it appears that procedures may be developed for the stepwise degradation of RNA in the presence of dipolar aprotic solvents which could improve the conditions with respect to nonspecific internal degradation of the polynucleotide chain.

References

Brown, D. M., and Read, A. P. (1965), *J. Chem. Soc.*, 2206.
Burton, K., and Petersen, G. B. (1960), *Biochem. J. 75*, 17.
Cavalieri, L. F., Bendich, A., Tinker, J. F., and Brown, G. B. (1948), *J. Amer. Chem. Soc. 70*, 3875.
Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1786.
Cohn, W. E., and Khym, J. X. (1962), *Colloq. Int. Cent. Nat. Rech. Sci. 106*, 217.
Cockerill, A. F. (1967), *J. Chem. Soc.*, 964.
Cordes, E. H., and Jencks, W. P. (1962), *J. Amer. Chem. Soc. 84*, 832.
Cowie, J. M. G., and Toporowski, P. M. (1961), *Can. J. Chem. 39*, 2240.
Fratiello, A. (1964), *J. Mol. Phys. 7*, 565.
Jencks, W. P. (1959), *J. Amer. Chem. Soc. 81*, 475.
Kamen, R. (1969), *Nature (London) 221*, 321.
Khym, J. X. (1963), *Biochemistry 2*, 344.
Khym, J. X., and Cohn, W. E. (1960), *J. Amer. Chem. Soc. 82*, 6380.
Khym, J. X., and Cohn, W. E. (1961), *J. Biol. Chem. 236*, PC9.
Khym, J. X., and Uziel, M. (1968), *Biochemistry 7*, 422.
Kolthoff, I. M., Chantooni, M. K., Jr., and Bhowmik, S. (1968), *J. Amer. Chem. Soc. 90*, 23.
Livingston, D. C. (1964), *Biochim. Biophys. Acta 87*, 538.
Neu, H. C., and Heppel, L. A. (1964), *J. Biol. Chem. 239*, 2927.
Parker, A. J. (1962), *Quart. Rev., Chem. Soc. 16*, 163.
Phillipsen, P., Thiebe, R., Wintermeyer, W., and Zachav, H. G. (1968), *Biochem. Biophys. Res. Commun. 33*, 922.
Steinschneider, A., and Fraenkel-Conrat, H. (1966), *Biochemistry 5*, 2735.
Theander, O. (1957), *Acta Chem. Scand. 11*, 717.
Uziel, M., and Khym, J. X. (1969), *Biochemistry 8*, 3254.
Weith, H. L., and Gilham, P. T. (1967), *J. Amer. Chem. Soc. 89*, 5473.
Whitfeld, P. R. (1965), *Biochim. Biophys. Acta 108*, 202.
Yu, R. J., and Bishop, C. T. (1967), *Can. J. Chem. 45*, 2195.

Charge Effect on the Interaction of Free Radicals from Phenazine Methosulfate with Deoxyribonucleic Acid*

Kazuyuki Akasaka† and Henry H. Dearman‡

ABSTRACT: The complexes of the neutral and of the protonated forms of the 5-methylphenazyl-2-nitrile free radical with deoxyribonucleic acid have been studied with ultraviolet–visible and electron spin resonance spectroscopy. For the uncharged free radical at pH 10 a binding constant $K_I = 4 \times 10^4$ M^{-1} was determined. The binding constant for the cation radical at pH 4.7 was found to be $K_I = 3 \times 10^6$ M^{-1}. Both these measurements were made in acetone–water mixed solvent (1:9, v/v) with Na^+ concentration 0.001 M. These quantities can be combined to obtain an estimate of the contribution of the charge to the binding free energy of 2.5 kcal mole^{-1}. This result is compared to previous estimates obtained for complexes of deoxyribonucleic acid with aminoacridine cations.

Binding of planar aromatic molecules to nucleic acids is a subject of considerable interest and the determination of the structure of resulting complexes has been the aim of many investigations in recent years. Much of this work has involved dye molecules of the aminoacridine class and a useful review of this subject is available (Blake and Peacocke, 1968). At neutral pH, most of the biologically active aminoacridines exist in the cationic form and bind to DNA in two clearly distinguishable ways. The type I complex is formed at low concentrations of the dye relative to nucleic acid phosphate and bears most resemblance to the proposed intercalated structure which provides an appealing model for the frame-shift mutational activity of these dyes (Lerman, 1961; Crick *et al.*, 1961). For high dye concentration in which the number of bound dyes is approximately equal to the number of polymer phosphates, the type II complex predominates and is characterized by binding on the outside of the double helix. Most of the investigations have been focused on the type I complex because of its obvious biological relevance.

It is a well established fact that an increase in ionic strength leads to a decrease in the extent of binding of both types of complexes (Drummond *et al.*, 1965). On the other hand, it also is clear that uncharged aromatic hydrocarbons and heterocyclic molecules likewise bind to nucleic acids and are thereby rendered soluble in aqueous solution (Boyland and Green, 1964; Nagata *et al.*, 1966). These observations prompted our interest in the contribution of the charge of the dye to the binding free energy of type I complexes. Previous estimates of the effect have been made by comparing the

* From the Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina 27514. *Received July 13, 1970.* This investigation was carried out under a research grant from the National Science Foundation (GB-16423). We were assisted by facilities provided by the University of North Carolina Material Research Center (Contract SD-100 with the Advanced Research Projects Agency).

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binding free energy of a given cationic dye at high ionic strength, where phosphate charge compensation by counter-ion is presumably complete, with the same quantity measured at low ionic strength (Chambron *et al.*, 1966a,b; Heilweil and Van Winkle, 1955). The validity of such a procedure is not completely clear since the nucleic acid itself undergoes complicated polyelectrolyte behavior with varying salt concentration. This concern is raised by the observation that binding constants for type I complexes of actinomycin D and related structures are markedly affected by ionic strength at a pH for which the intercalating phenoxazine moiety is uncharged (Müller and Crothers, 1968).

We have approached the problem by comparing the binding to DNA of the protonated and neutral forms of a dye at different pH but at constant low ionic strength. Following our previous studies, we have employed a free-radical species as ligand and utilized changes in the electron spin resonance spectra arising from complex formation to determine binding parameters (Ishizu *et al.*, 1969 (part I in series); Akasaka and Dearman, 1969 (part II in series)).[1] The free radical is MPCN[2] which is uncharged at pH 10 and protonated at pH 4.7. The binding of the parent *N*-methylphenazinium cation radical to native DNA is characterized by behavior very similar to that displayed by the aminoacridine cations. However, such is not the case for its binding to denatured DNA. Proflavine has been found to bind as well to single-stranded DNA as to the native form (Drummond *et al.*, 1965). The apparent lack of specificity for the helical structure led to the proposal of a modified intercalation model for the complex in which ionic attraction of the phosphates for the cation dye molecule is an important feature. However, the phenazine cations bind strongly only to the double-stranded DNA. It thus becomes important to determine the contribution of ionic attraction to the binding energy of the phenazinium cations and to compare it to the previous estimates of this quantity for the aminoacridines cations.

Materials and Methods

A detailed description of the general procedures employed in the electron spin resonance technique can be found in Ishizu *et al.* (1969). A minor modification required by this particular system is described in the following section. It was not possible to use the unsubstituted MP system for study of the charge effect because disproportionation of the free radical occurs in alkaline pH (Zaugg, 1964), which prevents the formation of the uncharged species. However, the 2-nitrile derivative MPCN is stable in both charged and uncharged forms (McIlwain, 1937). MPCN was prepared from phenazine methosulfate by McIlwain's method and was obtained as black needles melting in the range 145.5–148°. A C=N stretching mode at 2200 cm^{-1} was prominently displayed in the infrared spectrum. The purified material was adjudged to be 100% free radical by comparison of electron spin resonance intensity with that of DPPH as standard.

Salmon sperm DNA (Calbiochem, highly polymerized) was dialyzed extensively against 0.001 M NaCl solution and pH was adjusted to 4.7 by dilute HCl solution and to 10 by dilute NaOH solution. The lower pH was maintained with a sodium acetate buffer and the higher pH was maintained with a sodium phosphate buffer. In all cases the final sodium ion concentration was 0.001 M.

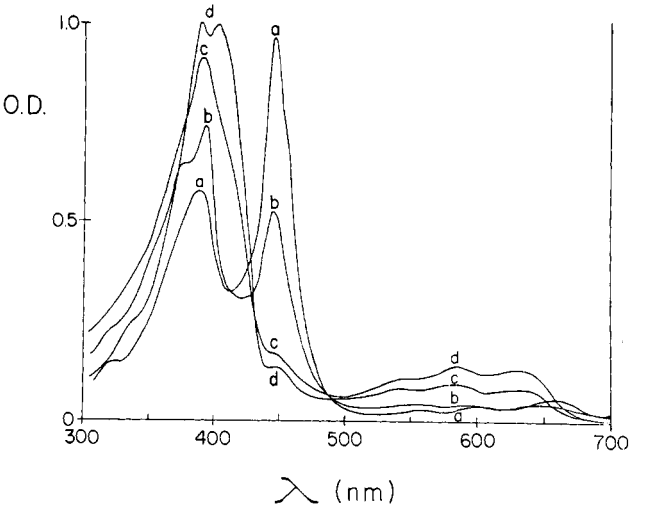

FIGURE 1: Absorption spectra of MPCN at various pH values in acetone–water (1:9, v/v) mixed solvent. Total concentration of MPCN is 3.6×10^{-5} M throughout and spectra were recorded in a 2-cm quartz cuvet. (a) pH 2, (b) pH 4.7, (c) pH 7, and (d) pH 10.

Although we have previously shown in Akasaka and Dearman (1969) that the neutral MPCN species is "solubilized" by aqueous solutions of DNA at pH 10, the substance is insoluble under these conditions in the absence of the nucleic acid. In order for binding parameters to be obtained by the electron paramagnetic resonance technique it was necessary to use a mixed solvent in which polymer and ligand are soluble in both low and high pH conditions. We employed the solvent mixture acetone–water (1:9, v/v). We went to considerable effort to ascertain whether DNA is denatured or otherwise significantly modified in the two pH conditions in this solvent system. Although the classical measurement of absorbance at 260 nm was complicated by the absorbance of the acetone in this region, all other physical tests failed to reveal denaturation. Such measurements included optical rotatory dispersion at 350 nm and sedimentation velocity experiments as well as internal checks. Further discussion of this point will follow.

Results

The molecular electronic spectra of the species in the equilibria involved in the two-electron reduction of *N*-methylphenazinium cation have been reported by Zaugg (1964). At low pH excellent isobestic points are displayed for each of the one-electron reduction steps confirming the presence of a single radical–cation intermediate. In aqueous solution above pH 8 rapid disproportionation of the uncharged phenazyl radical intermediate was observed. However, this species was found to be relatively stable in basic ethanol solution. The absorption spectrum of stable MPCN in basic ethanol resembles that of the phenazyl.

The absorption spectra of MPCN at various pH values in the acetone–water (1:9, v/v) solvent are considerably more complicated, as shown in Figure 1. The crystalline free

[1] Part III in this series.

[2] Abbreviations used are: MP^+, the 5-methylphenazinium cation; $MPH\cdot^+$, the 5-methylphenazinium cation radical; $MP\cdot$, the 5-methylphenazyl radical; MPH, fully reduced MP^+; $MPCN^+$, the 5-methylphenazinium-2-nitrile cation; $MPCNH\cdot^+$, the 5-methylphenazinium-2-nitrile cation radical; $MPCN\cdot$, the 5-methylphenazyl-2-nitrile radical; MPCNH, fully reduced MPCN. MP refers to all of the 5-methylphenazine species and MPCN refers to all the 5-methylphenazine-2-nitrile species which may be present in a mixture. See Figure 5.

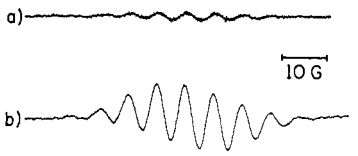

FIGURE 2: Electron spin resonance spectrum of MPCN at pH 4.7 in acetone–water (1:9, v/v) mixed solvent. (a) Low modulation amplitude (0.03 G) and (b) high modulation amplitude (0.3 G).

radical is soluble in this solvent over a wide pH range, but is insoluble at high pH in the absence of the acetone. The spectrum of MPCN at high pH (Figure 1d) closely resembles the spectrum of the free radical dissolved in a neutral organic solvent such as benzene or acetone except that in the organic solvent the peak at 403 nm appears at slightly lower wavelength as an unresolved shoulder. The spectrum at low pH (Figure 1a) is characterized by an intense absorption at 447 nm which is quite similar to that for $MPH\cdot^{+}$ reported by Zaugg (1964). In the intermediate pH range the appearance of peaks at 393 and 377 nm, as well as an increase in absorbance near 350 nm, is noted. A striking feature of the spectra in Figure 1, which is confirmed when a more gradual pH variation is carried out, is the absence of isobestic points in the 350–700-nm wavelength range. This indication of a complex equilibrium situation is further corroborated by measurements of the concentration of paramagnetic species as a function of pH. In general, the spectra of MPCN in high and low pH in the acetone–water mixed solvent are quite similar to the spectra of $MPH\cdot^{+}$ in aqueous solution and of the $MP\cdot$ in basic ethanol which were obtained by Zaugg (1964). Spectra of the unsubstituted *N*-methylphenazyl free-radical system in a common solvent as a function of pH are not available for comparison.

The electron spin resonance spectra of MPCN at pH 4.7 in the mixed solvent are shown in Figure 2 and at pH 10 in Figure 3. In both cases the electron spin resonance spectra under conditions of low and high modulation amplitude are presented. We were unable to improve the resolution of the low modulation spectrum of MPCN at pH 4.7. This situation reflects the presence either of unresolved hyperfine structure or an exchange phenomenon involving the proton attached to the nitrogen in the 10 position. The high modulation spectrum at pH 4.7 is virtually identical with that of MPH^{+}, being composed of nine lines with a 6.5-G splitting. This observation leads to the conclusion that 5-methylphenazyl-2-nitrile exists as the radical-cation $MPCNH\cdot^{+}$ at pH 4.7. Both the low and high modulation electron spin resonance spectra of MPCN at pH 10 are distinctly different from the corresponding spectra at pH 4.7. There are several lines of argument which support the contention that the molecule is

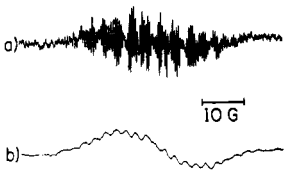

FIGURE 3: Electron spin resonance spectrum of MPCN at pH 10 in acetone–water (1:9, v/v) mixed solvent. (a) Low modulation amplitude (0.03 G) and (b) high modulation amplitude (0.3 G).

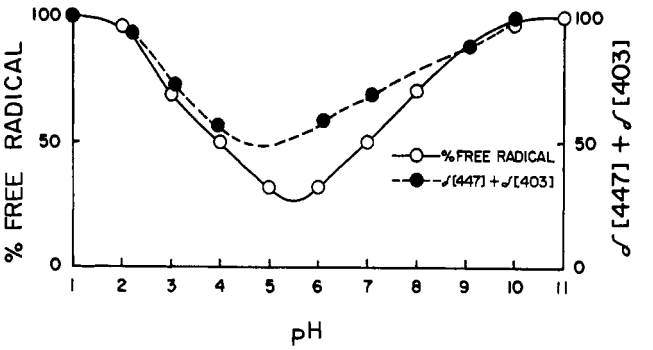

FIGURE 4: Free-radical concentration in a solution of MPCN in acetone–water (1:9, v/v) mixed solvent as a function of pH. Open circles refer to the integrated intensity of the electron spin resonance signal and corresponds to left-hand vertical axis. Closed circles refer to the sum of the absorbances at 403 and 447 nm which are assigned to the neutral and protonated free radical, respectively, and corresponds to the right-hand vertical axis. $\delta(447)$ is the percentage of the pH 1 absorbance at 447 nm and $\delta(403)$ is the percentage of the pH 10 absorbance at 403 nm observed at a given intermediate pH. Buffers of 0.05–0.1 M were used.

present at high pH in the uncharged form. Although the hyperfine pattern of MPCN in pure organic solvents such as benzene differs in detail from that shown in Figure 3a, the width of the high modulation spectrum is the same, namely 18.5 G. Solvent association at the nitrile substituent can be expected to produce slight modifications of the delocalized spin distribution which show up in the complicated high-resolution hyperfine pattern. However, the width is determined largely by the large coupling constants of the two ring nitrogens and protons either directly attached or in the methyl group. Variations in these quantities due to solvent effects are likely to be small. Evidence that the molecule does not undergo permanent chemical change is provided by the observation that extraction of the radical from the acetone–water mixed solvent at pH 10 by benzene leads to an electron spin resonance spectrum identical with that of a solution of the crystalline free radical dissolved directly in benzene. The electrical neutrality of the free radical is further substantiated by electrophoresis experiments with pH 10 solutions containing both MPCN and MP^{+}. Under conditions for which MP^{+} moved toward the cathode, MPCN did not move from the origin.

The absence of isosbestic points in the optical spectra suggests that there are species other than $MPCN\cdot$ and $MPCNH\cdot^{+}$ involved in the equilibrium at intermediate pH. The electron spin resonance spectral changes accompanying the lowering of pH also reflect this and indicate that these species are diamagnetic. The results are shown in Figure 4 in which the relative concentration of paramagnetic species as a function of pH is indicated. On the same graph a quantity proportional to the sum of the absorbances at 447 nm (assigned to $MPCNH\cdot^{+}$) and at 403 nm (assigned to $MPCN\cdot$) is also shown. $\delta(447)$ is the percentage of the pH 1 absorbance at 447 nm and $\delta(403)$ is the percentage of the pH 10 absorbance observed at a given intermediate pH. The rough parallel between these two measurements indicates the essential correctness of the assignments, and the lack of exact agreement between the two is due to errors in the optical data caused by overlapping spectra and absorbance at the two-wavelength maxima of the intermediates. The appearance of peaks at intermediate pH which correlate fairly well with those of the fully reduced and fully oxidized MP species led

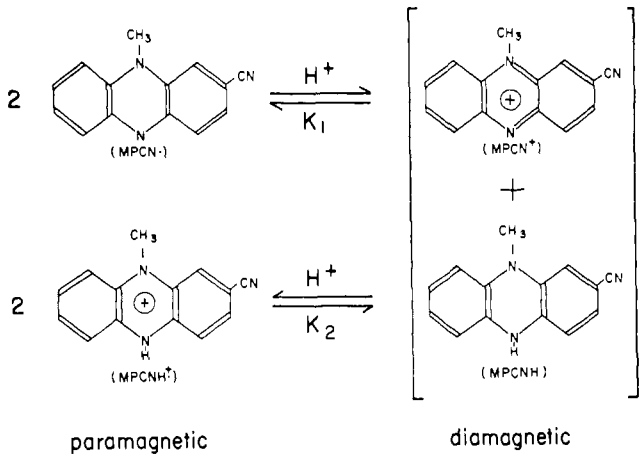

FIGURE 5: Proposed pH-dependent equilibria involving paramagnetic and diamagnetic MPCN species. Note the analogy to the usual two-electron Michaelis equilibrium (King, 1963).

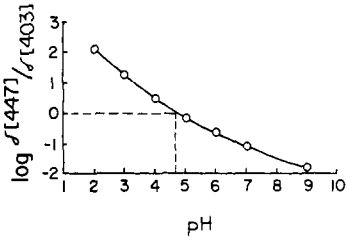

FIGURE 6: Log of the ratio of the change in absorbance at 447 nm (relative to that at pH 1) to the change in absorbance at 403 nm (relative to that at pH 10) *vs.* pH of MPCN in acetone–water (1:9, v/v) mixed solvent. $\delta(447)$ indicates the relative concentration of MPCNH·$^+$ and $\delta(403)$ indicates the relative concentration of MPCN·. Equation 1 predicts a linear plot. See text.

us to consider a pH-dependent disproportionation and to suggest the scheme shown in Figure 5. This process is directly analogous to a Michaelis equilibrium (King, 1963). This proposal can be tested since the indicated equilibrium expressions can be combined to obtain eq 1. We can make

$$\log (\text{MPCNH}\cdot^+)/(\text{MPCN}\cdot) = \log (K_1K_2)^{1/2} - \text{pH} \quad (1)$$

a rough estimate of the left-hand side of eq 1 by setting it equal to log $\delta(447)/\delta(403)$. A plot of this quantity *vs.* pH is given in Figure 6. We feel that the curvature of the plot is due to errors in the absorbances due to overlapping spectra.

By assuming that $K_1 = K_2$, we can obtain from the plot the value $K_1 = K_2 = 5 \times 10^4$ M. An alternative method for evaluating the equilibrium constants K_1 and K_2 consists of noting from Figure 4 the pH values at which the composition of the solution is 50% free radical (as measured by electron spin resonance), namely, pH 4 and 7. Simple calculation leads to the values $K_1 = 2.5 \times 10^6$ M and $K_2 = 4 \times 10^4$ M.

State of the DNA. Having established the charge of the ligand under our experimental conditions, the state of the nucleic acid in the mixed solvent at pH 4.7 and 10 must be assessed. However, with our low cationic concentration ($(Na^+) = 10^{-3}$ M) of the aqueous solution of the salmon sperm DNA, absorbance at 260 nm was observed to increase below pH 4.5 at 20°. Thus pH 4.7 was chosen for the experiment in the mixed solvent for the low pH. The stability of DNA to acid and basic denaturation in aqueous solution with relatively high ionic concentration has been previously demonstrated over the pH range 3–11 (*e.g.*, Lepecq and Paoletti, 1967). Our concern is for the effect of the 10% component of acetone in the solvent system. Although we are unable to determine the presence or absence of short single-stranded regions, all our evidence points to a native conformation under the conditions of the binding experiments. The absorbance of the acetone complicates the use of the classical criteron for double- or single-stranded structure, the hypochromism at 260 nm. We have carried out sedimentation velocity experiments and optical rotatory dispersion measurements at 350 nm, to the red of absorbance due to acetone. Both lines of experimentation showed identical behavior for DNA in the acetone–water mixed solvent at either of the pH values and DNA in aqueous solution at the same pH. In each case these experiments yielded quite different and easily distinguishable results for heat-denatured DNA in aqueous solution of the appropriate pH. For example, in the mixed solvent at pH 10, we obtained the value 565 ± 10° for $[\alpha]_{350}$, which compares favorably to the value 576° calculated from the Drude constants for native salmon sperm DNA reported by Samejima and Yang (1964).

Our previous binding studies with MP^+ and MPH·$^+$ also provide strong support to the conclusion that the DNA is unaffected by our experimental conditions. It was found that these two cations do not bind measureably to heat-denatured nucleic acid. It would be surprising if the 2-nitrile derivative displays a different behavior. Our previous work (Akasaka and Dearman, 1969) clearly demonstrates the binding of the neutral radical to DNA at pH 10 in the absence of acetone, but no measurable binding was observed to heat-denatured DNA under the same condition. We did find that denaturation occurred at pH 3 in the mixed solvent, again paralleling behavior in aqueous solution. Negligible binding of MPCNH·$^+$ to the acid-denatured DNA in that solution was observed. A further check is provided by an experiment in which it was found that the binding of MPH·$^+$ to DNA at pH 6 and Na^+ concentration 0.001 M is the same in the presence and in the absence of 10% acetone.

Binding of MPCNH·$^+$ and MPCN· to DNA. The review of Blake and Peacocke (1968) contains a good discussion of the validity and limitations of the Scatchard plot method of treating spectral measurement of the binding of dyes to nucleic acids. We employ the same notation as in Ishizu *et al.* (1969), namely, F, B, and P which denote the equilibrium molar concentration of unbound ligand, bound ligand, and DNA phosphorus. $\bar{B}$ is the ratio of B/P. Plots of the ratio $\bar{B}/F$ *vs.* $\bar{B}$ can be interpreted in terms of the binding equilibrium constants, K, and the maximum number of binding sites per DNA phosphates, n. The identification of two different sites for binding of heterocyclic cations depends on the existence of two linear regions in these plots.

Binding at pH 4.7. A detailed description of the way in which electron spin resonance measurements can be utilized to obtain values for $\bar{B}$ and F is given in Ishizu *et al.* (1969). The poor resolution of the low modulation spectrum shown in Figure 2 necessitates a minor modification of this technique. Since proton hyperfine structure is seen to make a small contribution to signal amplitude, it becomes necessary to use conditions of high modulation aplitude throughout the binding experiment. In our previous method, which worked well with the binding of the well-resolved spectrum of MPH·$^+$, F was determined by the intensity of the low modulation

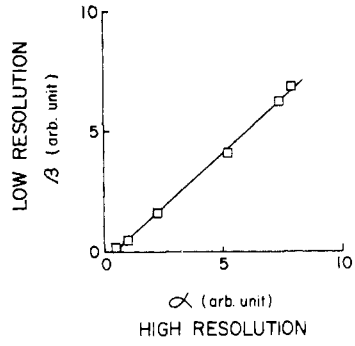

FIGURE 7: Relation of peak height, α, of electron spin resonance spectra of DNA–MPH·$^+$ mixtures under conditions of high resolution (low modulation amplitude, 0.03 G) to the peak height, β, under low resolution (high modulation amplitude, 0.3 G). The straight line indicates that either quantity can be used to measure the concentration of unbound radical. See text and Ishizu *et al.* (1969).

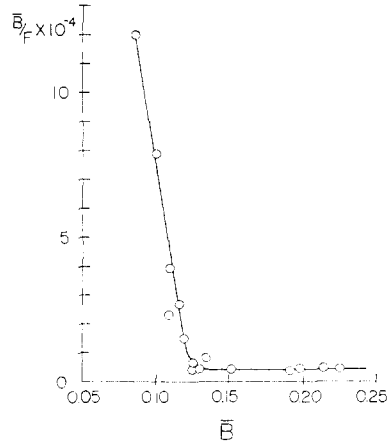

FIGURE 8: Plot of $\bar{B}/F$ *vs.* $\bar{B}$ for the interaction of MPCN· with native salmon sperm DNA in acetone–water (1:9, v/v) mixed solvent at pH 4.7 and Na^+ concentration 0.001 M. See Ishizu *et al.* (1969) for techniques and text for definitions.

spectrum of the radical-cation–DNA mixtures. It was noted, however, that an equally good estimate of this quantity could be obtained from the height of the center peak of the high modulation spectrum of the mixture. In other words the tumbling of the DNA–cation complex is slow compared to reciprocal of the anistropic part of the hyperfine coupling constants, expressed in frequency units, and the spectrum of the complex is practically structureless. Any decrease of structure in the spectrum of a mixture of bound and unbound radicals from that of the unbound directly indicates the amount of the bound radicals. This result is apparent from Figure 7 in which the height of the central hyperfine component of the low modulation spectrum of MPH·$^+$–DNA mixtures is plotted against the height of the central hyperfine component of the high modulation spectrum. Using this result we feel confident that an accurate estimate of F can be obtained just as well from the high modulation spectra of DNA–MPCNH·$^+$ mixtures. Having made this modification, a Scatchard plot of our results is constructed according to the previous method and is shown in Figure 8. The two straight-line regions which are characteristic of cation binding are apparent. We focus attention of the type I "tight-binding site" characterized by the steep region of the curve with $\bar{B}$ values less than 0.13. The parameters which characterized this species are found to be $n_1 = 0.12$ and $K_1 = 3 \times 10^6$ M^{-1}. The data are insufficiently precise to permit determination of the binding parameters for the type II complex but it is evident that the maximum number of binding sites per nucleotide is much larger and the binding equilibrium constant is smaller than for the type I case.

If the scheme for pH-dependent equilibria involving the various diamagnetic and paramagnetic forms of MPCN is correct, $MPCN^+$ and MPCNH are present along with MPCNH·$^+$ at pH 4.7. The electron spin resonance technique has the advantage that it measures only the paramagnetic species. On the other hand, our interpretation depends on the assumption that the diamagnetic forms bind with smaller equilibrium constants so as not to compete effectively with MPCNH·$^+$ for type I binding sites. There is precedence for such an assumption from our previous studies with the diamagnetic cation MP^+ and the paramagnetic MPH·$^+$ where it was found that the radical cation binds with an order of magnitude larger equilibrium constant than does the parent oxidized form. Thus, the formation of a complex with DNA shifts the Michaelis equilibrium strongly to the radical intermediate. This observation also indicates that binding of MPH, the fully reduced methylphenazinium species, is small. An even stronger shift of the equilibrium to MPCNH·$^+$ in presence of DNA was observed in the present case. The equilibrium characterized by K_2 in Figure 5 is directly analogous to the Michaelis equilibrium previously investigated, and it is clear that relative stabilization of MPCNH·$^+$ also occurs in the present case. The data in Figure 4 indicate that in the absence of nucleic acid the composition of the solution at pH 4.7 is 35% MPCNH·$^+$ and 65% diamagnetic form. If we assumed that both diamagnetic forms bind with the same equilibrium constant (this is an upper limit, since the uncharged species is expected to bind less well, *vide infra*) and that, as with MPH·$^+$, MPCNH·$^+$ binds with an equilibrium constant ten times as large, the composition of the solution of DNA complexes becomes 85% radical and 15% diamagnetic forms. Such a composition is consistent with our measurements of total radical concentration at low dye to polymer ratios.

Binding at pH 10. At this pH it is clear that the only ligand species which is present in significant concentration even in the absence of nucleic acid is MPCN·. This situation makes it possible to utilize both electron spin resonance and optical measurement in construction of the Scatchard plot and thus to obtain an internal check. The data from optical binding experiments along with those from the electron spin resonance measurements are included in the Scatchard plot in Figure 9. Good agreement is found between these independent measurements. Due to the small extent of binding, we are unable to extend the plot beyond the range of $\bar{B} = 0.05$, that is 1 radical/20 nucleotides. We are currently undertaking equilibrium dialysis experiments to obtain data in the more concentrated region. However, over the region accessible in the present work, the plot consists of a single straight line and leads to the binding parameters $n = 0.08$ and $K = 4 \times 10^4$ M.

Charge Contribution of Binding. Conversion of the equilibrium constants into binding free energy leads to the results given in Table I.

Our results show that the electrostatic attraction of the cation for the negative charge of the phosphodiester linkage contributes roughly 30% of the binding free energy. This contribution will undoubtedly vary from ligand to ligand

TABLE I: Binding Parameters for Complexes of 5-Methylphenazinium-2-nitrile Species with Native Salmon Sperm DNA.[a]

Radical	pH	n	$K_I \times 10^{-5}$ (M^{-1})	$\Delta G°$ (kcal mole^{-1})
MPCNH·$^+$	4.7	0.12	30	−8.7
MPCN·	10	0.08	0.40	−6.2
				$\Delta G°$ (charge) = −2.5

[a] All measurements were made with Na^+ concentration 0.001 M in acetone–water (1:9, v/v) mixed solvent at 20°.

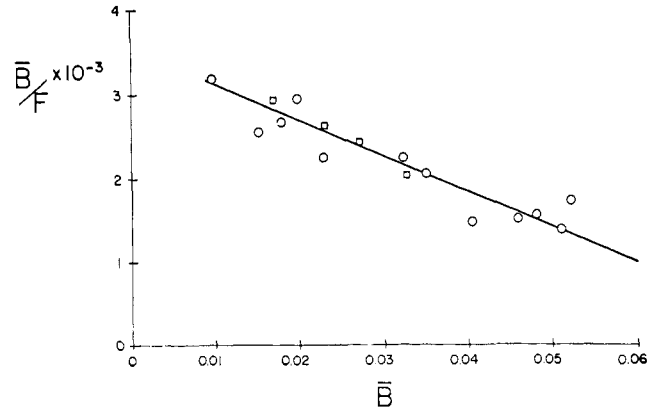

FIGURE 9: Plot of $\bar{B}/F$ *vs.* $\bar{B}$ for the interaction of MPCN· with native salmon sperm DNA in acetone–water (1:9, v/v) mixed solvent at pH 10 and Na^+ concentration 0.001 M. Circles are electron spin resonance data and squares are optical data. See Ishizu *et al.* (1969) for techniques and text for definitions.

since the distribution of positive charge over the molecular framework depends on the detailed electronic structure of the species. On the other hand, it seems unlikely that, in any case, it would account for more than 50% of the interaction energy.

Denatured DNA. Here, in agreement with our previous observations for the MP^+–MPH·$^+$ system, we were unable to detect appreciable binding of MPCNH·$^+$ or MPCN· to heat-denatured DNA.

Discussion

Under conditions of low ionic strength the difference in binding free energy of a charged and uncharged species of the same molecule should provide a measure of the contribution of the charge to the binding free energy. Several factors, however, make this approach somewhat equivocal. We believe that the contribution of these extraneous effects is small.

First, the effect of the mixed solvent on the conformation of the nucleic acid has already been considered. Our conclusion is that while we are unable to detect minor changes, the overall conformation of DNA is the same at high and low pH values in both aqueous buffer and in the mixed solvent. The similarity of n, the maximum number of binding sites, for charged and uncharged MPCN supports this contention. In aqueous buffer at pH 10, we have previously (Akasaka and Dearman, 1969) found that $n = 0.05 \pm 0.01$, very nearly the same value as in Table I. This estimate was obtained by measurement of "solublized" radical concentration by independent electron spin resonance and optical observations. The fact that this number is slightly lower than the value determined here may well be due to failure to establish equilibrium in the solubilization experiments. These experiments are carried out by shaking the nucleic acid solution with the solid free radical and were carried out up to 24 hr. However, such heterogeneous equilibria may well require a longer period for complete attainment of equilibrium. In short, the difference between $n = 0.05$ and $n = 0.08$ may be due to experimental error, failure to establish equilibrium, or to a slight increase in the number of binding sites caused by the effect of the acetone on the DNA. One would expect that if the acetone had an effect, it would be to cause denaturation. However, we have been unable to detect any binding of MPCNH·$^+$ or MPCN· to heat denatured DNA. Thus, if acetone were to produce local denatured regions, the number of binding sites per nucleotide would be expected to decrease rather than to increase as observed.

Also unclear is the state of MPCN· in the acetone (10%)–water (90%) mixed solvent. If the MPCN· molecule were surrounded by acetone rather than water molecules in the mixed solvent, then the observed free energy change in its complexing to DNA may not be directly compared with that of MPCNH·$^+$ complexing to DNA, since the latter will be surrounded mostly by water molecules in the mixed solvent. However, the electron spin resonance hyperfine pattern of MPCN· in the 10% acetone solution differs completely from that of MPCN· in pure acetone itself, and suggests strongly that MPCN· molecule is surrounded mostly by water molecules.

A second concern is that protonation of the ring nitrogen leads to such a strong perturbation to the electronic structure of the neutral ligand that interaction with the bases is quite different in the two species. From a Hückel molecular standpoint the Coulomb integral α for an sp^2 carbon atom becomes $\alpha_N = \alpha + h\beta$ for an aza nitrogen. Since the resonance integral, β, is a negative quantity, the more positive the value of h, the larger will be the energy required to remove a π electron from the nitrogen. The proton stabilizes the nitrogen π electron by the amount Δh and estimates place Δh at 0.4–0.6 unit.

The change in charge density at the kth atom in the π system of MPCN· by virtue of protonation at the 10 nitrogen is $\Delta q_k = (0.6\beta)\pi_{10,k}$ where $\pi_{10,k}$ is mutual polarizability of atom k and the aza nitrogen. Of course, the largest polarization effect is for $k = 10$ but for the other atoms $\pi_{10,k}$ is typically 0.1 (unit β^{-1}) or smaller. A precise estimate of this effect requires a more detailed calculation such as that of DeVoe and Tinoco (1962) and Gersch and Jordon (1964), but a simple Hückel calculation reveals that the π electron charge distribution is slightly more polar for MPCNH·$^+$. This indicates that the value of $\Delta G°$ (charge) may be slightly overestimated by our procedure.

We have made an attempt to compare our results to previous experimental and theoretical estimates of contribution of charge. Table II contains binding constants for type I complexes of several aminoacridine cations determined under conditions of both high and low ionic strength. It can be seen that values of 1–2 kcal mole^{-1} are typical for the difference in binding free energy at low and at high ionic strengths. Considering the difficulties involved in interpreting this as the

TABLE II [a]

Cation	Temp (°C)	pH	Ionic Strength (Na^+)	K_1 (M)	$\Delta G°$ (Charge) (kcal $mole^{-1}$)	Reference
Proflavine	20	5	0.01	2.5×10^6	−2.4	Chambron *et al.* (1966)
	20	5	1.0	5.0×10^4		
Proflavine	10	6.9	0.016	1.2×10^5	−0.64	Li and Crothers (1969)
	10	7.1	0.2	4.2×10^4		
Acriflavine	25	7.0	0.002	6.7×10^7	−1.9	Heilweil and Van Winkle (1955)
	25	6.0	0.3	2.8×10^6		
Ethidium bromide	23	7.5?	0.015	2.5×10^6	−2.0	Lepecq and Paoletti (1967)
	23	7.5?	1.0	8.2×10^4		

[a] Previous estimates of $\Delta G°$ (charge) by the measurement of equilibrium constants for binding at high and low ionic strength.

measure of electrostatic contribution as well as in our own, agreement between results is satisfactory. A decrease in equilibrium constant by a factor of 10–100 accounts for the observed decreased dye binding with increasing ionic strength. In the limit $K[P] \gg 1$, the fraction of unbound ligand varies inversely with the binding constant. At low ionic strength phosphate charge repulsion should extend the helix and also stiffen it. The first effect may make the intercalation process easier but if bending is required for accommodation of the ligand between adjacent base pairs, this process might be more difficult at low ionic strength. A near cancellation of these oppositing effects could explain why the results in Table II are in reasonable agreement with our results.

Gersch and Jordan (1964), using the approach of DeVoe and Tinoco (1962), compare the free energy of binding of proflavine calculated for an intercalated and a external stacked model. Unfortunately, it is not possible to compare these results directly to those obtained in this work since calculations are carried out only for the situation of one ligand per base pair. Also, no account was taken of ionic attraction for the phosphate negative charge. It is of interest to note that these authors estimate that the contributions of charge interaction between ligand and bases exceed contributions of nonionic terms such as London forces, dipole–dipole, etc., by about 50%. Inclusion of interaction with the phosphates in the calculation should increase the percentage contribution of charge attractions. This prediction is not borne out by either our results or the results in Table II. This disagreement may emphasize the presence of a large contribution from hydrophobic bonding.

Schildkraut and Lifson (1965) have developed a semiquantitative model for the effect of ionic strength on the interchain repulsion of the phosphate negative charges of DNA. The free energy of electrostatic interaction, per mole of nucleotide pairs, ΔG_e, is work done by removing a phosphate of charge λe on one strand against the electrostatic potential $\lambda\psi_1$ induced by the other strand and is given by $\Delta G_e = -\lambda^2 e\psi_1$. Here λ is the screening factor necessary on mathematical grounds if use of the Debye-Hückel approximation to the Poisson–Boltzmann equation is to be made. Furthermore, while the value of the potential ψ_1 is high within the helix, it is considerably lower at the phosphates due to the high local concentration of counterions adjacent in the bulk solution. These authors suggest a value $\lambda = 0.22$ which is supposed to be constant over a wide range of salt concentrations. This theory has been extended by Chambron *et al.* (1966b) to estimate the contribution of the charge of the dye to the increase in the melting temperature of proflavine–DNA complexes over that of uncomplexed DNA observed with increasing $\bar{B}$. These workers assume that the electrostatic potential at the NH^+ position of the dye is ψ_1 leading to the electrostatic free energy due to the charged dye of $+\psi_1\lambda e$ per mole. This procedure considers that high local concentration of counterions in the adjacent bulk solution causes a screening of the phosphate charge by the factor λ, but does not affect the potential inside the helix. The validity of this approximation is not clear. One then proceeds by utilizing the value for ψ obtained as computer solution of the "screened" Debye–Hückel equation by Schildkraut and Lifson (1965) who employed the coordinates of the phosphates reported by Landridge *et al.* (1957, 1960). Actually these authors report in their Table II and Figure 4 calculations of interchain electrostatic potential energy $\lambda^2 e\psi_1$ as a function of salt concentration. The estimate of −0.4 kcal (mole nucleotide pair)$^{-1}$ for 0.001 M salt is obtained from the graph in their Figure 4. This value has been recently utilized by Kleinwachter *et al.* (1969) with reference to the effects of proflavine on the melting temperature at this salt concentration. Since in the development of Chambron *et al.* (1966a,b), $\Delta G_e(\text{dye}) = -\Delta G_e/\lambda$, the estimate $\Delta G_e(\text{dye}) = 1.8$ kcal $mole^{-1}$ is obtained using $\lambda = 0.22$. This agreement with our experimental results is satisfactory considering the number and uncertainties of the approximations employed. The definition of ΔG_e was such that plus means stabilization.

Recently Li and Crothers (1969) have studied by temperature-jump techniques the kinetic of formation of the proflavine–DNA complex at $\bar{B} = 0.05$ in the tight-binding region. Their results are interpreted in terms of a consecutive mechanism in which an "outside complex" is initially formed as an intermediate to the intercalated complex.

$$P + DNA \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (P)_{out} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} (P)_{in}$$

$$K_{12} = \frac{k_{12}}{k_{21}} \qquad K_{23} = \frac{k_{23}}{k_{32}}$$

Here P is proflavine and $(P)_{out}$ and $(P)_{in}$ are the outside-

bound and intercalated form of the dye, respectively. It is found that the equilibrium constant K_{23} is markedly decreased with decreasing salt concentration. This result means that at low ionic strength, the percentage of outside complexes increases relative to intercalated complexes. It is argued that this occurs because the phosphate ionic attraction is diminished in the intercalated form due to steric factors. This difference becomes unimportant at high ionic strength where shielding of the phosphates by counterions predominates.

Neither the optical nor electron spin resonance techniques distinguish between molecules bound on the outside or in the inside of the helix. The free energy of formation of the complex which we measure is then composed of contributions both from the inside and outside complexes. One can argue that no outside complex of MPCN· with DNA should be present if ionic attraction is the major source of binding energy for such a complex, and the observed free energy of binding is, in fact, a measure of base–ligand nonelectrostatic forces. However, for the intercalated complex, $\Delta\bar{G}^\circ$ would be underestimated. The free energy of binding per mole is given by

$$\Delta\bar{G}^\circ = -X_{out}RT \ln K_{12} - X_{in}[RT \ln K_{12} + RT \ln K_{23}] \quad (2)$$

$$= -RT \ln K_{12} - \frac{RT \ln K_{23}}{1 + K_{23}}$$

where X_j is the mole fraction of complex j. Since our interpretation of the measured $\Delta\bar{G}^\circ$ is that it represents the binding free energy for a situation of 100% of the "inside" complex, addition of the quantity $(-RT \ln K_{23})/(1 + K_{23})$ to the measured $\Delta\bar{G}^\circ$ is necessary to account for the presence of outside complexes. One can easily show that the maximum value of this correction, assuming $K_{23} \geq 1$, is $-0.28RT$, or -0.18 kcal/mole at room temperature.

The decrease in K_{23} with decreasing salt concentration must be compensated by an increase in K_{12} to account for the salt effects. One cannot expect that K_{12} would increase indefinitely at even lower salt concentration. Thus to conform to the observation that the product $(K_{12}K_{23})$ is larger at 0.001 M NaCl than at higher concentrations, we conclude that $K_{23} > 1$ and do not concern ourselves with the possibility that the correction becomes positive. Thus we anticipate that the correction will always be neglible. It is indeed unfortunate that the high electrical resistances of solutions of low ionic strength prevent temperature-jump studies of the kinetics of the intercalation reaction in this region. It would be most interesting to measure the relaxation kinetics of the binding of an uncharged dye to DNA.

Summary

An estimate of the contribution of charge to the interaction of a dye with DNA has been obtained. At low pH the protonated 5-methylphenazyl-2-nitrile cation radical predominates, and at high pH the uncharged 5-methylphenazyl-2-nitrile radical is present exclusively in a water–acetone (1:9, v/v) mixed solvent. By a comparison of the equilibrium constants for binding of these two species to salmon sperm DNA at low ionic strength (0.001 M Na^+), we find that the charged radical binds with a 2.5 kcal greater free energy than that of the uncharged radical. This amounts to about 30% of the total binding free energy. These conclusions refer to the type I or intercalated dye–nucleic acid complex.

Acknowledgment

We are indebted to Professor J. J. Hermans of the Chemistry Department and Professor J. R. White of the Biochemistry Department, University of North Carolina, and to Professor K. Ishizu of the Chemistry Institute, Faculty of Science, Ehime University, Japan, for helpful discussions and suggestions and to Dr. M. T. Huang for assistance in carrying out several experiments.

References

Akasaka, K., and Dearman, H. H. (1969), *Biochem. Biophys. Res. Commun. 35*, 377.
Blake, A., and Peacocke, A. R. (1968), *Biopolymers 6*, 1225.
Boyland, E., and Green, B. (1964), *J. Mol. Biol. 9*, 589.
Chambron, J., Daune, M., and Sadron, C. (1966a), *Biochim. Biophys. Acta 123*, 306.
Chambron, J., Daune, M., and Sadron, C. (1966b), *Biochim. Biophys. Acta 123*, 319.
Crick, F. H. C., Barnett, L., Brenner, S., and Watts-Tobin, R. J. (1961), *Nature (London) 192*, 1227.
DeVoe, H., and Tinoco, I. (1962), *J. Mol. Biol. 4*, 500.
Drummond, D. S., Pritchard, N. J., Simpson-Gildemeister, V. F. W., and Peacocke, A. R. (1965), *Biopolymers 3*, 135.
Gersch, N. F., and Jordan, D. O. (1965), *J. Mol. Biol. 13*, 138.
Heilweil, H. G., and Van Winkle, Q. (1955), *J. Phys. Chem. 59*, 939.
Ishizu, K., Dearman, H. H., Huang, M. T., and White, J. R. (1969), *Biochemistry 8*, 1238.
King, T. E. (1963), *J. Biol. Chem. 238*, 4032.
Kleinwachter, V., Balcarova, F., and Bohacek, J. (1969), *Biochim. Biophys. Acta 174*, 188.
Landridge, R., Marvin, D. A., Seeds, D. E., Wilson, H. R., Hooper, C. W., Willeins, M. H. F., and Hamilton, L. D. (1957), *J. Biophys. Biochem. Cytol. 3*, 767.
Landridge, R., Marvin, D. A., Seeds, D. E., Wilson, H. R., Hooper, C. W., Willeins, M. H. F., and Hamilton, L. D. (1960), *J. Mol. Biol. 2*, 38.
Lepecq, J. B., and Paoletti, C. (1967), *J. Mol. Biol. 27*, 87.
Lerman, L. (1961), *J. Mol. Biol. 3*, 18.
Li, H. J., and Crothers, D. M. (1969), *J. Mol. Biol. 39*, 461.
McIlwain, H. (1937), *J. Chem. Soc., A* 1704.
Müller, W., and Crothers, D. N. (1968), *J. Mol. Biol. 35*, 251.
Nagata, C., Kodama, M., Tagashira, Y., and Immamura, A. (1966), *Biopolymers 4*, 409.
Samejima, T., and Yang, J. T. (1964), *Biochemistry 3*, 613.
Schildkraut, C., and Lifson, S. (1965), *Biopolymers 3*, 195.
Zaugg, W. S. (1964), *J. Biol. Chem. 239*, 3964.