfer from excited state ethidium to solvent appears unlikely, since the pKₐ for c7G is actually higher than for guanine itself (10.3 compared with pKₐ values of 4.5 and 9.3 for guanine) (21). In this regard the significant fluorescence given by poly[d(TTC)].poly[d(c7GAA)] is important. Possibly some ethidium is intercalating between the two T-A base pairs, and in this position the quenching by c7G is ineffective. In other words the ethidium must be stacked against and in electronic contact with c7G for quenching to occur. The possibility was also considered that ethidium was becoming attached to c7G through a photochemical reaction. Tm measurements showed that even after irradiation at 525 nm, ethidium could transfer from poly[d(TC)].poly[d(c7GA)] to other DNAs. Thus there is no evidence for the covalent binding of ethidium.

These results demonstrate that even a drug such as ethidium, which has been studied intensively for several decades, still harbors some secrets. The quenching of fluorescence by c7G was most unexpected but serves as a reminder that optical properties are distinctly sensitive to the environment of the chromophore.

### REFERENCES

Supplementary Material

Fluorescence Quenching of Ethidium by 7-Deazaguanine

L.J.P. Latimer and J.S. Lee

Materials and methods

Synthetic DNA. Duplex DNA was prepared by replication of an appropriate template with E. coli or M. leuconos DNA polymerase I as described previously (18). In general the dXTPs were added at 1 mM except for the duplexes which contained dGTP, which were used at 0.2 mM to eliminate this resource. The sequence of the modified DNAs were checked by using them as a template to reproduce the original parent polymer whose Tm could be compared with an authentic sample (19). Unmodified polymers could also be analyzed by their ability to form triple helices since this is only a property of poly(d(TC))poly(d(GA)).

Fluorescence. All fluorescence measurements were performed on a Turner model 420 spectrophotometer in a buffer of 10mM Tris- HCl pH 8.0, 0.1 mM EDTA and 50mM NaCl.

Results

Figure 1. Fluorescence excitation spectra for free Ethidium and for Ethidium bound to DNApoly(dTCl)poly(dGAc) family. --- free Ethidium; =poly(dTCl)poly(dGAc) gene no fluorescence. In all cases the excitation wavelength was set at 500nm and the Ethidium concentration was 0.1µg/ml (20mg/ml in the standard buffer). The fluorescence was excited at 495nm in excess so that excites the Ethidium was bound.

Figure 2. Melting curves for DNAs of the poly(dTCl)poly(dGAc) family of duplex DNA with 0% dye and with 0.2 µg/ml of Ethidium. The buffer was 10mM Tris-HCl pH 8.0 with 0.1 mM EDTA and 50mM NaCl.

Figure 3. Scatchard plot for the binding of Ethidium to DNAs of the poly(dTCl)poly(dGAc) family. See methods section for details. The insert shows the computed value of K, the intrinsic association constant, and n, the number of base-pairs occupied by the binding of one drug molecule.

Figure 4. UV absorption spectra for Ethidium alone (1 µM) and in the presence of an excess of DNAs. The Mw of the poly(dTCl)poly(dGAc) family. --- free Ethidium; poly(dTCl)poly(dGAc) family, ---poly(dTCl)poly(dGAc)Excess; poly(dTCl)poly(dGAc)Excess.

Under these conditions greater than 95% of the Ethidium is bound to the DNA. The spectrum for poly(dTCl)poly(dGAc)Excess is similar to that for calf thymus DNA and compared to unbound dye has an absorbance at 510 nm greater than 95% of the maximum from 480nm to 520nm and a 30% decrease in the extinction coefficient (1). For poly(dTCl)poly(dGAc)Excess the effect is a further reduction in the extinction coefficient with little change in the maximum while for poly(dTCl)poly(dGAc)Excess the maximum occurs at 600nm.

