Topography of Nucleic Acid Helices in Solutions. Stoichiometry and Specificity of the Interaction of Reporter Molecules with Nucleic Acid Helices

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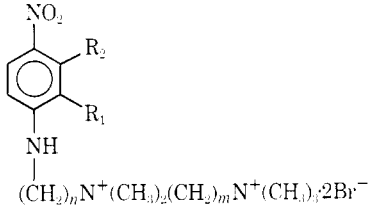
ABSTRACT: The stoichiometry of the interaction of reporter molecules, **1**, 2,4-$(NO_2)_2C_6H_5NH(CH_2)_2N^+H(CH_3)_2 \cdot Br^-$, and **2**, 2,4-$(NO_2)_2C_6H_5NH(CH_2)_2N^+(CH_3)_2(CH_2)_3N^+(CH_3)_3 . 2Br^-$, with salmon sperm DNA is reported. A spectrophotometric technique is used to determine the apparent binding constant, k'_R. Moreover, proton magnetic resonance studies on the free and DNA-bound reporter molecules, together with viscosity measurements of DNA solution in the presence and absence of reporter molecules, permit the following conclusions: (1) H-bonding, electrostatic, and "hydrophobic type" forces are involved in the binding process; (2) a site-binding model adequately explains the nucleic acid reporter complex; (3) pmr studies show that the rate of tumbling of the reporter molecule in the DNA complex is considerably diminished with a concomitant upfield chemical shift which is probably indicative of shielding due to an aromatic ring current effect; the results are consistent with an intercalation model; (4) viscosity measurements support the pmr studies and indicate that the reporter molecules **1** and **2** are intercalated.

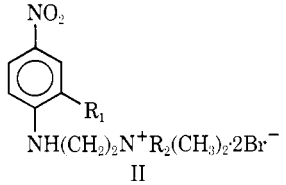
Considerable information concerning the nature and specificity of interactions of reporter molecules, I, with nucleic acid systems has been obtained.[2]



I

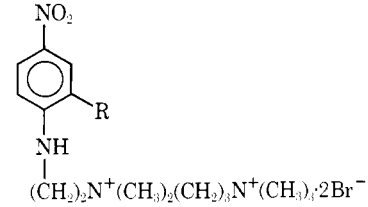
For example, an induced circular dichroism (CD) and large hypochromism are observed in the nucleic acid bound reporter molecules. It is found that as the ratio of the polynucleotide phosphate, P, to reporter concentration, R, *i.e.*, P/R, approaches 0, the induced CD and the per cent hypochromicity approach 0.[2a,b] In addition, as the P/R ratio approaches ∞, the induced CD and per cent hypochromicity approach a limiting value characteristic of the polynucleotides as well as the reporter molecules. This behavior is indicative of a simple site-binding complex between the nucleic acid systems and the reporter molecules.

This paper describes the effect of temperature and univalent ions on the binding of reporter molecules **1** and **2** to salmon sperm DNA. In addition, the effect of reporter molecules **1**–**4** on the viscosity of a DNA solution was studied. These results, together with proton magnetic resonance studies on the free and DNA-bound reporter molecules **1**–**5**, are reported and discussed.



II

1, $R_1 = NO_2$; $R_2 = H$
4, $R_1 = CH_3$; $R_2 = H$
5, $R_1 = CH_3$; $R_2 = CH_3$



III

2, $R = NO_2$
3, $R = CH_3$

Results and Discussion

Binding Studies. The reporter molecules of the general structure I have been shown to exhibit large hypochromism upon binding to nucleic acid systems.[2] Figure 1 shows the effect of increasing concentrations of salmon sperm DNA on the absorption of reporters **1** and **2**. The hypochromic effect has been attributed to an intensity interchange between the low-energy 4-nitroaniline transition of the reporter molecule **2** with the higher energy transition of the purine and pyrimidine bases of the nucleic acid systems.[2] In order to determine the apparent binding constant, k'_R, of the reporter to the nucleic acid system a very simple treatment was utilized to describe the binding process. The treatment ignores the effect of the electrostatic potential and near-

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(2) (a) E. J. Gabbay, *J. Amer. Chem. Soc.*, **90**, 6574 (1968); (b) E. J. Gabbay and J. Mitschele, *Biochem. Biophys. Res. Commun.*, **34**, 53 (1969); (c) E. J. Gabbay, *J. Amer. Chem. Soc.*, **91**, 5136 (1969); (d) E. J. Gabbay and M. Malin, *J. Biol. Chem.*, submitted for publication; (e) E. J. Gabbay, B. L. Gaffney, and L. Wilson, *Biochem. Biophys. Res. Commun.*, **35**, 854 (1969).

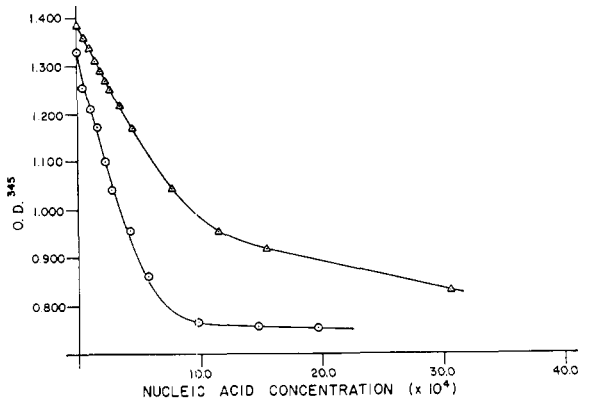


Figure 1. The effect of increasing concentration of DNA on the optical density of 10^{-4} *M* solution of reporter molecules *1* (△—△) and *2* (○—○) in 0.01 *M* sodium phosphate buffer at 25°.

est neighbor interaction of adjacent phosphate anions. The results obtained in this manner are consistent and appear to justify the simplification, as will be shown subsequently. The over-all binding process can be broken up into two separate equilibria

$$R_f^{+y} + P^{-y} \overset{k_R}{\rightleftharpoons} R_bP \quad (1)$$

$$M^+ + P^- \overset{k_m}{\rightleftharpoons} MP \quad (2)$$

where R_f, P^-, and M^+ are the free reporter molecule, polynucleotide phosphate, and metal ion, respectively. The extent of binding of the reporter molecule is expected to be dependent on the metal ion concentration. An apparent binding constant, k'_R, can be described which would be relative to the metal ion concentration

$$k'_R = R_bP/[R_f^{+y}][P^{-y}] \quad (3)$$

If the degree of binding, β, is defined according to Lawley[3]

$$\beta = R_bP/P_T^{-y}$$

where (P_T^{-y}) is the total polynucleotide phosphate in solutions, then by eq 1 and 2

$$\beta = k_R(R_f^{+y})/1 + k_R(R_f^{+y}) + k_m(M^+)$$

The last equation is the form of the Langmuir adsorption isotherm for the competitive binding of reporter molecule and metal cations.

In order to relate k_m and k_R to the apparent binding constant, k'_R, it is necessary to put k'_R in terms of β. From eq 3, it may be shown that

$$k_R' = \beta/(R_f^{+y})(1 - \beta) \quad (4)$$

and

$$k'_R = k_R/1 + k_m(M^+) \quad (5)$$

Values of k'_R are easily obtained from the experimental curves shown in Figure 2. For example, at any point along the curve, the following expression applies

$$R_0\epsilon_p = X\epsilon_{R_b}R_0 + (1 - X)\epsilon_{R_f}R_0 \quad (6)$$

where R_0, ϵ_p, ϵ_{Rb}, ϵ_{Rf}, and X represent the total reporter concentration, observed molar extinction coefficient at that point, molar extinction coefficient of the bound reporter, molar extinction coefficient of the free reporter molecule, and the fraction of the reporter molecule bound, respectively. Equation 6 presupposes that the free and bound reporter molecules obey Beer's law. For the experimental conditions, this restriction has been met. The bound and free[2] reporter molecules **1** and **2** are found to obey Beer's law in the range of 10^{-5}–10^{-4} *M*. Figures 2a and 2b show the effect of increasing concentration of reporters **1** and **2**, respectively, in the presence of excess DNA. The results clearly indicate that the DNA-bound reporter molecules, **1** and **2**, obey Beer's law.

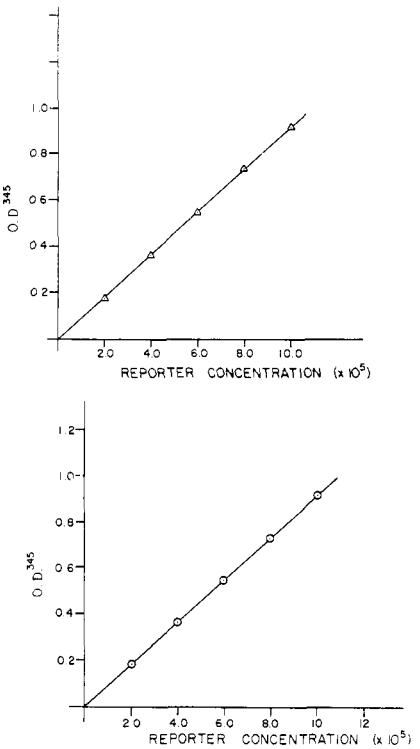


Figure 2. (a) The effect of increasing concentration of reporter molecule *1* on the optical density at 345 mμ in the presence of excess salmon sperm DNA (4.6×10^{-3} mol of P/l). (b) Same as (a) but for reporter *2*.

Values of k'_R may be calculated from the relationship

$$k'_R = X/(1 - X)[(1/P_T^{-y}) - XR_0] \quad (7)$$

Values of k'_R can be obtained at different M^+ ion concentrations, and eq 5 may be solved to obtain k_m and the absolute binding constant, k_R.

It should be emphasized again that this treatment of the data is overly simplified and ignores the effects of the electrostatic potential and nearest neighbor interactions. However, it is reasonable to ignore these effects since the calculated values of the apparent binding constant, k'_R, are relatively constant in the region where the reporter molecule is bound to the extent of 25–75%. Table I shows the values which were calculated for DNA and reporter molecules **1** and **2** at various temperatures and Na^+ ion concentrations. Each value of k'_R represents an average of at least 18 points, with the data taken in the mid range of the spectral titration curves (Figure 1) at three different wavelengths. In most cases, the k'_R values at high and low values of

(3) (a) P. D. Lawley, *Biochem. Biophys. Acta.*, **19**, 160 (1956); (b) *ibid.*, **19**, 328 (1956).

TABLE I
THE EFFECT OF TEMPERATURE AND [Na^+] ON THE INTERACTION BETWEEN SALMON TESTES DNA AND REPORTER MOLECULES 1 AND 2

Reporter molecule	Temp, °C	[Na^+]	$k'_R \times 10^{-2}$
1	25	0.01	12.5 ± 0.5
1	25	0.02	8.6 ± 0.5
2	25	0.01	90 ± 14
2	25	0.02	65 ± 4
2	25	0.04	38 ± 3
1	35	0.01	12.5 ± 0.1
1	35	0.02	9.0 ± 0.4
2	35	0.01	91 ± 15
2	35	0.02	62 ± 3
2	35	0.04	38 ± 3
1	45	0.01	12.2 ± 0.4
1	45	0.02	8.4 ± 0.6
2	45	0.01	93 ± 15
2	45	0.02	63 ± 4
2	45	0.04	38 ± 3

($X/1 - X$) were not included in the average k'_R because they deviated considerably from the relatively constant values throughout the middle range. This deviation is expected due to the fact that the spectral changes in these regions are small and subsequent errors associated with ($X/1 - X$) values are substantial.

From the results shown in Table I, the following conclusions are most obvious: (1) the divalent reporter molecule **2** binds about ten times more strongly than the monovalent reporter molecule **1**; (2) increasing the Na^+ concentration causes a decrease in the apparent binding constant for both reporter molecules; (3) temperature changes (25–45°) have little effect on the binding of the reporter molecules to salmon sperm DNA; (4) finally, it is possible to calculate k_m and k_R by simultaneous equations using two sets of values for k'_R. The results are shown in Table II. Although a number of simplifications are inherent in this treatment, it is satisfying that a fairly constant value of k_m is obtained for DNA. The monovalent and divalent reporter molecules, **1** and **2**, respectively, are 25 and 200 times as strongly bound as sodium ion to DNA. This conclusion is obtained from the calculated values of the absolute binding constant, k_R, assuming a k_m value of approximately 85. It is interesting to note that a similar value of k_m for the binding of Na^+ to DNA was also calculated by Ross and Scruggs[4] using electrophoretic techniques.

TABLE II
CALCULATED VALUES FOR k_m AND k_R FOR THE Na^+, REPORTER MOLECULE, AND SALMON SPERM DNA

Reporter	Temp, °C	[Na^+]	k_m	$k_R \times 10^{-2}$ [a]
1	25	0.01	83	24
1	25	0.02		
2	25	0.01	62	
2	25	0.02	84 123	173
2	25	0.04		
1	35	0.01	70	24
1	35	0.02		
2	35	0.01	88	
2	35	0.02	86 85	164
2	35	0.04		
1	45	0.01	83	24
1	45	0.02		
2	45	0.01	90	
2	45	0.02	93 96	175
2	45	0.04		

[a] In the calculation of k_R, an average k_m value at each temperature was used.

Viscosity Measurements. A number of investigators have demonstrated that planar molecules such as acridine orange, ethidium bromide, and proflavine may intercalate between base pairs in DNA.[5] This phenomenon leads to an increase in the length of the helix with a concomitant increase in the viscosity of the solution. In order to define the nature of the binding process of reporter molecules **1** and **2** to DNA, viscosity measurements were made, and the results are shown in Table III. It is noted that the intrinsic viscosity of the DNA-bound reporter **1** and **2** complexes relative to pure DNA are increased by 0.17 and 0.39, respectively. Thus, intercalation is strongly indicated. It is probably safe to assume that (a) intercalation of each of the reporter molecules produces the same increase in the length of the double helix, and (b) the relative intrinsic viscosity increments, 0.17 and 0.39 for DNA reporter **1** and **2** complexes, respectively, may be taken as a measure of the equilibrium intercalated reporter $\rightleftharpoons$ externally bound reporter. In other words, the mono-

TABLE III
THE RELATIVE, INTRINISC VISCOSITIES OF DNA–REPORTER COMPLEXES TO PURE DNA[a,b]

Reporter	$[\eta]/[\eta_0]$
1	1.17
2	1.39
3	1.24
4	1.06

[a] The intrinsic viscosity of pure DNA, $[\eta_0]$, and the DNA–reporter complex, $[\eta]$, were calculated using the equation $\eta_{sp}/c = [\eta] + k'[\eta]^2c$ and assuming that the value of the Huggins constant, k', is the same for DNA and the DNA–reporter complex. This assumption is justifiable and has been recently shown by Ross and Scruggs[6] to apply for DNA and DNA–dye complexes under similar ionic strength conditions. [b] Viscosity measurements were carried out using 1.13×10^{-3} M in P/l. of DNA and 1.0×10^{-4} M in reporter molecule in 0.025 M sodium phosphate buffer (pH 6.50) at 25° using a Zimm viscometer. Under these conditions, the reporter molecules are fully bound to DNA as evidenced by spectrophotometric titration studies.

(4) P. D. Ross and R. L. Scruggs, *Biopolymers*, 2, 79 (1964).
(5) (a) L. S. Lerman, *J. Mol. Biol.*, 3, 18 (1961); (b) D. S. Drummond, N. J. Pritchard, V. F. W. Simpson-Gildemeister, and A. R. Peacocke, *Biopolymers*, 4, 971 (1966); (c) G. Cohen and H. Eisenberg, *ibid.*, 8, 45 (1969); (d) R. W. Armstrong, T. Kurucsev, and U. P. Strauss, in preparation.

TABLE IV
CHEMICAL SHIFTS (PARTS PER MILLION) FROM DSS (±0.01) AND LINE WIDTH AT HALF-HEIGHT IN HERTZ ($\Delta\nu^{1/2}$) OF FREE AND DNA-BOUND REPORTER MOLECULES **1**–**5** AT VARIOUS TEMPERATURES[a,b]

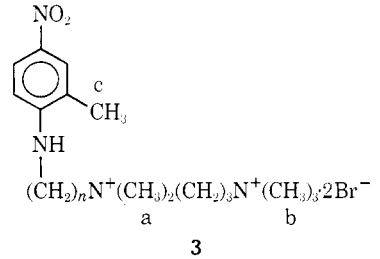
System	Deriv	R_1	R_2	R	Temp, °C	a signal[c] $\delta(\Delta\nu^{1/2})$	b signal[c] $\delta(\Delta\nu^{1/2})$	c signal[c] $\delta(\Delta\nu^{1/2})$
1	II	NO_2	H		32	3.03 (2.0)		
1	II	NO_2	H		90	2.99 (1.5)		
1–DNA	II	NO_2	H		32			
1–DNA	II	NO_2	H		58	2.90 (14)		
1–DNA	II	NO_2	H		90	2.97 (3.0)		
2	III			NO_2	32	3.32 (2.0)	3.21 (1.7)	
2	III			NO_2	90	3.32 (2.0)	3.21 (2.0)	
2–DNA	III			NO_2	32	3.20 (14)[d]		
2–DNA	III			NO_2	58	3.20 (5.5)[d]		
2–DNA	III			NO_2	90	3.25 (5.0)	3.21 (3.2)	
3	III			CH_3	32	3.30 (2.3)	3.16 (1.8)	2.22 (2.5)
3	III			CH_3	90	3.29 (1.8)	3.17 (1.7)	2.24 (2.2)
3–DNA	III			CH_3	32	3.17 (15)[d]		
3–DNA	III			CH_3	58	3.18 (4.8)[d]		
3–DNA	III			CH_3	70	3.22 (3.5)	3.16 (2.8)	1.70 (9.0)
3–DNA	III			CH_3	90	3.29 (2.5)	3.18 (2.0)	2.03 (4.0)
4	II	CH_3	H		25	2.99 (1.5)		2.22 (2.0)
4	II	CH_3	H		90	2.97 (1.5)		2.22 (2.0)
4–DNA	II	CH_3	H		25			
4–DNA	II	CH_3	H		58	2.90 (7.0)		
4–DNA	II	CH_3	H		90	2.95 (1.5)		2.09 (2.5)
5	II	CH_3	CH_3		25	3.23 (2.0)		2.16 (2.5)
5	II	CH_3	CH_3		90	3.24 (2.0)		2.21 (2.0)
5–DNA	II	CH_3	CH_3		25	3.22 (18)		
5–DNA	II	CH_3	CH_3		51	3.22 (5.0)		1.60 (>20)
5–DNA	II	CH_3	CH_3		88	3.26 (2.0)		2.18 (2.0)

[a] Sonicated low molecular weight salmon sperm DNA were used at 0.16 mol of P/l. in D_2O in 10^{-4} *M* sodium phosphate buffer (pD 7.0 ± 0.2). The concentration of reporter molecule was 0.02*M*. [b] Spectra were taken on a Varian A-60A. [c] The a signal is assigned to the protons of the monovalent ammonium methyl groups and the nonterminal protons of the divalent ammonium methyl groups. The b signal is assigned to the protons of the terminal ammonium methyl groups of the divalent reporter molecules. The c signal is assigned to the protons of the ring methyl group. [d] The chemical shifts for protons a and b are similar and lead to a coalesced signal.

valent reporter **1** is not as extensively intercalated as the divalent reporter **2** to DNA. It is of interest to observe that the divalent and monovalent reporter molecules **3** and **4** also exhibit a similar behavior, *i.e.*, a greater increase in the relative, intrinsic viscosity for the DNA–**3** than the DNA–**4** complex. The interaction with DNA of these reporter molecules, which contain a methyl group substituent at the 2 position of the 4-nitroaniline ring, have also been studied by pmr spectroscopy. The results of these studies are entirely consistent with an intercalated complex (see below). It should be added that the ultraviolet absorption and induced circular dichroism spectra are consistent with the interpretation that the divalent reporter molecules are more extensively intercalated to DNA than the monovalent reporter molecules, since the extent of hypochromism and the induced CD are greater for the former.[2]

It should be noted that Gabbay[2c] had originally concluded from studies of molecular framework models of the DNA–reporter complex that it would be difficult to intercalate the 4-nitroaniline ring and maintain electrostatic binding of the diammonium side chain of **2** to adjacent phosphate anions of DNA. Clearly, the viscosity data, as well as the pmr data to be cited below, indicate that intercalation does, in fact, occur. Careful reexamination of framework models indicates that it is possible to intercalate the 4-nitroaniline ring of reporter **2**. However, the side chain diammonium group cannot bind to adjacent phosphate anions of DNA on the periphery as was originally assumed, but rather in closer proximity to the major or minor grooves.

Proton Magnetic Resonance Studies. In recent studies utilizing pmr spectroscopy, it has been shown by Gabbay, *et al.*,[2e] that considerable line broadening is observed for the proton signals in the bound DNA– and RNA–reporter complexes. This phenomenon has been explained in terms of restricted rotations about the plane of the 4-nitroaniline ring of the nucleic acid-bound reporter molecule, leading to incomplete averaging of the magnetic environment and substantial line broadening. Moreover, the partial pmr spectrum of the DNA bound reporter **3** complex shows that the c-methyl proton signal is shifted upfield by 0.54 ppm and is extensively broadened, whereas the upfield shift and line broadening are considerably less for the a-methyl protons and negligible for the b-methyl protons signals (Table IV). The pmr studies thus strongly indicate



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that the 4-nitroaniline ring system of the reporter molecule **3** is, in fact, intercalated. Both the line broadening of the c-methyl protons, indicative of restricted rotation,[6] and the upfield chemical shift of the c-CH_3 proton signals, indicative of anisotropic shielding by aromatic ring currents,[6] support this conclusion.

Table IV summarizes the temperature-dependent partial pmr spectra of the free and DNA-bound reporter molecules **1–5**. The results in terms of chemical shifts from sodium 2,2-dimethyl-2-silapentanesulfonate (DSS) and line width in hertz at half height, $\Delta\nu_{1/2}$, are reported. Several interesting points may be made. (1) The partial pmr spectra of the DNA-bound monovalent and divalent reporter molecules **1–5** show considerable variation in the chemical shifts and line width of the a-, b-, and c-methyl protons. As can be seen from Table IV, the resonance signal of the N-methyl protons (a-CH_3) of the tertiary ammonium salts, reporters **1** and **4** complexes of DNA, is considerably broadened and is indistinguishable from base line noise at 32°. These results may be explained in terms of restricted rotations of the $-N^+H(CH_3)_2$, as compared with the quaternary ammonium group, $-N^+(CH_3)_3$, of reporter **5**, since the resonance signal of the latter is clearly distinguishable from the base line noise. The results are strongly suggestive of H-bonding interactions between $\geqslant N^+H$ and H-bond acceptor in DNA. (2) The ring methyl signal (c-CH_3) of the DNA–reporters **3**, **4**, and **5** is indistinguishable from the base line noise at 25–30°. Moreover, as the temperature is increased the pmr signal of the ring methyl protons appears as a broad band which is shifted upfield by 0.4–0.6 ppm. The anisotropic shielding of the ring methyl protons is probably due to the influence of a nearby aromatic ring current phenomenon.[6] The data, therefore, are strongly suggestive of an intercalated complex of reporter molecules with DNA.

Experimental Section

Native salmon testes deoxyribonucleic acid (sodium salt) was obtained from Calbiochem (lot SDNA 6FA). Solutions of DNA were made in 0.001 *M* sodium phosphate buffer and stored at 0–4° at 4.6–5.9 × 10^{-3} mol of P/l. Concentrations of DNA were calculated using $\epsilon_{max}^{257} = 6500$ and the optical density of a DNA solution prepared by diluting the stock solution in 0.1 *M* sodium phosphate buffer and measured with a Cary 14 spectrometer at 25 ± 0.2°. Stock solutions of the reporters **1** and **2** were made to 10^{-3} *M* using distilled water.

In a typical binding study experiment, stock solutions of DNA were diluted by a factor of 1/3 to prepare a stock solution of nucleic acid reporter complex which contained 10^{-4} *M* reporter of 0.01 *M* sodium phosphate buffer. The spectral titration curve was obtained by using a diluent solution which contained the reporter molecule at 10^{-4} *M* and phosphate buffer at 0.01 *M* in Na^+.

The reporter concentration was maintained at 10^{-4} *M* and the phosphate buffer concentration was maintained at 10^{-2} while the nucleic acid concentration was gradually lowered from 20–40 × 10^{-4} to 0.50–1.0 × 10^{-4} mol of P/l. The binding of reporter molecule **2** to the nucleic acid was studied at three different ionic strengths; 0.01, 0.02, and 0.04 *M* in Na^+. The studies with reporter molecule **1** were carried out at 0.01 and 0.02 *M* in Na^+.

For the determination of the binding curves, optical density measurements were made using matched 1-cm quartz cuvettes. It was found that there was no adsorption to glass containers or cuvettes by either the reporter or the nucleic acid reporter complex. Optical density measurements were taken on either a Gilford Model 240 or Beckman DU-2 spectrophotometers. Both instruments were equipped with a Gilford automatic cuvette positioner and their cell compartments were thermostated by a Haake constant-temperature circulator.

For each set of three samples, the measurements were recorded at 25, 35, and 45 ± 0.2°. Equilibration times of 5 min at 25°, 10 min at 35 and 45° were allowed. The correction for increase in volume due to temperature was not necessary since it was found that these did not cause significant variations throughout the range of optical densities studied. Optical densities were recorded at 340, 345, and 350 mμ.

Concentration studies were also performed for the free and bound reporter molecules to determine whether or not Beer's law was obeyed. For example, for the free reporter molecules **1** and **2** in solution, absorption spectra were recorded in the range of 10^{-4} to 10^{-3} *M* using the Cary 14 spectrometer. In both cases, it was found that Beer's law was strictly obeyed.

Studies for the bound reporter molecule were performed in the following manner. Stock solutions of reporter molecules **1** and **2** were prepared at 0.03 *M*. A DNA solution at 46 × 10^{-4} *M* in 0.01 *M* sodium phosphate buffer was prepared in a 10-mm cuvette. Five successive 2-μl additions of the stock reporter molecule solutions were made into the cuvette by means of a Hamilton microsyringe and the solution was mixed with a mechanical stirrer. Absorption spectra were recorded on a Cary 14 spectrometer.

Acknowledgment. This work was supported by Grant GM 15308 from the U. S. Public Health Service.

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