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$^{155}Eu^{3+}$ as a Probe of Metal Ion and Cationic Drug Binding Sites on Native and Heat-denatured DNA

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Abstract

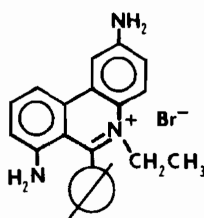
A flow-dialysis apparatus suitable for the study of high affinity metal ion binding sites in macromolecules has been utilized to study $^{155}Eu^{3+}$ exchange processes, as a function of pH, in both 'native' and 'heat-denatured' DNA. 'Free exchange' of $^{155}Eu^{3+}$ was found to occur at a significantly faster rate at pH = 7.0 than at pH = 6.0 for both forms of DNA; while non-radioactive Eu^{3+}-induced 'displacement' of bound $^{155}Eu^{3+}$ occurred at a significantly faster rate at pH = 6.0 than at pH = 7.0 for both species of DNA. These results are consistent with a greater 'entropic' driving force for metal ion:DNA complexation at the lower pH value. The effect of ethidium bromide on $^{155}Eu^{3+}$ exchange was also examined as a function of pH. The intercalating agent was found to accelerate $^{155}Eu^{3+}$ displacement at pH = 6.0 and decelerate displacement at pH = 7.0. All three sets of experiments (*i.e.*, free-exchange of bound $^{155}Eu^{3+}$, Eu^{3+}-induced displacement of bound $^{155}Eu^{3+}$ and ethidium ion-induced displacement of bound $^{155}Eu^{3+}$) indicate that the $^{155}Eu^{3+}$ ion can serve as a useful probe of metal ion and drug binding sites in nucleic acid polymers, and constitutes a particularly sensitive probe at pH = 6.0.

Introduction

Since Manning [1] introduced the 'polyelectrolyte theory' of cation binding to DNA, there have been many conflicting opinions concerning the relative importance of the different modes of metal ion interactions with the DNA helical structure [2–5]. Basically, four types of interactions are thought to exist: (1) direct coordination of the metal ion to a phosphate group or groups, (2) unrestricted movement of hydrated metal ion complexes along the phosphate backbone, (3) metal ion interactions with basic sites located on heterocyclic bases, and (4) diffuse 'atmospheric' associations. It may well be that all four types of metal ion-DNA interactions contribute to the observed unusual stability of the DNA helix.

Several trivalent lanthanide ion species have been successfully employed in numerous studies of cation binding to biological macromolecules [6–9]. For example, both Tb^{3+} and Eu^{3+} have been employed as fluorescent probes of alkaline earth binding sites in DNA [9]. We have used the radioactive forms of these same ions (*i.e.*, $^{160}Tb^{3+}$ and $^{155}Eu^{3+}$) to specifically probe calcium ion binding sites in a number of proteins, including carp muscle calcium binding parvalbumin [10, 11], bovine brain calmodulin [12, 13] and the acetylcholine receptor protein from both *Torpedo californica* and *Drosophila melanogaster* [14, 15]. In this paper we report on the use of $^{155}Eu^{3+}$ to probe cation binding sites on both native and heat-denatured DNA, in the presence and absence of the DNA-binding drug ethidium bromide.

The cationic dye, ethidium bromide (2,7-diamino-5-ethyl-6-phenyl phenanthridium bromide), belongs



Ethidium Bromide

a class of chemical agents which bind to DNA and inhibit function [16–18]. Interactions between ethidium bromide and DNA molecules are well documented [19–22], and these studies have shown that ethidium bromide interacts with DNA primarily through the intercalation of the planar phenanthridinium ring system between stacked base pairs of the DNA helix.

The rationale for employing an intercalating cationic drug such as ethidium bromide in a study of metal ion binding sites in DNA is as follows: the binding of the cationic drug, either to the negatively

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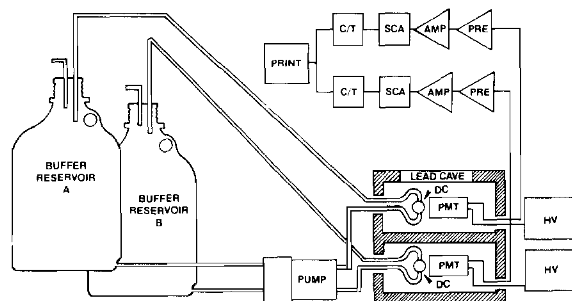
charged outer (cylinder) phosphate groups of DNA by simple electrostatic forces or between the base pairs of DNA by inter-calative associations, should have a dramatic effect on the binding of cationic radionuclides to electronegative sites on the DNA. In addition, these DNA–drug associations should be sensitive to the pH of the buffer used to examine radionuclide exchange as DNA is a polyelectrolyte with many ionizable groups. Finally, by performing experiments on native as well as heat-denatured DNA it might be possible to discriminate between ethidium bromide-induced displacement from the outer phosphate 'shell' *versus* the electro-negative centers on various bases in the central core of DNA, since the extent of base stacking should be greatly diminished in the heat-denatured DNA, thus precluding extensive ethidium bromide intercalation.

The results presented in this paper represent the first application of our flow dialysis/gamma ray nuclear counting apparatus to the problem of metal ion binding to DNA biopolymers. Employing this apparatus radionuclide displacement (from DNA) is followed continuously by monitoring the gamma ray emission from $^{155}Eu^{3+}$-labeled DNA as 'chase buffer' flows through the sample chamber of the dialysis apparatus. In the current work, $^{155}Eu^{3+}$ exchange in both 'native' and 'heat-denatured' DNA was investigated under a variety of conditions: (1) as the pH of the chase buffer was changed, (2) as competing, non-radioactive Eu^{3+} was added to the chase buffer and (3) as increasing concentrations of the cationic, intercalating drug ethidium bromide was added to the chase buffer. The results of our experiments have revealed that there are significant differences in metal ion exchange rates at a particular pH value in native *versus* heat-denatured DNA in the absence of ethidium bromide. Of particular interest physiologically, however, is the marked difference in metal ion exchange profiles at pH = 6.0 *versus* pH = 7.0 for both forms of DNA in the absence and presence of ethidium bromide, an effect which can be explained in part on the basis of differing degrees of metal ion solvation at differing pH values.



CT Counter timer
SCA Single Channel Analyzer
AMP Amplifier
PRE Pre-Amplifier
HV High Voltage Supply
PMT Photo Multiplier Tube
DC Dialysis Cell

Fig. 1. The dual-chambered flow dialysis apparatus employed in ^{155}Eu(III) displacement experiments. Identical DNA samples, previously labelled with ^{155}Eu(III), are loaded into identical dialysis cells (DC). Samples are effectively separated from their respective buffer reservoirs by a semi-permeable cellulose membrane. With the aid of a peristaltic pump, the samples are allowed to dialyze against washout buffer (20 mmol cacodylate, 10 mmol KCl, pH 6 or 7 according to specific experimental protocol) until a stable ^{155}Eu(III) exchange rate is observed. One buffer reservoir is then perturbed by the addition of competing ligand (either non-radioactive Eu(III) or ethidium bromide). ^{155}Eu(III) displacement is simultaneously monitored from both compartments by independent photomultiplier tubes (PMT). Experimental output from both compartments is processed and relayed to a line printer giving recorded counts over a pre-set time interval (dwell-time = 2000 s). One dialysis cell is left unperturbed to monitor sample integrity during an experiment. The entire system is housed in a lead cave in order to eliminate outside interferring radiation.

Methods

Rat liver DNA samples were prepared according to a modified version of the procedure of Marmur [23]. DNA isolated from rat liver was stored as frozen aliquots (2.5 mg/ml) in 5 mmol TRIS-HCl buffer (pH = 7.0), containing 100 mmol KCl. Prior to experimentation an aliquot of frozen stock DNA was thawed and dialyzed overnight against 20 mmol sodium cacodylate buffer (pH = 6.0 or 7.0, depending on the particular experiment), containing 10 mmol KCl. $^{155}EuCl_3$ was obtained from New England Nuclear (Cambridge, Massachusetts, U.S.A.). Ethidium bromide was obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.), and was used without further purification.

Nuclear gamma-ray scintillation counting was performed with a dual chambered flow dialysis apparatus designed and constructed in our laboratory. A schematic diagram of the equipment is shown in Fig. 1. Each flow cell is positioned 0.5 cm in front of the gamma-ray scintillation crystal in order to continuously monitor $^{155}Eu^{3+}$ release from a previously labeled DNA sample. Each compartment contained a 3 inch by 3 inch cylindrical Teledyne NaI (Tl) scintillation crystal dectector optically coupled to a photomultiplier tube. The output of each photomultiplier tube was amplified using an Ortec No. 113 preamplifier and further amplified with an Elscint CAV-3 linear amplifier. In order to minimize the effect of background radiation and to effectively integrate the $^{155}Eu^{3+}$ 0.0865 MeV gamma-ray peak, single channel analyzers (Ortec 420-A) were employed, in order to focus on pulses corresponding to 0.0865 ± 0.025 MeV. Logic pulses from the single channel analyzers were routed to Ortec 776 counter/timers. Data accumulated for

dwell times of 2000 seconds were recorded using an Ortec 777A line printer. $^{155}Eu^{3+}$ exchange half lives were obtained using a least squares decay curve analysis program implemented on a VAX 11/780 computer.

The protocol for a typical kinetic experiment involved overnight incubation of a sample of DNA (1.25 mg) in a solution containing 2.5 μCi $^{155}EuCl_3$ at 5 °C. A 0.5 ml aliquot of the incubate was then placed in the sample compartment of the flow dialysis apparatus. The sample compartment was separated from the buffer reservoir by a cellulose membrane (pore size = 20 Å). The buffer typically contained 20 mmol cacodylate and 10 mmol KCl, and the pH was adjusted according to the specific experimental protocol. At the start of all experiments the sample was dialyzed against buffer and the loss of DNA-bound $^{155}Eu^{3+}$ was followed continuously as a function of time. A constant buffer flow rate of 150 ml/min was maintained through the flow dialysis cell with a peristaltic pump. Experiments with heat-denatured DNA were performed in a similar manner, except that following the incubation in $^{155}EuCl_3$, the DNA samples were heat-denatured in a boiling water bath for 10 min followed by rapid cooling to prevent reanealing of the DNA strands. Experiments with ethidium bromide were performed by adding specified amounts of an ethidium bromide stock solution to the 'chase buffer' reservoir. $^{155}Eu^{3+}$ displacement was then followed as a function of time with increasing concentrations of ethidium bromide.

Results and Discussion

Non-radioactive Eu^{3+} Induced $^{155}Eu^{3+}$ Displacement from DNA

Figure 2 depicts the displacement of $^{155}Eu^{3+}$ from rat liver DNA at pH = 6.0 in both native and heat-denatured forms, while Fig. 3 depicts the analogous displacement at pH = 7.0. For each form of DNA (*i.e.*, native or heat-denatured) at a particular pH value, two exchange profiles were recorded: (1) an exchange profile associated with a 'chase' buffer containing no competing metal ions (*i.e.*, the curve marked 'background' in both figures) and (2) an exchange profile illustrating induced displacement of DNA bound $^{155}Eu^{3+}$ by non-radioactive $EuCl_3$ added to the 'chase' buffer. The following conclusions can be drawn from the results depicted in Figs. 2a and 2b:

(1) $^{155}Eu^{3+}$ 'free exchange' is significantly slower at pH = 6.0 than at pH = 7.0 for both native and heat-denatured DNA

There is general agreement in the literature [24], that the binding of trivalent lanthanides such as Eu^{3+} (and Tb^{3+}) to organic ligands occurs with a large entropy of complexation (*i.e.*, ΔS is large and positive). This is consistent with the requirement for both lanthanide ion and organic ligand (DNA in our case) to undergo extensive 'desolvation' upon complex formation. Desolvation of the lanthanide ion component of the complex appears to be particularly important in determining the overall free-

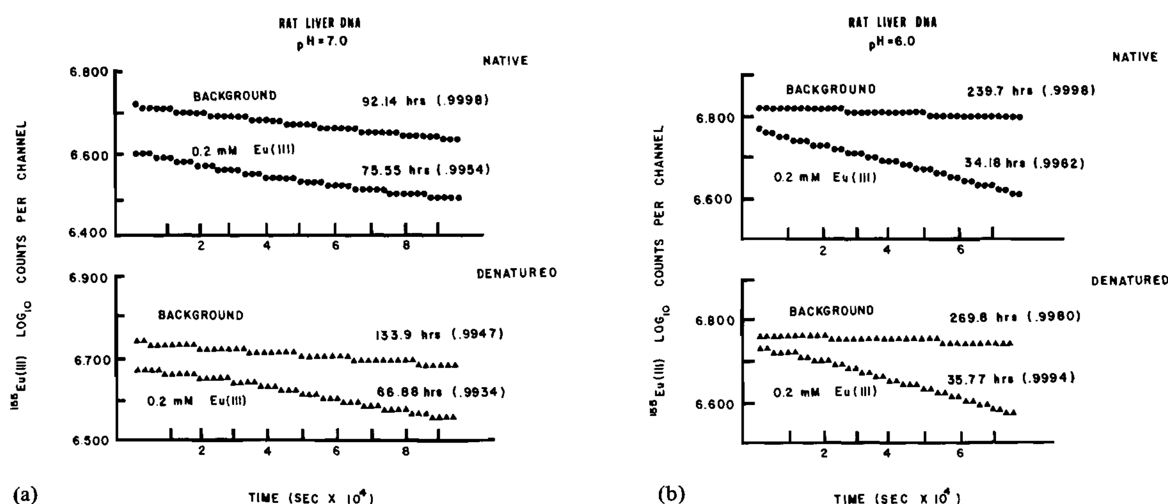


Fig. 2. Time *versus* activity plots showing ^{155}Eu(III) displacement from native and heat-denatured samples of rat liver DNA, at pH = 6 (a) and pH = 7 (b). The ^{155}Eu(III) exchange half-life (h) for each displacement profile has been calculated from the slope of the line and is shown to the right of the profile. The numbers in parentheses represent correlation coefficients.

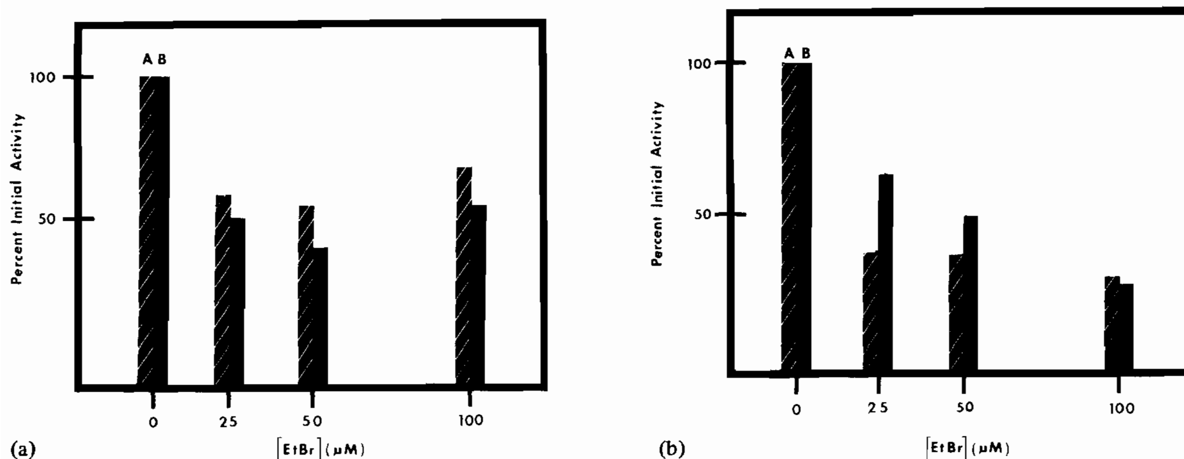
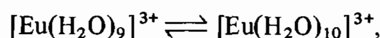


Fig. 3. Bar graphs summarizing the effect of ethidium bromide on $^{155}Eu(III)$ displacement from native (a) and heat-denatured (b) rat liver DNA at pH = 6.0. The stripped bars, labelled 'A', and the solid bars, labelled 'B', correspond to experiments performed on two different samples of rat liver DNA.

energy change for complexation because of its high degree of solvation in the absence of ligand [24]. In aqueous solution, Eu^{3+} ion exists as an equilibrium mixture,



$$[Eu(H_2O)_9]^{3+} \rightleftharpoons [Eu(H_2O)_{10}]^{3+},$$

with 9.6 waters, on the average, bound to each Eu^{3+} ion. Most of these coordinated waters leave the first coordination sphere of the lanthanide ion during the complexation event, which, in fact, constitutes the favorable 'entropic driving force' for complexation (see Horocks *et al.* [25] for a method of quantitating numbers of waters released upon lanthanide ion binding to a wide variety of complexing agents). It is further known that the extent of solvation of lanthanide ions in aqueous solution is pH-dependent [26]. While the pH-dependency is typically not great [26], it may be significant enough to account for our observed results. There are fewer water molecules comprising the first 'coordination sphere' of the ionizable groups in DNA (see Manning [1] for a complete discussion of solvated DNA), and, in the pH range being examined in the current study, little change is expected in the degree of solvation of these ionizable groups. [NOTE: In the pH range 6.0 to 7.0, all four bases of DNA are neutral, the closest pK_a being over 2.0 pH units removed. In addition, the phosphate groups of the DNA phosphodiester backbone are essentially 100% ionized in the examined pH range.] Thus, in light of the pK_a values of the various ionizable groups in DNA, as well as the expected pH-dependency of Eu^{3+} solvation, we believe that the observed differences in $^{155}Eu^{3+}$ displacement rates from DNA at the two pH values examined (Figs. 2a and 2b) can not be attributed to different states of ionization of the nucleic acid bases or the DNA phosphate groups, but rather must be linked with a differential Eu^{3+} desolvation at pH = 6.0 and 7.0, leading to a greater entropic effect at the lower pH value. Finally, we address the question of where the $^{155}Eu^{3+}$ is binding on the DNA molecule. Evidence exists in the literature which suggests that trivalent lanthanides bind preferentially to charged phosphate moieties near guanine bases [27] in both double-helical and single-stranded DNA [28]. While lanthanide ion binding to single-stranded DNA occurs with somewhat lower affinity, such binding has been shown to be associated with a sensitization of lanthanide ion (specifically, Tb^{3+}) luminescence [28, 29] (*i.e.*, base-paired nucleic acids induce no emission enhancement, while Tb^{3+} binding to unpaired residues produce substantial enhancement in Tb^{3+} emission). In fact, techniques employing Tb^{3+} emission spectroscopy have been developed to assess single-strand content of DNA [9, 30]. Assuming Eu^{3+} to be similar to Tb^{3+} in binding site preference, we interpret the data presented in Figs. 2a and 2b to be indicative of high affinity binding of $^{155}Eu^{3+}$ to the phosphate groups comprising the outer cylindrical shell of DNA at pH = 6.0.

(2) Non-radioactive Eu^{3+}-induced $^{155}Eu^{3+}$ 'displacement' is significantly faster at pH = 6.0 than at pH = 7.0 for both native and heat-denatured DNA

This result is consistent with the interpretation given above. If the rate-limiting step in the binding of lanthanides to sites on DNA is desolvation of the lanthanide ion, and if desolvation is entropically

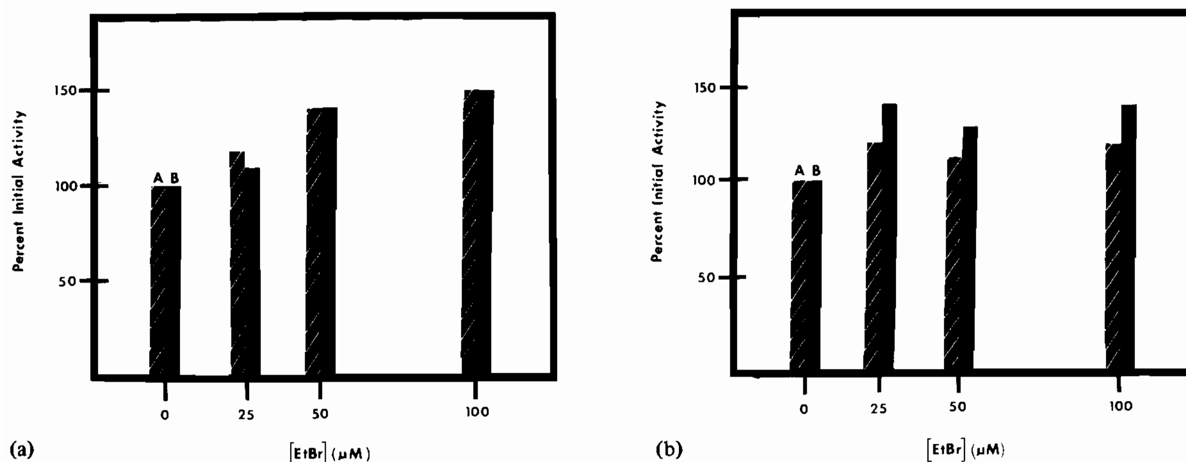


Fig. 4. Bar graphs summarizing the effect of ethidium bromide on $^{155}Eu(III)$ displacement from native (a) and heat-denatured (b) rat liver DNA at pH = 7.0. The stripped bars, labelled 'A', and the solid bars, labelled 'B', correspond to experiments performed on two different samples of rat liver DNA.

more favored at pH = 6.0, then we would expect non-radioactive Eu^{3+} in the pH = 6.0 'chase' buffer to more readily interact with and displace DNA-bound $^{155}Eu^{3+}$. An additional contributor to the increased $^{155}Eu^{3+}$ exchange half-life at pH = 7.0 (*versus* pH = 6.0) is increased interaction of non-radioactive Eu^{3+} ions with solvent hydroxide ions.

(3) $^{155}Eu^{3+}$ *is a better probe of cation binding sites at pH = 6.0 than at pH = 7.0*

From a comparison of $^{155}Eu^{3+}$ exchange profiles obtained for chase buffers with and without competing nonradioactive metal ion at the two pH values examined, it is clear that the exchange experiments conducted at pH = 6.0 reveal greater sensitivity than those conducted at pH = 7.0. Therefore, experiments on ternary systems (*i.e.*, experiments designed to follow cationic drug binding to DNA by monitoring $^{155}Eu^{3+}$ displacement) might prove more informative if performed at pH = 6.0 (see next section).

Ethidium Ion-induced $^{155}Eu^{3+}$ *Displacement from DNA*

The final phase of the present work concerns the potential utility of the radionuclide probe, $^{155}Eu^{3+}$, and our nuclear counting apparatus for examining the mode of interaction of an intercalating drug with native and heat-denatured DNA. Figures 3 and 4 show the effect of increasing concentrations of ethidium bromide on $^{155}Eu^{3+}$ displacement from both native and heat-denatured DNA at pH values of 6.0 and 7.0, respectively. The following conclusions can be drawn from the results depicted in Figs. 3 and 4:

(1) At pH = 6.0 ethidium ion accelerates $^{155}Eu^{3+}$ *displacement*

For both native (Fig. 3a) and heat-denatured (Fig. 3b) DNA, ethidium bromide (at all concentrations employed) causes significant displacement of DNA-bound $^{155}Eu^{3+}$ relative to the control (*i.e.*, $^{155}Eu^{3+}$ displacement associated with 'chase' buffer containing no ethidium bromide). In addition, the exchange profile for ethidium ion-induced $^{155}Eu^{3+}$ displacement at pH = 6.0 is somewhat dependent on the form of DNA employed. For both samples of native DNA (*i.e.*, 'A' and 'B' in Fig. 3a) $^{155}Eu^{3+}$ displacement was observed to pass through a 'minimum' at 50 μM ethidium bromide. This effect was not observed for either sample of heat-denatured DNA (see Fig. 3b). For heat-denatured DNA, the $^{155}Eu^{3+}$ exchange half-life continued to decrease with increasing concentration of ethidium bromide in the 'chase' buffer. Thus, once again, to reinforce the conclusion made in the previous section, our probe system seems very 'well-behaved' at pH = 6.0. The results are readily interpretable. The cationic ethidium ion is binding by rather strong intercalative forces to the DNA polyelectrolyte and is displacing the cationic radionuclide $^{155}Eu^{3+}$ near the drug binding sites. When large quantities of ethidium ion bind to the highly structured native DNA, it seems reasonable that some $^{155}Eu^{3+}$ ions become 'trapped' between the bound ethidium ions and the solvated external 'cylindrical' walls of the DNA polyelectrolyte, giving rise to a larger $^{155}Eu^{3+}$ exchange half-life (*i.e.*, giving rise to the 'minimum' in the exchange profile shown in Fig. 3a). The more extended, less highly-structured heat-denatured DNA presumably would allow more ethidium bromide to bind (some

probably by purely electrostatic associations with the negative phosphate groups of the phosphodiester backbone), prior to steric blockage of exchange, hence the lack of a minimum in the exchange profile.

(2) At pH = 7.0 ethidium ion decelerates $^{155}Eu^{3+}$ displacement

For both native (Fig. 4a) and heat-denatured (Fig. 4b) DNA, ethidium bromide (at all concentrations employed) causes significant retardation of $^{155}Eu^{3+}$ displacement relative to the control (*i.e.*, $^{155}Eu^{3+}$ displacement associated with 'chase' buffer containing no ethidium bromide). There are a number of possible explanations for the observed decrease in $^{155}Eu^{3+}$ exchange half-lives:

(i) A greater proportion of ethidium ion might bind to the phosphate backbone at pH = 7.0 than at pH = 6.0, sterically 'trapping' $^{155}Eu^{3+}$ ions. Consideration of the relevant pK_a values, however, would seem to preclude this interpretation;

(ii) Intercalative associations between ethidium ion and DNA might be different at pH = 7.0 than at pH = 6.0, leading to the 'opening up' of DNA helices at pH = 7.0, making available more 'internal' sites for $^{155}Eu^{3+}$ binding. This explanation would carry more weight if evidence were available suggesting the existence of different conformations for the ethidium ion at the two pH values; however, comparison of the carbon-13 NMR spectra of ethidium ion in aqueous solution over the relevant pH range reveals no evidence of such conformational change [31];

(iii) The hydrated form of $^{155}Eu^{3+}$ at pH = 7.0 might have an unusually high affinity for the amino functions of the ethidium ion itself. Lenkinski *et al.* [24] have demonstrated that Ca^{2+} and the lanthanide ion Tb^{3+} both bind (at pH = 7.0) to the primary amine moiety of the diaminopropionamide portion of the intercalative antibiotic bleomycin. Carbon-13 NMR analyses on the nature of complexes formed between Eu^{3+} and ethidium ion as a function of pH might be informative in this regard, and such experiments are currently in progress.

In summary, we have shown that our flow dialysis/gamma nuclear counting apparatus (together with a suitably chosen lanthanide radionuclide probe) can be used effectively to investigate the binding of both metal ions and intercalative drugs to macromolecular DNA. We have further indicated the responsiveness of the technique to the pH-dependency of the binding of both metal ions and intercalative drugs to DNA. Finally, the results presented here indicate that the technique employed is particularly sensitive to intercalative drug binding at pH = 6.0, and suggests that a complete spectroscopic (i.e., NMR) investigation at this pH would be most informative.

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