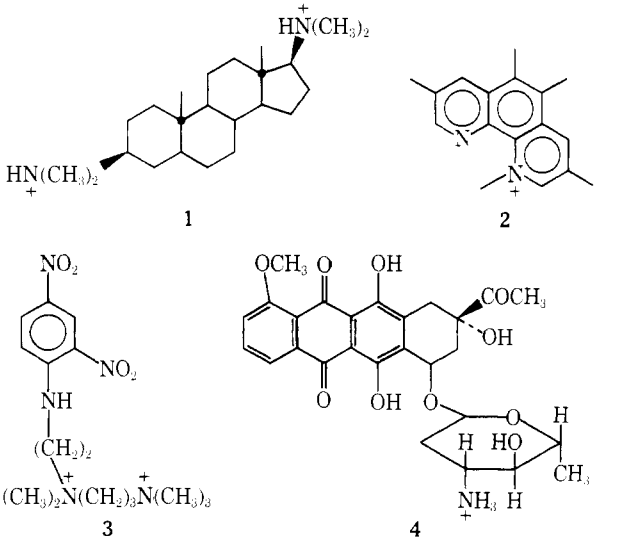
Binding of Manganese(II) to DNA and the Competitive Effects of Metal Ions and Organic Cations. An Electron Paramagnetic Resonance Study†

Jacques Reuben* and Edmond J. Gabbay‡

ABSTRACT: The binding of manganese(II) to DNA was studied by monitoring the concentration of free Mn^{2+} by electron paramagnetic resonance (EPR). It was found that the association constant of the Mn-DNA complex depends upon the degree of saturation. The competitive effects of magnesium, calcium, sodium, and a number of organic cations including the antibiotic drug daunomycin were analyzed and the parameters describing the cation-DNA interaction were evaluated. It was found that the association constant as well as the parameter describing its dependence upon the degree of saturation decrease along the series Mn, Mg, Ca, Na. Differences in the extent of interaction with the base nitrogens (N-7) are suggested as the possible mechanisms leading to these observations. The EPR spectrum of the manganese-DNA complex was found to be similar to that of manganese-nucleotide complexes suggesting a similar mode of coordination. A comparison of the results of competitive and direct binding studies reveals some salient features of the small molecule-DNA interaction and leads to the conclusion that manganese binds at the major groove of the DNA helix.

The interaction of metal ions with DNA and with nucleic acids in general is of primary importance in maintaining the conformational stability of the macromolecules and in controlling their biological function. These interactions have been studied by a variety of physical methods and seem to be qualitatively fairly well understood (Steiner and Beers, 1961; Felsenfeld and Miles, 1967; Izatt et al., 1971; Von Hippel and McGhee, 1972; Eichhorn, 1973; Eisenberg, 1974). Detailed quantitation of metal ion-DNA interactions in terms of binding constants and number of binding sites has recently been attempted (Daune, 1970; Sander and Ts'o, 1971; Clement et al., 1973). The methods used included equilibrium perturbations, e.g., ultracentrifugation, and potentiometry utilizing ion selective electrodes. We approached the problem of binding of manganese(II) to DNA by electron paramagnetic resonance (EPR). The manganese aquo ion has a characteristic and relatively sharp EPR spectrum, whereas the spectrum of bound manganese is usually much broader and of low intensity (Cohn and Townsend, 1954). Thus, the spectral intensity may serve as a direct measure of free manganese in solution. In this way the binding of manganese to proteins (cf., e.g., Reuben and Cohn, 1970), to tRNA (Danchin and Guéron, 1970), and to "activated" low molecular weight (42,000) DNA (Slater et al., 1972) has been investigated. Moreover, it should be possible by this method to study the interaction of other cations competing or interfering with the binding of manganese to DNA.

Reported in this paper are the results of our studies of the binding of manganese to DNA and the competitive effects of magnesium, calcium, sodium, and a number of organic cations including the antibiotic drug daunomycin. The organic molecules **1**, **2**, and **3** and the drug **4** were chosen since considerable information concerning their interaction specificity with DNA is available (cf. Gabbay and Glaser, 1971; Gabbay et al., 1973; Passero et al., 1970; Pigram et al., 1972, for **1–4**, respectively) and hence selective competition with manganese might give further insight into their mode of interaction with DNA.



Experimental Section

All solutions containing DNA were prepared in 1 m*M* piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes)[1] buffer at pH 6.8 made with deionized triply distilled water and stored at 4°. Salmon testes DNA (lot no. 47B-7740, Sigma Chemical Co., St. Louis, Mo.) was sonicated following a previously published procedure (Gabbay and De Paolis, 1971). The initial solution was prepared by allowing 930 mg of the so-

† From the Department of Isotope Research (J.R.) and Chemical Immunology (E.J.G.), the Weizmann Institute of Science, Rehovot, Israel. *Received September 6, 1974.*

‡ Recipient of a National Institutes of Health Career Development Award (GM 18653). On sabbatical leave from the Department of Chemistry, University of Florida, Gainesville, Fla.

[1] Abbreviations used are: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EPR, electron paramagnetic resonance.

dium salt of DNA to dissolve overnight in 100 ml of buffer solution at 4°. After sonication the solution was filtered, placed in a Visking dialysis bag (previously washed with an EDTA solution), and dialyzed at 4° as follows: first against 2 l. of 0.5 m*M* EDTA (pH 7.1) for 2 hr and then against three consecutive changes of 2 l. of buffer for 2, 2, and 14 hr, respectively. Finally the solution was lyophilized and a white solid obtained. The sonic treatment results in a homogeneous preparation of an average molecular weight of 5×10^5 (Cohen and Eisenberg, 1966). The native state of the DNA was checked by the hyperchromicity at 260 nm upon melting of the helix. The sonicated DNA showed a 36% hyperchromism, in agreement with the literature value (Reichman et al., 1954). Concentrations of DNA are expressed in terms of the phosphate (base) content and were determined spectrophotometrically using a Cary 15 spectrophotometer.

Daunomycin hydrochloride was a gift from Dr. Ronald Levy. The chloride salts of compounds **1, 2,** and **3** were prepared according to the previously published procedures (cf., respectively, Gabbay and Glaser, 1971; Gabbay et al., 1973; Passero et al., 1970). The metal chlorides used in this study were reagents of the highest purity available.

EPR measurements were carried out with a Varian E-12 spectrometer operating at the X-band. Determinations of free manganese were done at room temperature (23 ± 1°) following the procedure of Reuben and Cohn (1970) and applying corrections for the contribution of the bound manganese (vide infra). Titrations were done at constant DNA concentration (0.46–0.50 m*M* phosphate) and varying manganese concentrations (0.06–1.2 m*M*). Competitive binding studies were carried out at constant concentration of the competing cation. In addition, for magnesium, calcium, and sodium, measurements were done at constant manganese concentration (0.2 or 0.4 m*M*) and varying concentrations of the competing cation. The results are expressed in terms of the Scatchard plot parameters $\bar{\nu}_{Mn}$ and $\bar{\nu}_{Mn}/[Mn]_f$ explained in the next section. The EPR spectrum of the manganese–DNA complex was recorded with a sample containing 1 m*M* $MnCl_2$ and 24.0 m*M* DNA–phosphate.

Results

The EPR Spectrum of Manganese–DNA. The literature reports (Clement et al., 1973) as well as our own preliminary experiments have shown that manganese binding to DNA is strong. Therefore, it was of interest to examine the EPR spectrum of the manganese–DNA complex which may have structural implications and also should show the extent to which it might contribute to the measured signal intensity attributed to free manganese. The EPR spectrum of bound manganese was observed with a solution containing 1 m*M* $MnCl_2$ and a large excess of DNA and is shown in Figure 1. The spectrum is similar to the spectra reported for nucleotide complexes of manganese (Reed et al., 1971; Reed and Cohn, 1972) and for Mn–tRNA (Danchin and Guéron, 1970). Its peak to peak amplitude is about 10% that of aquo manganese recorded under similar instrumental conditions and was taken into account in determining the concentrations of free manganese in the binding studies.

Scatchard Plots and Competition. Before presenting the results of the binding studies we briefly outline the approach to the data analysis. The Scatchard equation (Scatchard, 1949) for metal ion binding to a lattice of binding sites, e.g. DNA, has the form

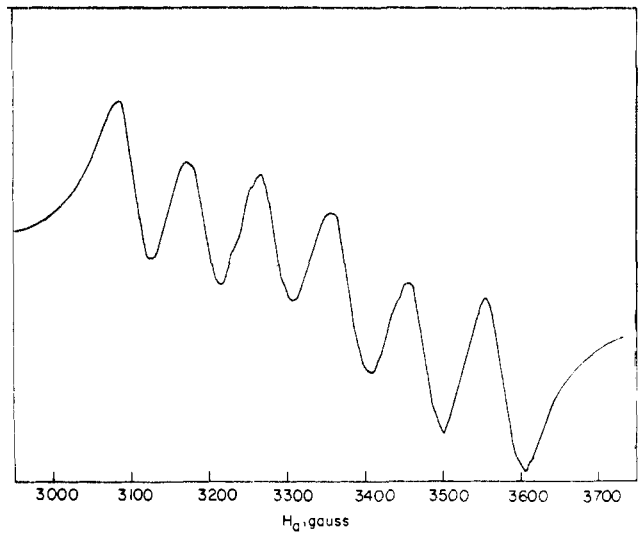


FIGURE 1: The EPR spectrum at X-band of the manganese–DNA complex. (The base-line inclination is an instrumental artifact.)

$$\bar{\nu}_a/[A]_f = K_a(1/n_a - \bar{\nu}_a) \quad (1)$$

where $\bar{\nu}_a = [A]_b/[P]_t$, A denoting the metal ion, the subscripts f, b, and t indicating free, bound and total, respectively, $[P]_t$ is the total phosphate (base) concentration, n_a is the number of phosphates (bases) involved in the binding of one metal ion of type A, and K_a is the association constant. In fact $[P]_t - n_a[A]_b$ is the concentration of free binding sites. Therefore, in the presence of competing ions of type B, eq 1 will be transformed into

$$\bar{\nu}_a/[A]_f = K_a(1/n_a - \bar{\nu}_a - n_b\bar{\nu}_b/n_a) \quad (2)$$

where $\bar{\nu}_b = [B]_b/[P]_t$. Denoting $\bar{\nu}_t = \bar{\nu}_a + n_b\bar{\nu}_b/n_a$ one obtains:

$$\bar{\nu}_a/[A]_f = K_a(1/n_a - \bar{\nu}_t) \quad (3)$$

It should be emphasized that in the present studies the term $n_b\bar{\nu}_b/n_a$ represents only that fraction of bound molecules of type B which competes or interferes with the binding of manganese to DNA. In general this particular fraction may be smaller than the total number of B molecules bound to DNA.

In the absence of competition a plot of $\bar{\nu}_a/[A]_f$ against $\bar{\nu}_a$ will be linear according to eq 1. In the presence of competition a linear plot will be obtained only if $\bar{\nu}_t$ is used as the abscissa; otherwise the plot will be curved. The Scatchard plots for the binding of small molecules and ions to polynucleotides are usually curved due to interactions between the bound species. There are several approximate treatments of this case (Steiner and Beers, 1961). A simple one suggested by Scatchard (1949) involves the expression of the association constant as $K_a = K_a^0 \exp(-W\bar{\nu}_a)$ leading to

$$\bar{\nu}_a/[A]_f = K_a^0 \exp(-W\bar{\nu}_a)(1/n_a - \bar{\nu}_a) \quad (4)$$

For the case involving competition one has $K_a = K_a^0 \exp(-W\bar{\nu}_t)$ obtaining the general expression

$$\bar{\nu}_a/[A]_f = K_a^0 \exp(-W\bar{\nu}_t)(1/n_a - \bar{\nu}_t) \quad (5)$$

Equation 5 was used to analyze the experimental results throughout the present study.

Binding of Manganese in the Presence of Sodium. The Scatchard plots for the binding of manganese in the presence of sodium are shown in Figure 2. Curved plots are obtained at all sodium concentrations studied. It is seen that the values of $\bar{\nu}_{Mn}$ exceed 0.33, indicating $n_{Mn} < 3$. A value

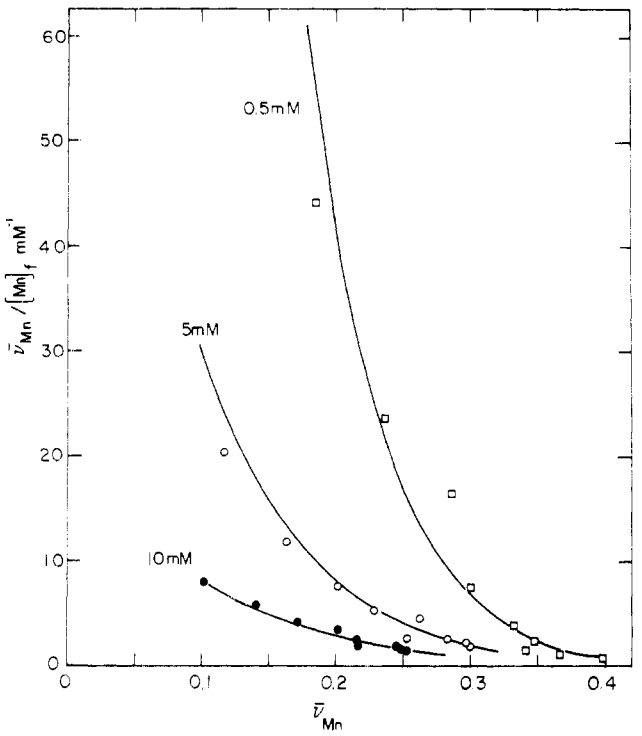


FIGURE 2: The Scatchard plots for the binding of manganese to DNA at different sodium ion concentrations. Curves are calculated (see text).

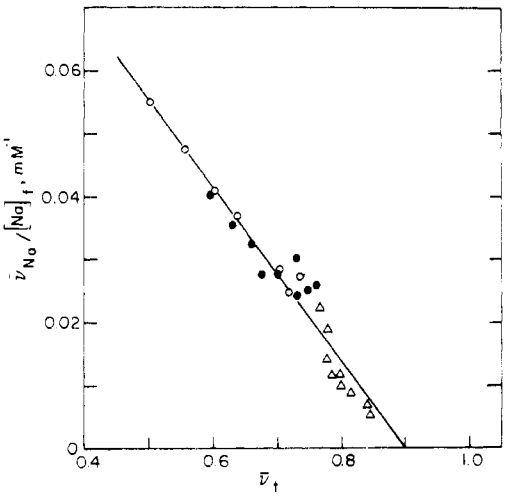


FIGURE 3: The Scatchard plot for the interaction of sodium ions with DNA in the presence of bound manganese: (○) manganese titration at 5 mM sodium; (●) manganese titration at 10 mM sodium; (△) NaCl titration at 0.4 mM manganese. Line calculated by least squares.

of $n_{Mn} = 2$ was therefore assumed in the calculations. The data obtained at the lowest sodium concentration were replotted according to the logarithmic form of eq 4. A plot of ln $[\bar{\nu}_{Mn}/[Mn]_f(0.5 - \bar{\nu}_{Mn})]$ against $\bar{\nu}_{Mn}$ resulted in a straight line from which the constants $K_{Mn}^0 = 2.93 \times 10^6$ M^{-1} and $W_{Mn} = 15.0$ were obtained. Since all the competition experiments were carried out in the presence of 0.5 mM sodium, these constants were used throughout in the analysis of the data. The above values of K_{Mn}^0 and W_{Mn} were used with eq 5 to analyze the data at higher sodium concentrations (5 and 10 mM) and the results of a NaCl titration (5–100 mM) at constant manganese concentration (0.4 mM). Thus, values $\bar{\nu}_f$ were obtained from which $\bar{\nu}_{Na}$

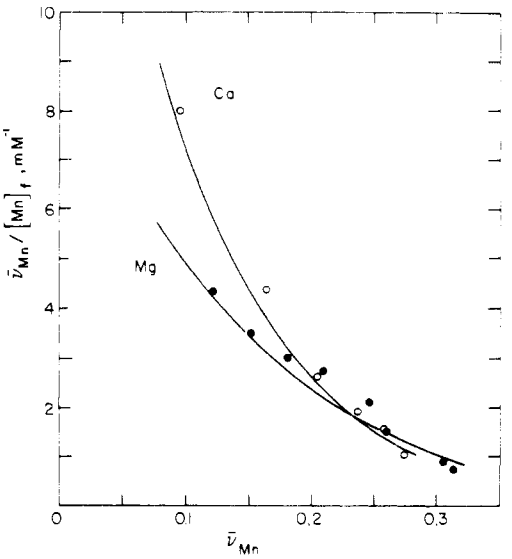


FIGURE 4: The Scatchard plots for the binding of manganese to DNA in the presence of 0.2 mM calcium or magnesium. Curves are calculated (see text).

were calculated. The results are plotted in Figure 3. It is seen that the Scatchard plot is linear, suggesting that $W_{Na} = 0$. A least-squares treatment of the data gave $K^0 = 140$ M^{-1} and $n_{Na} = 1.11$. Finally a simultaneous fit of the three sets of data shown in Figure 2 was attempted. With the assumption that $n_{Na} = 1.0$ and $n_{Mn} = 2$ the following constants were obtained: $K_{Mn}^0 = 3.64 \times 10^6$ M^{-1}, $W_{Mn} = 15.1$, $K_{Na}^0 = 100$ M^{-1}, and $W_{Na} = 0$. The lines corresponding to this set of constants are shown by the smooth curves in Figure 2. The two sets of constants are in good agreement with each other. The level of confidence one may have in them is given by the variance between the two sets (±20%).

The Effects of Magnesium and Calcium. The Scatchard plots for the binding of manganese in the presence of 0.2 mM magnesium and calcium, respectively, are shown in Figure 4. In addition, titrations were carried out with $MgCl_2$ and $CaCl_2$ (0.5–1.0 mM) at a constant manganese concentration (0.2 mM). Values of $\bar{\nu}_t$ and of $\bar{\nu}_L$, where L = Mg or Ca, were calculated in a manner similar to that described for sodium in the preceding paragraph. The Scatchard plots of the results thus obtained are shown in Figure 5. The plots for both magnesium and calcium show curvature suggesting that $W_L \neq 0$. The values of n_L could not be determined unequivocally and $n_{Mg} = n_{Ca} = 2$ were assumed in the calculations. The constants K_L^0 and W_L were obtained from a least-squares analysis of the data recalculated to conform to the logarithmic form of eq 5. The values obtained are given in Table I. The smooth curves drawn in Figures 4 and 5 were calculated with eq 5 using these constants.[2]

The Effects of Organic Cations. The effects of the organ-

[2] It should be pointed out that in competitive binding experiments, the accuracy in determining saturation-dependent phenomena, e.g. the value of W, depends upon the range of saturation ($\bar{\nu}_t$) experimentally covered. The competitive effects of magnesium and calcium in our study cover a relatively narrow range of $\bar{\nu}_t$ (cf. Figure 5) and therefore little weight can be placed on the differences observed in the values of W, in particular when these differences are small. This also results in rather flattened curves for manganese (cf. Figure 4), where the curvature is due mostly to competition (cf. eq 3 and the comments following it).

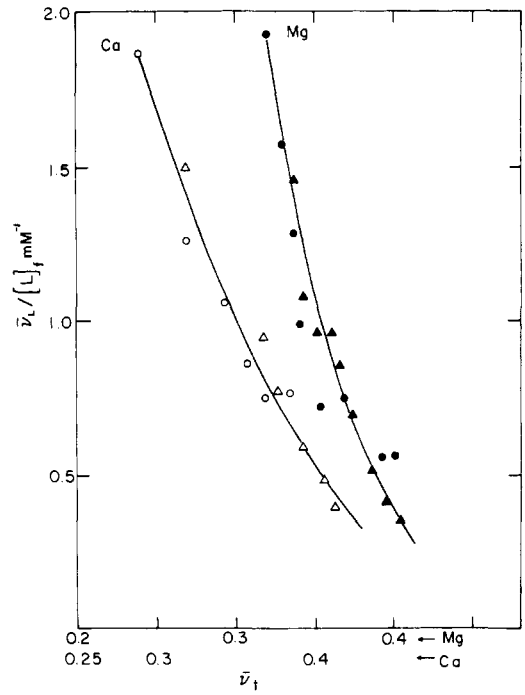


FIGURE 5: The Scatchard plots for the binding of calcium and magnesium to DNA in the presence of bound manganese: circles, manganese titrations at 0.2 mM calcium or magnesium; triangles, $CaCl_2$ or $MgCl_2$ titrations at 0.2 mM manganese. Curves are calculated (see text). Abscissas displaced for clarity.

Table I: Apparent Binding Parameters of Cations to DNA at 23 ± 1°.

Cation	n	K^0, M^{-1}	W
Mn			
a	2	3.64×10^6	15.1
b	2	2.93×10^6	15.0
Mg	2	6.30×10^5	12.75
Ca	2	3.3×10^4	4.53
Na			
a	1	1.0×10^2	0
c	1.11	1.4×10^2	0
1	2	9.9×10^5	14.3
2	1.7	8.9×10^3	0
3	3.0	7.3×10^3	0
4	1.71	1.6×10^4	0

[a] From simultaneous fit of the manganese titrations at 0.5, 5, and 10 mM sodium using eq 5. [b] Obtained from the manganese titration at 0.5 mM sodium using eq 4. These values were used in the analysis of the results of the competition experiments. [c] From a least-squares analysis of data with eq 3.

ic cations **1**, **2**, **3**, and **4** on the binding of manganese to DNA were also investigated. The Scatchard plots for manganese in the presence of 0.1 mM of the competing cations are shown in Figures 6 and 7. It is seen that all of them compete effectively with manganese. The data analysis was carried out by the above-mentioned procedures. It was found that the minimum values of n_L conforming with the data are $n_L > 1$ for compounds **1**, **2**, and **4** and $n_L \simeq 3$ for compound **3**. The plots of $\bar{\nu}_L/[L]_f$ vs. $\bar{\nu}_t$ were linear for compounds **2**, **3**, and **4**, suggesting that $W_L = 0$ for them. A

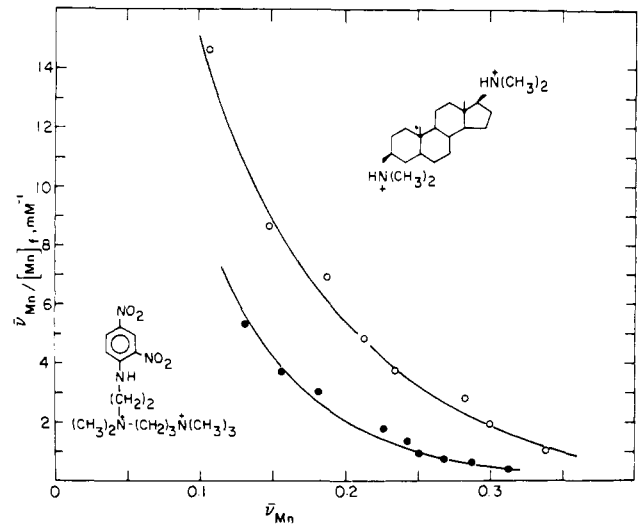


FIGURE 6: The Scatchard plots for the binding of manganese to DNA in the presence of 0.1 mM of compounds **1** and **3**. Curves are calculated (see text).

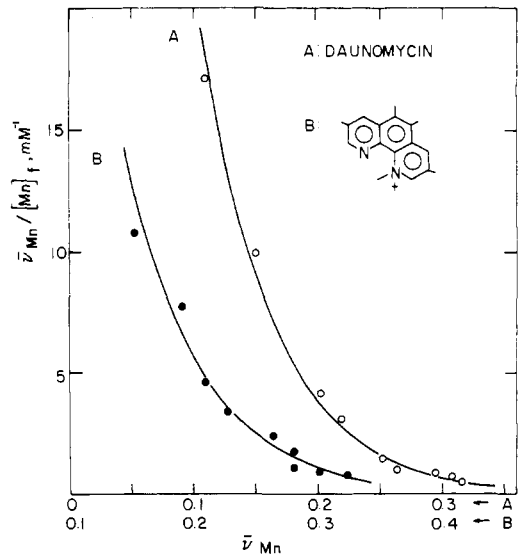


FIGURE 7: The Scatchard plots for the binding of manganese to DNA in the presence of 0.1 mM of compounds **2** or **4**. Curves are calculated (see text). Abscissas displaced for clarity.

similar plot for the steroidal diamine **1** was curved. The constants that gave the smallest deviation of assumed and least-squares calculated value of n_L are summarized in Table I. The smooth curves in Figures 6 and 7 were calculated with eq 5 using these constants.

Discussion

The binding of cations to DNA has previously been analyzed using the exponential form of the Scatchard equation, eq 4, on several occasions (Daune, 1970; Sander and Ts'o, 1971; Clement et al., 1973). The present study seems to be the first time that the competitive effects of other cations, in particular sodium, are being quantitatively considered and apparent binding constants derived. For the divalent metal ions the order of the association constants at zero saturation, K^0, is Mn > Mg > Ca in agreement with many other physical measurements (Felsenfeld and Miles, 1967; von Hippel and McGhee, 1972, and references cited therein). The interaction of sodium with DNA is much weaker and

seems to be independent of saturation ($W_{Na} = 0$). Scatchard's original derivation of eq 4 was for *spherical* macromolecules with net charge spread uniformly over their surface (Scatchard, 1949). In that case the parameter W has an interpretation in the frame of the Debye-Hückel theory. For the cation-DNA interaction W may be seen as originating primarily from electrostatic repulsion. A crude estimate for divalent cations gives $4.2 < W < 8.1$ (Clement et al., 1973). Most of the values obtained in the present study are outside this range.[2] Clement et al. (1973) found that for calf-thymus DNA $W_{Mn} = 6.25$ and $W_{Mg} = 6.25$ and 5.5 determined by two different methods and different sodium ion concentrations. We also note that the values of the apparent association constant, K^0, for manganese and magnesium are higher than those reported in the literature (Daune, 1970; Sander and Ts'o, 1971; Clement et al., 1973). These discrepancies probably reflect the neglect of considering the competitive effects of sodium ions in previous studies.

There are three major mechanisms by which cation binding to DNA could be affected by the extent of saturation (coverage): (a) mutual steric interference between bound species; this mechanism will have the effect of increasing the value of n and is likely to set a lower limit for it; (b) electrostatic repulsion between bound cations with incompletely neutralized charge; this interaction is likely to raise the value of W; (c) local conformational changes induced by the formation of the cation-DNA complex. These may affect all the quantities describing the binding (n, K^0, and W). Conformational changes induced by binding of cations to DNA give rise to changes in the melting temperature, in the ultraviolet absorption, and in the circular dichroism (cf., e.g., Luck and Zimmer, 1972). It seems unlikely that subtle conformational changes will have pronounced effects on the electrostatic interaction of cations with the lattice of phosphate groups of DNA; however, they may affect the interaction with the bases. A plausible origin of the differences in the association constants for the metal ions seems to be in the different extent of their interaction with the nitrogen atoms of the bases. Thus, e.g., it has recently been found that the binding of manganese alters the circular dichroism of DNA at 270 nm, whereas that of magnesium does not (Luck and Zimmer, 1972). The EPR spectrum of the manganese-DNA complex as observed in the present study is very similar to that of manganese-nucleotide complexes (Reed et al., 1971; Reed and Cohn, 1972). Recent proton (Glassman et al., 1971; Anderson et al., 1971) and carbon-13 (Kotowycz and Hayamizu, 1973; Lam et al., 1974) nuclear magnetic resonance (NMR) studies have indicated that manganese interacts with the N-7 base nitrogen of purine nucleotides and nucleosides. The differences in the binding characteristics obtained in the present study suggest that the relative importance of coordination to the base nitrogen (N-7) decreases along the series Mn^{2+}, Mg^{2+}, Ca^{2+}, Na^+. Further comments regarding the nature of the sodium-DNA interaction are presented together with the results of NMR study of alkali metal nuclei (Reuben et al., 1975). At this point we note only that the apparent association constant for sodium obtained in the present study is in good agreement with those obtained from other competitive binding studies (Passero et al., 1970) and from ^{23}Na NMR (Reuben et al., 1975).

The constants describing the binding of calcium and of the organic cations **2–4** obtained by us via competition with manganese are lower than those previously determined using direct methods (Daune, 1970; Sanford et al., 1975; Gabbay and Levy, 1975). This apparent discrepancy suggests that not all of the DNA binding sites for these cations can accommodate manganese and that the mode of the manganese-DNA interaction is different (vide infra).

It has been shown that the phenanthrolinium cation **2**, the dinitroaniline ring of **3**, and the anthracycline ring of daunomycin **4** intercalate between base pairs of DNA (Gabbay et al., 1973; Passero et al., 1970; Pigram et al., 1972) and evidence has been presented which suggests that the diammonium side chain of **3** (Gabbay and Sanford, 1974) and the amino sugar moiety of daunomycin (Gabbay and Levy, 1974) lie in the minor groove of the DNA helix. It is thus reasonable to conclude from the present competition studies that the binding site for manganese is probably *not* between base pairs or in the minor groove. On the other hand the steroidal diamine **1** is the only one of the organic cations investigated, for which the association constant as determined by equilibrium dialysis (Sanford et al., 1975) is of the same order of magnitude as that obtained now by competition with manganese, suggesting that these two cations occupy similar sites on DNA. Evidence presented by Sanford et al. (1975) shows that the steroidal diamine **1** binds to the major groove of the DNA helix. These facts, together with the EPR and NMR spectral evidence (vide supra) for the manganese interaction with the base N-7 (located in the major groove), lead to the conclusion that the plausible locale for the manganese binding site on DNA is the major groove of the helix.

The present work has shown that, via comparison of results of competitive and direct binding studies, salient features of the interaction specificities of small molecules with DNA can be revealed.

References

Anderson, J. A., Kuntz, G. P. P., Evans, H. H., and Swift, T. J. (1971), *Biochemistry 10,* 4368.

Clement, R. M., Sturm, J., and Daune, M. P. (1973), *Biopolymers 12,* 405.

Cohen, G., and Eisenberg, H. (1966), *Biopolymers 4,* 429.

Cohn, M., and Townsend, J. (1954), *Nature (London) 173,* 1090.

Danchin, A., and Guéron, M. (1970), *Eur. J. Biochem. 16,* 532.

Daune, M. (1970), *Stud. Biophys. 24/25,* 287.

Eichhorn, G. L. (1973), in Inorganic Biochemistry, Eichhorn, G. L., Ed., Amsterdam, Elsevier, Chapter 34.

Eisenberg, H. (1974), in Basic Principles in Nucleic Acid Chemistry, Vol. II, Ts'o, P. O. P., Ed., New York, N.Y., Academic Press, Chapter 3.

Felsenfeld, G., and Miles, H. T. (1967), *Annu. Rev. Biochem. 36,* 407.

Gabbay, E. J., and De Paolis, A. (1971), *J. Am. Chem. Soc. 93,* 562.

Gabbay, E. J., and Glaser, R. (1971), *Biochemistry 10,* 1665.

Gabbay, E. J., and Levy, R. (1975), *Biochem. Pharmacol.* (1975), submitted for publication.

Gabbay, E. J., and Sanford, K. (1974), *Bioorg. Chem. 3,* 91.

Gabbay, E. J., Scofield, R., and Baxter, C. S. (1973), *J. Am. Chem. Soc. 95,* 7850.

Glassman, T. A., Cooper, C., Harrison, L. W., and Swift, T. J. (1971); *Biochemistry 10,* 843.

Izatt, R. M., Christensen, J. J., and Ryttig, J. H. (1971),

Chem. Rev. 71, 439.
Kotowycz, G., and Hayamizu, K. (1973), *Biochemistry 12,* 517.
Lam, Y. F., Kuntz, G. P. P., and Kotowycz, G. (1974), *J. Am. Chem. Soc. 96,* 1834.
Luck, G., and Zimmer, C. (1972), *Eur. J. Biochem. 29,* 528.
Passero, F., Gabbay, E. J., Gaffney, B. L., and Kurucsev, T. (1970), *Macromolecules 3,* 158.
Pigram, W. J., Fuller, W., and Hamilton, L. D. (1972), *Nature (London) New Biol. 235,* 17.
Reed, G. H., and Cohn, M. (1972), *J. Biol. Chem. 247,* 3073.
Reed, G. H., Leigh, J. S., Jr., and Pearson, J. E. (1971), *J. Chem. Phys. 55,* 3311.
Reichman, M. E., Rice, S. A., Thomas, C. A., and Doty, P. (1954), *J. Am. Chem. Soc. 76,* 3047.
Reuben, J., and Cohn, M. (1970), *J. Biol. Chem. 245,* 6539.
Reuben, J., Shporer, M., and Gabbay, E. J. (1975), *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Sander, C., and Ts'o, P. O. P. (1971), *J. Mol. Biol. 55,* 1.
Sanford, K., Scofield, R. E., Adawadkar, P. D., and Gabbay, E. J. (1975), *Arch. Biochem. Biophys.,* submitted for publication.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Slater, J. P., Tamir, I., Loeb, L. A., and Mildvan, A. S. (1972), *J. Biol. Chem. 247,* 6784.
Steiner, R. F., and Beers, R. F., Jr. (1961), Polyneucleotides, Amsterdam, Elsevier, Chapter 10.
Von Hippel, P. H., and McGhee, J. D. (1972), *Annu. Rev. Biochem. 41,* 231.

Endonuclease II of *Escherichia coli:* Degradation of γ-Irradiated DNA†

D. M. Kirtikar, J. Slaughter, and D. A. Goldthwait*,‡

ABSTRACT: Irradiation of DNA in a nitrogen atmosphere with ^{60}Co γ-radiation produces at least two types of damage. The first type leads to single strand breaks in the DNA observed *after* exposure to alkali. This type of alkali-labile bond will be designated a spontaneous break. The second type of damage to DNA is an alteration which makes the DNA susceptible to phosphodiester bond hydrolysis by a 1600-fold purified preparation of endonuclease II of *Escherichia coli* and is designated an enzyme-sensitive site. This site is not alkali-labile. After irradiation, preincubation of the DNA either for days at 0° or for 4 hr at 37° increases both the spontaneous breaks and the enzyme sensitive sites. There is a greater increase of spontaneous breaks when the preincubation is in O_2 compared to N_2. The increase of enzyme sensitive sites due to the preincubation is not altered significantly by O_2. The increase of spontaneous breaks during the preincubation is almost completely prevented by addition of either $NaBH_4$ or NH_2OH after the irradiation. The treatment can be before or after the preincubation. This effect indicates that these breaks are due to alkali-labile bonds possibly produced by depurination or depyrimidination reactions. That the spontaneous breaks are due primarily to alkali-labile bonds is supported by an experiment in which formamide gradients were used. Neither $NaBH_4$ nor NH_2OH has any effect on the enzyme sensitive sites. Addition of β-mercaptoethanol (0.5 M) at the start of the preincubation prevents in part the appearance of both spontaneous breaks and enzyme-sensitive sites. It has no effect when added at the end of the preincubation. Catalase added before the preincubation has no effect on either type of damage. It is postulated that the spontaneous breaks occur because purine or pyrimidine radicals are formed (possibly hydroxyl radicals) which can then interact with oxygen to produce unstable intermediates. These intermediates then undergo either depurination or depyrimidination. The subsequent alkali catalyzed β-elimination reaction of depurinated or depyrimindinated DNA is prevented by $NaBH_4$ or NH_2OH. An alternative hypothesis would involve damage to the sugar rather than to bases. The enzyme-sensitive sites represent another form of base damage which is not oxygen dependent. The chemical nature of either form of primary damage is not known.

Enzymatic repair of DNA treated with physical or chemical agents can occur through the replacement of altered bases. One of the first steps involves an endonuclease. Two endonucleases have been described to date which recognize such altered DNA. One endonuclease recognizes thymine dimers (Kaplan *et al.,* 1971) and probably also DNA reacted with one or more specific chemical carcinogens (Kondo and Kato, 1968, Van Lancker and Tomura, 1974). Endonuclease II of *Escherichia coli* recognizes DNA which has reacted with various alkylating agents (Kirtikar and Goldthwait, 1974). Both of these enzymes produce endonucleolytic phosphodiester bond hydrolysis near the altered bases.

Endonuclease II of *E. coli* has been purified 1600-fold (Hadi *et al.,* 1973) on the basis of its ability to hydrolyze phosphodiester bonds in DNA alkylated with methyl methanesulfonate. It also degrades DNA reacted with ni-

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received September 4, 1974.* Supported by grants from the National Institutes of Health (CA-11322), The Health Fund of Greater Cleveland, and the Cuyahoga Unit of the American Cancer Society.

‡ National Institutes of Health Research Career Award Fellowship K6-6M-21444.