Interaction of the 5-Methylphenazinium Cation Radical with Deoxyribonucleic Acid*

K. Ishizu,† H. H. Dearman,‡ M. T. Huang, and J. R. White

ABSTRACT: The complexes of deoxyribonucleic acid with the cation 5-methylphenazinium and the 5-methylphenazinium cation radical were studied by ultraviolet-visible and electron spin resonance spectrometry. The 5-methylphenazinium cation was less strongly bound than the 5-methylphenazinium cation radical (association constants were, respectively, 0.4×10^6 and 2.6×10^6 M^{-1}) and had fewer binding sites per deoxyribonucleic acid nucleotide (0.13 *vs.* 0.26). In addition a weaker type of association was found for each cation, the binding by the radical again being the stronger (association constants 0.6×10^4 and 2.3×10^4 M^{-1}). The binding was reversed by raising the ionic strength, or, more strongly, by the presence of divalent cations. These characteristics are shared by proflavine, ethidium bromide, and other cationic ligands for which an intercalative model for the strong binding has been proposed. Association of the the 5-methylphenazinium cation radical with deoxyribonucleic acid strikingly stabilizes it against air oxidation and causes a shift in the Michaelis equilibrium toward the semireduced species. Single-stranded (heat-denatured) deoxyribonucleic acid shows very little tendency to bind either cation strongly, an observation which may be related to the weakness of these species as frame-shift mutagens.

Interest is increasing in the biological activity of synthetic and naturally occurring phenazine derivatives. Of these the most widely known is the compound 5-methylphenazinium methyl sulfate, which has been used as an electron-transfer agent in flavoprotein assays (Singer, 1963) and in the study of photosynthetic systems (Geller and Lipmann, 1960; Cost *et al.*, 1967). At sufficiently high concentrations phenazine methosulfate is bactericidal, largely because it stimulates the generation of hydrogen peroxide. At sublethal concentrations in cultures of *Escherichia coli* it rapidly stops the synthesis of all macromolecules by accepting electrons and shunting them directly to O_2 without formation of ATP (M. T. Huang and J. R. White, unpublished data). This action of phenazine methosulfate has made it a useful tool in studying the bactericidal effects of the antibiotics streptonigrin and mitomycin C, which are activated by intracellular reduction, and whose lethality is antagonized by phenazine methosulfate (White and White, 1965). Observations made with electron spin resonance spectroscopy have shown that the MP^+ [1] cation is reduced in anaerobic suspensions of bacterial cells to a readily observed free-radical form (White and Dearman, 1965; Ishizu *et al.*, 1968).

A number of naturally occurring derivatives of phenazine have been isolated, some of which have antibiotic activity (Umezawa, 1964). Certain synthetic derivatives, such as 1,3-diamino-5-methylphenazinium chloride, have shown promise as antitumor agents (Anstall *et al.*, 1967).

In view of the growing interest in phenazine derivatives as biological agents, we have investigated the interaction of phenazine methosulfate with nucleic acids (Ishizu *et al.*, 1966). We report here the effect of nucleic acids on the optical spectrum of MP^+. In addition the stability in aqueous buffers of the free radical derived from the MP^+ cation has enabled us to study its complex with DNA by effects on the electron spin resonance spectrum of the radical. This technique was used by Ohnishi and McConnell (1965) in studying the complex between DNA and the chlorpromazine radical cation. Our results are compared with previous studies of nucleic acid complexes with the structurally similar acridine dyes.

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[1] Abbreviations used: MP^+, the 5-methylphenazinium cation (Figure 4); $MPH\cdot^+$, the 5-methylphenazinium cation radical; $MP\cdot$, the 5-methylphenazyl radical; MPH, fully reduced MP^+. MP represents all of the preceding species which may be present in a mixture.

Materials and Methods

Preparation of Samples for Optical and Electron Spin Resonance Spectrometry. Stock solutions of salmon sperm DNA (Calbiochem) and *Escherichia coli* sRNA (General Biochemicals) were prepared by extensive dialysis against 0.001 M NaCl. Phenazine methosulfate (Sigma Chemical Co.) was used without further purification. Denatured DNA was prepared from the stock solution (1 mg/ml) by heating at 100° for 15 min and then cooling rapidly to 0°. Denaturation caused an increase in absorbance at 260 mμ of about 30%. Deaeration of solutions was accomplished by bubbling nitrogen through them for several minutes. A fresh stock solution of phenazine methosulfate in sodium acetate buffer (0.001 M, pH 6.0) was prepared each day, and the stock solution was kept at 0° in the dark. Sodium borohydride (12.5 mg) was dissolved in 250 ml of deionized water and kept in ice.

In order to prepare samples for electron spin resonance observations under anaerobic conditions, a Varian aqueous sample cell was fitted at the top and bottom with stopcocks through which the cell could be purged with nitrogen. Sealed vertically to the upper stopcock was a length of glass tubing in which the sample was prepared and deaerated prior to introduction into the sample cell. Phenazine methosulfate solution (1 ml; 0.012 M) and sodium acetate buffer (0.8 ml; 0.001 M, pH 6.0) were placed in the sample preparation tube. After the mixture had been deaerated for 2 min, it was reduced with 0.6 ml of sodium borohydride solution. Upon reduction the color of the solution changed from yellow to green. The pH was maintained at 6.0 by the addition of 0.1 ml of 0.01 M acetic acid. After 3 min of further deaeration 1.5 ml of previously deaerated DNA solution was mixed with the solution of reduced phenazine methosulfate, and the green color turned to golden yellow. After further deaeration the radical solution was introduced into the cell, and electron spin resonance measurements were carried out at room temperature with a Varian Model 4502 spectrometer operating with 100-kc magnetic field modulation.

For observation of the optical spectrum 3 ml of the radical–DNA mixture were prepared in a standard quartz cuvet with a 1-cm light path. After deaeration the cuvet was fitted with a tapered glass stopper, and the visible spectrum was recorded with a Cary Model 14 spectrophotometer.

Standardization of the Radical Concentration. In order to establish the relationship between the radical concentration and electron spin resonance signal intensity, electron spin resonance spectra were taken of radicals generated in 0.2 M aqueous acetic acid (pH 3.4). At this pH, according to Zaugg (1964), phenazine methosulfate can be reduced almost quantitatively to the free radical.

The electron spin resonance spectrum was recorded at low modulation (setting 20, 0.03 G), and the signal intensity, measured as the height in centimeters of the hyperfine line in the center of the recorded spectrum, was plotted against the concentration of radicals (Figure 1). A strictly linear relationship was obtained over the entire range of radical concentrations for which observations were made. No qualitative difference could be detected between the electron spin resonance hyperfine structure observed at pH 3.0 and that observed at pH 6.0. Therefore it was assumed that the relationship between radical concentration and signal intensity which holds at pH 3.4 is also valid for estimating radical concentrations at pH 6.0. The absorption of radicals complexed with DNA is broadened so much (due to their association with the large, slowly tumbling DNA molecules) that at low modulation the signal is indistinguishable from the noise level; hence only the concentration of unbound radical can be determined from the signal intensity of the electron spin resonance hyperfine line. In order to observe radicals bound to DNA, it is necessary to carry out the electron spin resonance experiments under conditions of high modulation (0.3 G, setting 200). The total concentration of radicals (both bound and unbound) is then proportional to the total amount of absorbed microwave energy. To estimate the absorbed energy the recorded spectrum (which is the first derivative of the absorption spectrum) was integrated, and the area under the resultant curve was evaluated graphically.

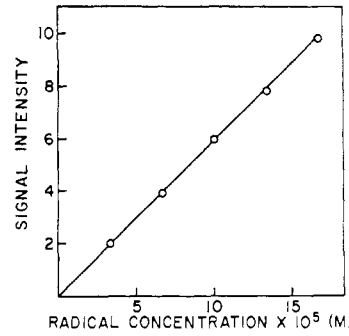

FIGURE 1: Relationship between electron spin resonance signal intensity and $MPH\cdot^{+}$ radical concentration. MP^{+} (5 mg) was dissolved in 50 ml of 0.2 M acetic acid. The solution was deaerated by nitrogen bubbling for 10 min. MP^{+} was reduced with excess sodium borohydride (60 mg). The radical solution was diluted under nitrogen with 0.2 M deaerated acetic acid to give the concentrations shown on the graph. The radical solutions were transferred anaerobically to the aqueous sample cell of the electron spin resonance spectrometer and low-modulation spectra were recorded at room temperature.

Determination of the Binding Parameters. The association constant for complex formation, k, and the maximum number of binding sites per mononucleotide, n, are evaluated from plots of $\bar{B}/F$ against $\bar{B}$, where $\bar{B}$ is the average number of bound ligands per nucleotide, and F is the molar concentration of free ligand. If all binding sites are equivalent and noninteracting, such plots are straight lines, according to the expression $\bar{B}/F = k(n - \bar{B})$. Optical spectrophotometry was used to study complex formation between MP^{+} and DNA. The fraction of the total ligand bound, α, was calculated from the expression $\alpha = (A_f - A)/(A_f - A_b)$, where A_f and A_b are the absorbances of the ligand when it is present entirely in the free or bound form, respectively, and A is the observed absorbance of the mixture.

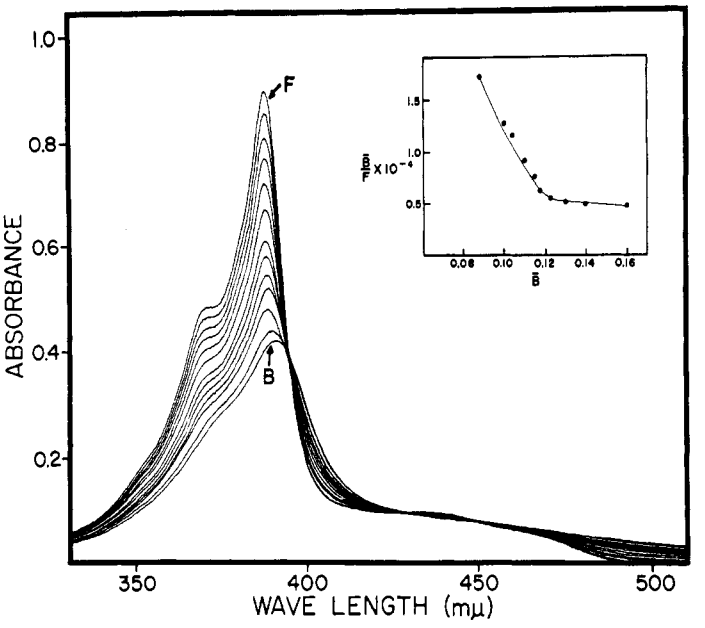

FIGURE 2: Absorption spectra of MP^+ in the presence of native DNA. Solutions of MP^+ were mixed with native salmon sperm DNA in sodium acetate buffer (0.001 M, pH 6.0) to give various molar ratios r = DNA:MP^+. The curves reading from top to bottom were characterized by values of r = 0(F), 0.5, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 15, and 30(B), where the concentration of MP^+ was always equal to 3.6×10^{-5} M. The absorption spectra were recorded in a standard 1-cm quartz cuvet. Inset: $\bar{B}/F$ *vs.* $\bar{B}$ plot of data derived from these spectra according to the procedure described in Methods.

$\bar{B}$ and F were determined from the relations $\bar{B} = (T/P)$, and $F = T(1 - \alpha)$, where T is the total molar concentration of ligand and P is the molar concentration of DNA nucleotide.

To study complex formation between $MPH\cdot^+$ and DNA, electron spin resonance observations were interpreted as follows. The concentration, F, of unbound radical was determined from the signal intensity of the central hyperfine line recorded at a modulation amplitude of 0.03 G. The total radical concentration, T, was determined by integration of the spectrum recorded with 0.3-G modulation. The average number of ligands per nucleotide was calculated as $\bar{B} = (T - F)/P$.

Results

Binding of MP^+ to DNA. The optical absorption spectra of solutions containing DNA and various concentrations of MP^+ are shown in Figure 2. In the absence of DNA (curve F) the molar absorptivity of MP^+ at the 388 maximum is 25,500 M^{-1} cm^{-1}. This value is comparable with 26,300 M^{-1} cm^{-1} reported by Zaugg (1964). The stepwise addition of DNA causes a progressive red shift and depression of this absorption maximum. An isosbestic point occurs at 394 mμ. No further change in the MP^+ spectrum is noted beyond a DNA:MP^+ mole ratio of 30:1 (curve B), at which point complete binding is assumed to occur. The fraction of total ligand which is bound was calculated from the data at 388 mμ using the procedure described in Materials and Methods, and the binding plot is shown in Figure 2 (inset). The two linear regions of this plot can be interpreted as disclosing the presence of two distinct types of site on the nucleic acid, one involving a small number of ligands with strong binding (type I) and the other a larger number with weaker binding (type II). The parameters which characterize the binding of MP^+ to native DNA are presented in Table I.

TABLE I: Binding Parameters for Complexes of 5-Methylphenazinium Species with Nucleic Acids.[a]

Nucleic Acid	Ligand	n_I	$k_I \times 10^{-6}$ M^{-1}	n_{II}	$k_{II} \times 10^{-4}$ M^{-1}
DNA	MP^+	0.13	0.4	0.60	0.6
DNA	$MPH\cdot^+$	0.26	2.6	0.70	2.3
dDNA	$MPH\cdot^+$			1.20	0.6
RNA	$MPH\cdot^+$	0.15	2.8	1.0	0.9

[a] All measurements were made at ionic strength, 0.001 M, pH 6.0. The MP^+ studies were carried out using ultraviolet-visible spectrometry, the others using electron spin resonance spectrometry. The dDNA was heat denatured.

The difference in effect on the MP^+ absorption spectrum of native and heat-denatured DNA is apparent from Figure 3. Curve B, resulting from addition of denatured DNA, is much more similar to that of the uncomplexed dye (curve A) than to the spectrum of the complex of MP^+ with native DNA (curve C). In fact, the small depression of curve B relative to curve A may be caused by slight adventitious renaturation upon cooling. Although we were thus unable to determine binding constants for MP^+ with denatured DNA by use of the technique previously described, we interpret the results shown in Figure 3 to indicate much weaker binding.

Interaction of $MPH\cdot^+$ with Nucleic Acids. The addition of sodium borohydride to a solution of phenazine methosulfate leads to the establishment of a Michaelis equilibrium involving the fully oxidized, fully reduced, and semireduced 5-methylphenazinium species, and the scheme proposed by King (1963) is shown in Figure 4. The electron spin resonance spectrum of the

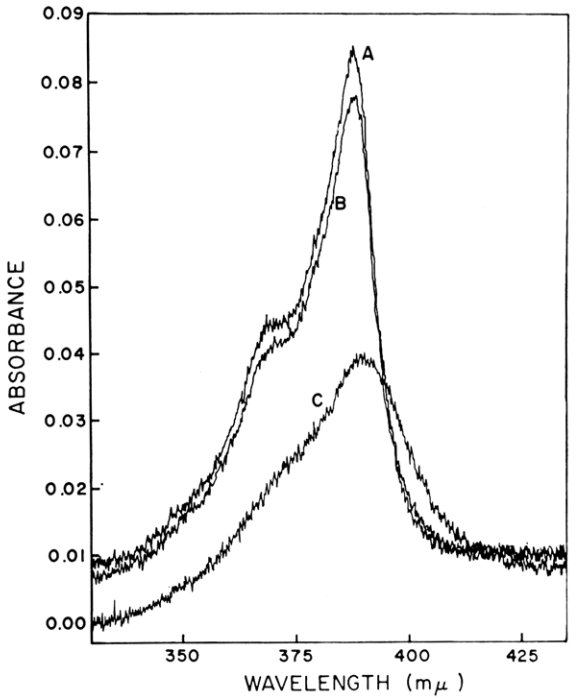

FIGURE 3: Comparison of the effects of native and denatured DNA on the spectrum of phenazine methosulfate. Concentrations were: phenazine methosulfate, 3.3×10^{-6} M; DNA, 70 μg/ml. The corresponding ratio MP^+:DNA nucleotide is 1:65. Curve A: phenazine methosulfate; curve B: phenazine methosulfate + denatured DNA; curve C: phenazine methosulfate + native DNA.

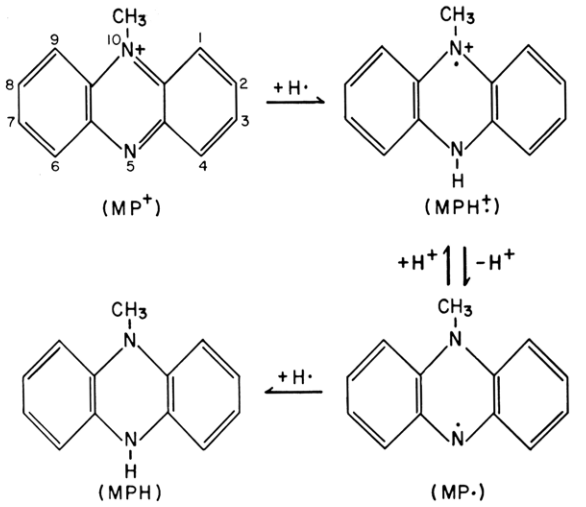

FIGURE 4: A proposed mechanism for the reduction of MP^+ by two successive one-electron transfer reactions (King, 1963). The symbols in parentheses are the abbreviations used in the text to designate the corresponding molecular species.

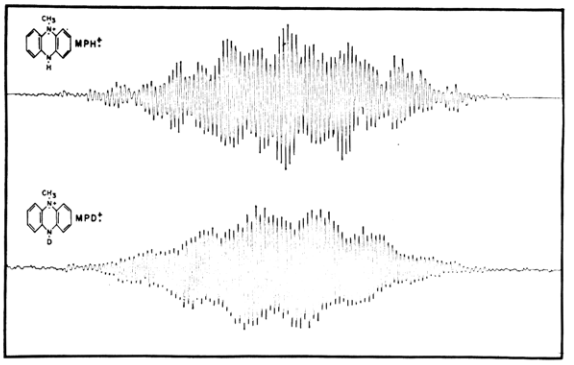

FIGURE 5: High-resolution electron spin resonance spectra of the radicals $MPH\cdot^+$ and $MPD\cdot^+$. MP^+ (0.75 mg) was dissolved in 10 ml of 0.1 M CH_3COOH–H_2O or CH_3COOH–D_2O solution. Solutions of the radicals were generated at pH 3.4 by reduction with 1.0 mg of sodium borohydride using anaerobic conditions as described under Figure 1. The electron spin resonance spectra were recorded at room temperature under low-modulation conditions (0.03 G).

radical obtained at pH 3.4 is shown in Figure 5. An identical spectrum is obtained at pH 6.0, the pH at which most of our studies were carried out. It is necessary to determine whether the semireduced species present at the pH employed in this work is the cation $MPH\cdot^+$ or the uncharged MP·, since a strong ionic interaction with the nucleic acid would be expected only for the cationic species shown in the King scheme. Proof that the protonated cation is the paramagnetic species present in the pH range 3.4–6.0 is obtained from a comparison of the proton hyperfine splittings in the electron spin resonance spectrum of the radical in H_2O and in D_2O. The N_5 position is the only one for which proton exchange with the solvent is likely to occur. Hence (Figure 5) the marked change in the hyperfine splitting pattern caused by introduction of the deuterated solvent demonstrates that the species is $MPH\cdot^+$ in H_2O and $MPD\cdot^+$ in D_2O. A complete analysis (to be published elsewhere) of the hyperfine splittings in these two cation radicals and the deuteriomethyl cogeners fully supports this conclusion.

Zaugg (1964) found that MP^+ can be reduced quantitatively to the cation radical at pH 3, while the yield of $MPH\cdot^+$ at pH 6 is about 40%. From the relative strengths of the electron spin resonance signals at these pH values we find a radical yield of 45% at pH 6.0 in the absence of nucleic acid, in good agreement with Zaugg's measurements. Upon addition of DNA at the same pH the over-all concentration of free radicals is markedly increased. The absorption spectra of semireduced phenazine methosulfate at pH 5.9 and 3.4 are shown in Figure 6 (curves A and C) along with the spectrum at pH 5.9 in the presence of DNA (curve B). DNA causes a decrease in the intensity of the absorption of MP^+ at 388 mμ and an enhancement of the absorptions of the cation radical at 371 and 446 mμ, indicating that under these conditions of preparation the presence of DNA favors stability of the radical $MPH\cdot^+$ relative to the MP^+ species. A red shift of approximately 6 mμ in the radical absorption is also noted. The absorbance of the radical in the mixture becomes comparable with that of the radical alone at pH 3.4. While these spectra give clear evidence for interaction

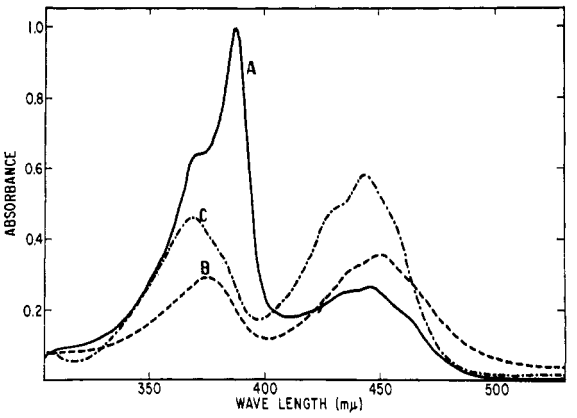

FIGURE 6: Absorption spectra of the radical $MPH^{\cdot+}$, free and bound to native DNA. MP^+ solution (1.0 ml; 2.0×10^{-4} M) and sodium acetate buffer solution (0.23 ml; 0.001 M, pH 6.0) were placed in a standard quartz cuvet, and the mixture was deaerated by nitrogen bubbling for 2 min. MP^+ was reduced to $MPH^{\cdot+}$ by addition of 0.24 ml of sodium borohydride (0.05 mg/ml). The pH value was adjusted to 6.0 with 0.03 ml of acetic acid (0.01 M). Following an additional 3 min of deaeration, 1.5 ml of deaerated sodium acetate buffer solution or DNA solution (0.686 mg/ml) was mixed with the radical solution. After further deaeration the cuvet was fitted with a glass stopper and the spectrum was recorded at room temperature. The final concentration of MP^+ was 6.6×10^{-5} M, pH 5.9. (A) No DNA, pH 5.9; (B) DNA present, pH 5.9, molar ratio MP^+: DNA = 1:16; (C) no DNA, pH 3.4.

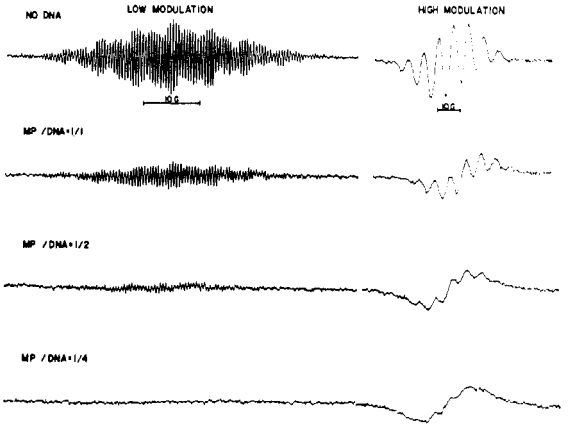

FIGURE 7: Electron spin resonance spectra of $MPH^{\cdot+}$ bound to native DNA. $MPH^{\cdot+}$ solution (2.5 ml) was prepared at pH 6.0 as described in Materials and Methods; 1.5 ml of deaerated sodium acetate buffer solution (0.001 M, pH 6.0) or various concentration of calf thymus DNA solutions were added to the $MPH^{\cdot+}$ solution. In each case the total concentration of the methylphenazinium species was equal to 3.0×10^{-4} M. The electron spin resonance spectra were recorded at room temperature at low modulation (0.03 G) and at high modulation (0.3 G).

between the radical and DNA, they are not very suitable for quantitative binding studies because the various species have overlapping spectra. Such studies proved feasible using electron spin resonance spectra.

The electron spin resonance spectra of the DNA–$MPH^{\cdot+}$ mixtures under two conditions of modulation are shown in Figure 7. When recorded under low-modulation conditions, a decrease in the sharp, structured signal of free $MPH^{\cdot+}$ with increasing DNA concentrations is observed. When a high-modulation amplitude is used, the broad signal due to the bound radical is superimposed on the modulation-broadened spectrum of the unbound species. At high DNA concentration only the broad signal of the bound radical is observed. The great difference in width and detailed hyperfine splittings for the bound and unbound radical is due to the differences in the Debye correlation times of the $MPH^{\cdot+}$ ion and the DNA. (The Debye correlation time of a molecule is a measure of its rotational mobility.) When the ion is free, a correlation time of 10^{-10} sec is expected, and this fast tumbling rate averages out anisotropies in both the hyperfine and the Zeeman interactions, leading to well-resolved signals. However, when the radical is bound to a polymer with a correlation time in the range 10^{-4}–10^{-5} sec, these anisotropies do not average to zero but lead to a smearing out of the hyperfine pattern and a broadening of the spectrum. A spin–lattice relaxation time of 4×10^{-9} sec due to these effects is calculated from the line width (17 G) of the DNA–$MPH^{\cdot+}$ electron spin resonance spectrum.

The increase in total radical concentration caused by

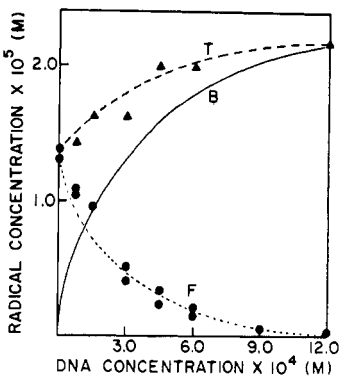

FIGURE 8: Dependence of the concentrations of bound, free, and total $MPH^{\cdot+}$ upon the concentration of DNA. The total concentration, T, of $MPH^{\cdot+}$ was determined from the intensity of the electron spin resonance signal at 0.3-G modulation. The concentration, F, of free $MPH^{\cdot+}$ was determined from the signal intensity at 0.03-G modulation. The concentration of the bound radical, B, was determined by difference as $B = T - F$. Experimental conditions for the electron spin resonance measurements were as described in Figure 7.

DNA complexing, which was revealed by the optical spectrum, is also observed in the electron spin resonance experiments. Figure 8 shows the concentrations of free (F) and bound (B) forms of the radical in the presence of various concentrations of DNA. At high nucleic acid concentration more than 80% of the total 5-methylphenazinium is in the free-radical form. Thus the binding of the radical cation to DNA is stronger than that of the oxidized cation, and the Michaelis equilibrium between the various oxidation states of 5-methylphenazinium is displaced toward the radical.

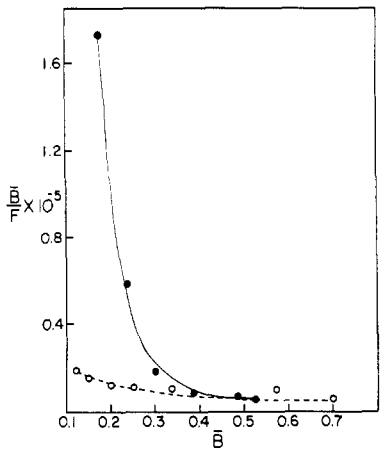

FIGURE 9: Plots of $\bar{B}/F$ against $\bar{B}$ for the interaction of MPH·$^+$ with native and denatured DNA. The curve for native DNA (●—●) was derived from the data of Figure 8. The curve for denatured DNA (○—○) was derived from data obtained using the experimental procedure of Figure 7.

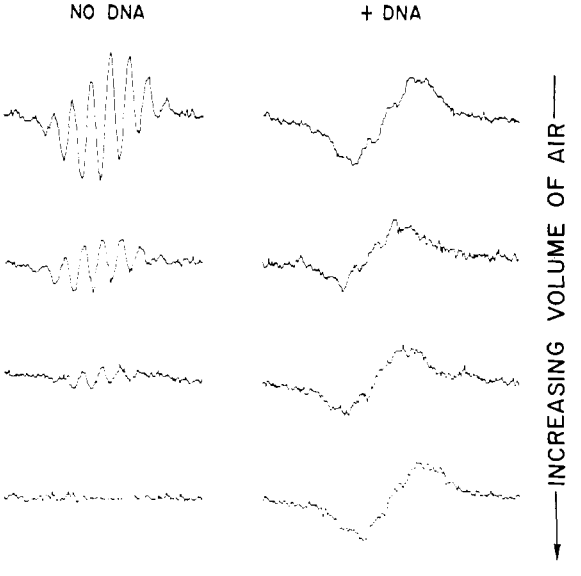

FIGURE 10: Decay of electron spin resonance signal intensity of MPH·$^+$ unbound or bound to DNA upon exposure to air oxidation. The MPH·$^+$ and MPH·$^+$–DNA solutions were prepared under the experimental conditions described in Materials and Methods. Initial 5-methylphenazinium concentration was 3.0×10^{-4} M, MP:DNA ratio was 1:4, and total volume was 4 ml at pH 6.0. The electron spin resonance signal was recorded under high-modulation conditions (0.3 G) without air oxidation or after passage of air through the solution. From top to bottom the curves show the signal after (a) no exposure to air; (b) passage of 8 ml of air; (c) 12 ml of air; (d) 16 ml of air. Rate of air flow was 1.3 ml/min.

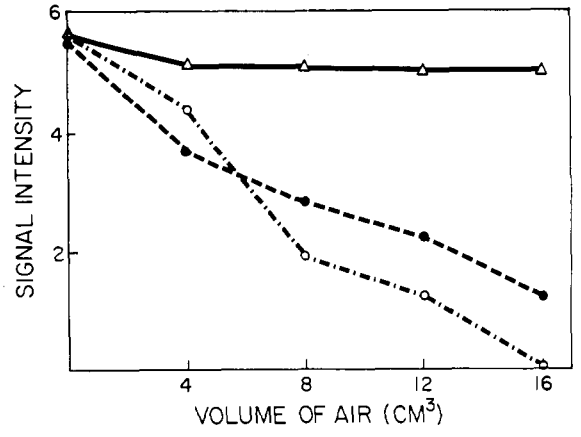

FIGURE 11: Effect of Mg^{2+} on the stability of the DNA–MPH·$^+$ complex to air oxidation. The electron spin resonance signal intensities of the MPH·$^+$ radical, prepared as in Figure 10, are expressed in arbitrary units. DNA present (△—△); DNA absent (○—·—○); DNA and $MgCl_2$ (0.01 M) present (●— —●).

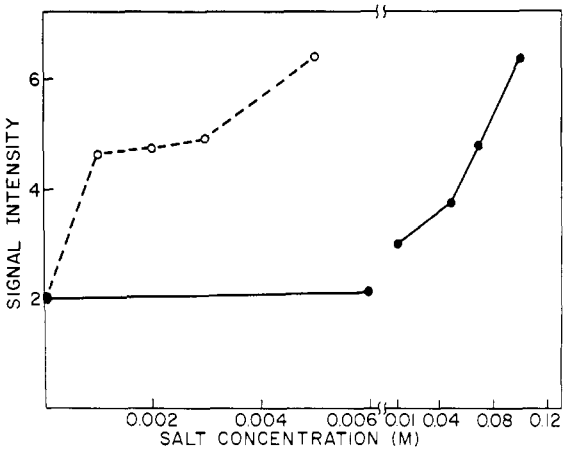

FIGURE 12: Effect of $MgCl_2$ and NaCl on the low-modulation electron spin resonance signal of MPH·$^+$ in the presence of DNA. Phenazine methosulfate solution (1.0 ml) was placed in the reaction tube along with 0.8 ml of sodium acetate buffer (0.001 M, pH 6.0) containing NaCl or $MgCl_2$. Reduction was carried out with sodium borohydride, and DNA was added as described in Materials and Methods. Final volume was 4.0 ml; the concentration of 5-methylphenazinium species was 3.0×10^{-4} M; the ratio MP:DNA was 1:2. Electron spin resonance observations were carried out under low-modulation conditions (0.03 G), so the signal observed is that of unbound MPH·$^+$. NaCl (●—●) and $MgCl_2$ (○—○).

Analysis of the data of Figure 8 leads to the binding plot shown in Figure 9, in which the small change in concentration of DNA binding sites caused by binding with the oxidized form, MP^+, has been neglected. For native DNA the binding curve can again be resolved into two linear regions indicating type I and type II binding sites. However, denatured DNA gives rise to only a single site characteristic of type II. In another experiment RNA showed the presence of two sites; however, the number of binding sites per nucleotide for the two types is different from native DNA. The binding parameters derived from the electron spin resonance studies are listed in Table I.

Air Oxidation of MPH·$^+$. The strong association of the cation radical with nucleic acids leads to a stabilization of the species with respect to oxidation by O_2.

TABLE II: Relative Mutagenicity of Phenazine Methosulfate and Proflavine.

Mutagen	Reversion Frequency	Rel
Proflavine	4×10^{-6}	4000
Phenazine methosulfate	3×10^{-8}	30
Spontaneous	10^{-9}	1

When air is bubbled through a solution containing $MPH\cdot^+$, the rapid oxidation of the cation radical is shown both by the decrease in the electron spin resonance signal and by the increase in the absorbance of MP^+ at 388 mμ. As shown in Figure 10, the presence of DNA in the same solution suppresses this behavior.

Effects of Metal Cations on the DNA–MPH·⁺ Complex. The inhibition of complex formation by Mg^{2+} is indicated in Figure 11 by the rapid oxidation of $MPH\cdot^+$ in solutions containing both DNA and $MgCl_2$. Further evidence for replacement of $MPH\cdot^+$ at the DNA binding sites by mono- and divalent cations is found in Figure 12. A gradual increase in the concentration of unbound radical cations is caused by the addition of sodium chloride up to a concentration of about 0.1 M. However, the presence of magnesium ions causes a sharp decrease in bound radicals even at low concentrations of $MgCl_2$. Mg^{2+} seems to have an inhibitory effect on complex formation about ten times that of Na^+. Ca^{2+} was similar to Mg^{2+} in this respect.

Mutagenesis. We have tested the mutagenic activity of phenazine methosulfate for reversion of an r_{II} mutant of T4 bacteriophage to the wild phenotype. The phage strain employed was a frame-shift mutant induced by 9-aminoacridine in the la region of the A cistron (S. Brenner, private communication). We compared the reversion frequency of phenazine methosulfate and proflavine at concentrations of 10 μg/ml, with the results shown in Table II.

Techniques and procedures employed in these experiments were the same as those described by Orgel and Brenner (1961). Although the mutagenicity of phenazine methosulfate is low, it is significantly above the spontaneous rate and is necessarily of the frame-shift type. At this time we have not established the oxidation state of the mutagenic species. Previously (White and Dearman, 1965) we have shown that MP^+ can be semireduced by suspensions of *E. coli*. However, since the present experiments were carried out in aerated solutions, the oxidized form is likely to be present in preponderant concentrations.

Molecular Orbital Calculations. In an attempt to develop an understanding of the relative stability of DNA complexes of MP^+ and $MPH\cdot^+$ we have carried out simple Hückel molecular orbital calculations of the π electron energies and charge densities; the results are presented in Figure 13. The Hückel parameters, α and β, were modified for nitrogen by the relations: $\alpha_N = \alpha + h\beta$ and $\beta_N = \beta$. The parameters which were employed are tabulated as follows.

Type of Nitrogen	h	Reference
>N:	+0.6	Streitwieser (1961)
$>N^+-H$	+1.2	Barton and Fraenkel (1964)
$>N^+-CH_3$	+0.9	K. Ishizu, J. Safdy, and H. H. Dearman (unpublished data)

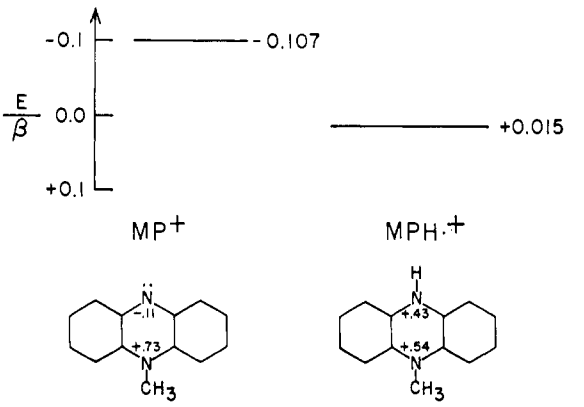

FIGURE 13: Energy of lowest unfilled molecular orbital and charge densities calculated for MP^+ and $MPH\cdot^+$ by simple Hückel molecular orbital theory. Parameters used in the calculation of these quantities are described in the text. Note that the parameter β is a negative quantity.

We find that the lowest unfilled π molecular orbital of $MPH\cdot^+$ is 0.12β ($\sim$ 0.9 kcal) lower than the corresponding orbital in MP^+. Also in Figure 13 are included calculated π-electron charge densities at the nitrogens. The balance of the unit positive charge is distributed over the rings, but no other position is calculated to have a charge density greater than 0.1. These results are dependent upon the choice of one-electron parameters for nitrogen. We feel that the order of the h parameter is correct and that the order of the calculated energy levels is more trustworthy than the absolute magnitude. The h value of the protonated aza nitrogen is more positive than for the unprotonated because the nonbonded electrons, stabilized by the proton, are less effective in shielding the π electrons from the nuclear charge. The value for the methylated nitrogen is intermediate due to the mitigating influence of the inductive effect of the methyl group.

Both the distribution of positive charge and the energy of the lowest molecular orbital capable of accepting an electron may be important in determining the free energy of binding of a heterocyclic aromatic cation to polynucleotides. Hückel calculations for proflavine have been carried out by Pullman and Pullman (1963), who found that the lowest vacant orbital occurs at -0.278β. The charge is somewhat more delocalized in proflavine, the charge at the protonated aza nitrogen being +0.36 and that at the amino groups being +0.13. There is also calculated to be a +0.22 charge at the carbon in the 10 position.

Discussion

The binding curves of MP^+ to native DNA are qualitatively similar to those of other planar aromatic cations. A region of tight binding exists at low cation concentration (denoted I by Peacocke and Skerrett (1956) in discussing the binding of proflavine to DNA), and a weaker complex is present at a cation concentration which approaches that necessary for one ligand per mononucleotide (denoted II). For the three-ring aminoacridinium and ethidium salts (Waring, 1965), k_I is of the order of $10^6\ M^{-1}$ and $n_I \leqq 0.25$. The two-ring cation, chloroquine, is found to have a binding constant to native DNA of the order of $10^4\ M^{-1}$ and a similar number of binding sites per mononucleotide (Cohen and Yielding, 1965). For all of these ligands the extent of formation of the type I complex depends strongly upon the concentration of inorganic cations in the medium, the number of binding sites per nucleotide decreasing with increasing ionic strength. The ionic strength employed here, $\mu = 0.001$, represents a situation in which competition for type I binding sites by inorganic cations is negligible.

The structure of the DNA–acridinium complexes of type I is of particular interest because models which have been advanced serve as the molecular basis of the well-known mutagenic action of many of these heterocyclic aromatic cations. The planar cation is pictured as aligning itself perpendicular (or almost so) to the helix axis between adjacent bases or base pairs. Stability accrues both from ionic interaction of the positively charged nitrogen and the negatively charged phosphate and from interaction of the π electrons of the ligand with almost parallel bases above and below. A frame-shift mutation occurs when the intercalated ligand causes an error in chromosome replication either by the insertion or deletion of a base pair. These mutations form a mutually exclusive set when compared with mutations caused by agents known to react with or substitute for the bases.

The original intercalation model proposed by Lerman (1961) showed overlap of the ligand with both members of a base pair, a configuration which would imply greatly reduced binding by denatured DNA. Experiments by Drummond *et al.* (1965), recently reinterpreted by Blake and Peacocke (1968), have shown that there are as many binding sites for aminoacridinium cations in denatured DNA and that the binding constants are similar to those for the native structure. Pritchard *et al.* (1966) have proposed a modification of the intercalation model for the single-stranded polymer, in which the left ring of the ligand interacts with the base below its plane and right ring interacts with the base above the plane. The charged nitrogen in the center ring is free to interact with the phosphate. The binding of aminoacridines, for example, proflavine, to denatured DNA is in striking contrast with the behavior of both oxidation states of 5-methylphenazinium under comparable conditions. We have been unable to obtain convincing evidence for the existence of a type I complex between single-stranded DNA and MP^+ or $MPH\cdot^+$, although these complexes may be demonstrable at lower ligand concentrations than were permitted by our detection techniques. The absence of a spectral shift on the addition of denatured DNA to MP^+ suggests that a type I complex does not exist in this case. There is some indication of a more strongly bound complex at the lowest $\bar{B}:F$ ratios in the $MPH\cdot^+$ curve (Figure 9), but even here the number of binding sites per nucleotide will be smaller than for the native case.

The failure of 5-methylphenazinium to bind to single-stranded DNA with the ease of proflavine may explain the relative mutagenic activities, since the DNA must be at least locally single-stranded during replication.

A further difference between the present system and the aminoacridines is in tendency for self-association, either in solution or bound to the nucleic acid. Acridine orange, in particular, forms dimers on DNA at dye:nucleotide ratios only slightly larger than the n_I found for 5-methylphenazinium (Bradley and Wolf, 1959). Proflavine obeys Beer's law in solution only at concentrations less than 2.5×10^{-5} M (Peacocke and Skerrett, 1956), whereas we have observed that MP^+ obeys Beer's law to a concentration of at least 8×10^{-4} M, and no new bands appear in MP^+–DNA mixtures. Also, the observed linearity of the electron spin resonance signal with concentration of $MPH\cdot^+$ shows that self-association of this species is not significant. Spin cancellation by dimer formation would lead to a deviation from linearity for this observation. Also, if spin cancellation did not occur, exchange narrowing would cause a loss of integrated intensity since the wings of the absorption would be depressed and lost in the noise level.

There are several reasons why $MPH\cdot^+$ binds more strongly to native DNA than does MP^+. If transfer of electrons from the bases to the ligand is an important source of binding energy, the lower energy vacant orbital of $MPH\cdot^+$ leads to a greater affinity for electrons. In fact, the calculated energy difference of $0.122\ \beta$ for these orbitals in MP^+ and $MPH\cdot^+$ has the value of 0.9 kcal, which is surprisingly close to the difference in free energy of formation of the two complexes of 1.1 kcal.

If we assume that there is no binding by the fully reduced species, MPH, the free energy of the Michaelis equilibrium of 5-methylphenazinium in the presence of native DNA can be evaluated relative to that of the equilibrium in the absence of the nucleic acid. Use of the reported equilibrium constants leads to the results $\Delta G°(\text{DNA}) - \Delta G° \sim -16$ kcal for the reaction

$$H^+ + (MP\text{–}DNA)^+ + MPH + DNA \rightleftarrows 2(MPH\cdot\text{–}DNA)^+$$

compared with

$$H^+ + MP^+ + MPH \rightleftarrows 2MPH\cdot^+$$

The binding of uncharged free radicals will be the subject of a future paper (K. Akasaka and H. H. Dearman, unpublished results). "Solubilization" of uncharged species is observed, but binding constants are considerably smaller than those reported here. We have no direct evidence that the fully reduced species is MPH. However the absorption spectrum of this species in nonpolar solvents is consistent with this assignment. Also, in

experiments with aqueous solvent, precipitation of a colorless solid was occasionally observed in the reduction solution. In addition to stabilizing the semireduced species DNA may offer $MPH\cdot^{+}$ steric protection from oxygen attack. The modified intercalation model of Pritchard *et al.* (1966) emphasizes the interaction of the positive charge on the ring with the negative phosphate group. Inspection of the charge densities shows that most of the positive charge in MP^{+} resides on the nitrogen bound to the methyl group. Presumably the size of the methyl group would prevent as close an approach as that of the protonated nitrogen in proflavine or in $MPH\cdot^{+}$.

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References

Anstall, H. B., Ahearn, M. J., and Jardine, J. H. (1967), *Biochem. Pharmacol. 16*, 561.

Barton, B. L., and Fraenkel, G. K. (1964), *J. Chem. Phys. 41*, 1455.

Blake, A., and Peacocke, A. R. (1968), *Biopolymers 6*, 1225.

Bradley, D. F., and Wolf, M. K. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 944.

Cohen, S. N., and Yielding, K. L. (1965), *J. Biol. Chem. 240*, 3123.

Cost, K., Bolton, J. R., and Frenkel, A. W. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 868.

Drummond, D. S., Simpson-Gildemeister, V. F. W., and Peacocke, A. R. (1965), *Biopolymers 3*, 135.

Geller, D. M., and Lipmann, F. (1960), *J. Biol. Chem. 235*, 2478.

Ishizu, K., Dearman, H. H., Huang, M. T., and White, J. R. (1966), *Science 154*, 421.

Ishizu, K., Dearman, H. H., Huang, M. T., and White, J. R. (1968), *Biochim. Biophys. Acta 165*, 283.

King, T. E. (1963), *J. Biol. Chem. 238*, 4032.

Lerman, L. S. (1961), *J. Mol. Biol. 3*, 18.

Ohnishi, S., and McConnell, H. M. (1965), *J. Am. Chem. Soc. 87*, 2293.

Orgel, A., and Brenner, S. (1961), *J. Mol. Biol. 3*, 762.

Peacocke, A. R., and Skerrett, J. N. H. (1956), *Trans. Faraday Soc. 52*, 261.

Pritchard, N. J., Blake, A., and Peacocke, A. R. (1966), *Nature 212*, 1360.

Pullman, B., and Pullman, A. (1963), Quantum Biochemistry, New York, N. Y., Wiley, p 681.

Singer, T. P. (1963), *Enzymes 7*, 345.

Streitwieser, A., Jr. (1961), Molecular Orbital Theory for Organic Chemists, New York, N. Y., Wiley, p 120.

Umezawa, H. (1964), Recent Advances in Chemistry and Biochemistry of Antibiotics, Tokyo, Microbial Chemistry Research Foundation, p 176.

Waring, M. J. (1965), *J. Mol. Biol. 13*, 269.

White, J. R., and Dearman, H. H. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 887.

White, J. R., and White, H. L. (1965), *in* Antimicrobial Agents and Chemotherapy, Sylvester, J. C., Ed., Washington, D. C., American Society for Microbiology, p 495.

Zaugg, W. S. (1964), *J. Biol. Chem. 239*, 3964.