Compounds, Elsevier, New York (in press).
Hill, D. L., Smith, T.-W., Johnson, T. P., & Struck, R. F. (1978) *Cancer Res. 38*, 2438–2442.
Huff, J. E. (1983) *EHP, Environ. Health Perspect. 47*, 359–363.
Inskeep, P. B., & Guengerich, F. P. (1984) *Carcinogenesis (London) 5*, 805–808.
Leonard, N. J. (1983) *CRC Crit. Rev. Biochem. 15*, 125–199.
Letz, G. A., Pond, S. A., Osterloh, J. D., Wade, R. L., & Becker, C. E. (1984) *JAMA, J. Am. Med. Assoc. 252*, 2428–2431.
Liebler, D. C., Meredith, M. J., & Guengerich, F. P. (1985) *Cancer Res. 45*, 186–193.
MacFarland, R. T., Gandolfi, A. J., & Sipes, I. G. (1984) *Drug Chem. Toxicol. (1984) 1*, 213–227.
Nachtomi, E. (1970) *Biochem. Pharmacol. 19*, 2853–2860.
National Toxicology Program (1982) *Carcinogenic Bioassay of 1,2-Dibromoethane in F344 Rats and B6C3F$_1$ Mice (Inhalation Study)*, U.S. Department of Health and Human Services, Washington, DC.
Olson, W. A., Habermann, R. T., Weisburger, E. K., Ward, J. M., & Weisburger, J. H. (1972) *J. Natl. Cancer Inst. (U.S.) 51*, 1993–1995.
Ozawa, N., & Guengerich, F. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5266–5270.
Rannug, U. (1980) *Mutat. Res. 76*, 269–295.
Rannug, U., Sundvall, A., & Ramel, C. (1978) *Chem.-Biol. Interact. 20*, 1–16.
Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., & Potter, D. W. (1980) *Anal. Biochem. 106*, 55–62.
Storer, R. D., & Conolly, R. B. (1985) *Toxicol. Appl. Pharmacol. 77*, 36–46.
Sun, M. (1984) *Science (Washington, D.C.) 223*, 464–466.
Sundheimer, D. W., White, R. D., Brendel, K., & Sipes, I. G. (1982) *Carcinogenesis (London) 3*, 1129–1133.
Tomasi, A., Albano, E., Dianzani, M. U., Slater, T. F., & Vannini, V. (1983) *FEBS Lett. 160*, 191–194.
van Bladeren, P. J., Breimer, D. D., van Huijgevoort, J. A. T. C. M., Vermuelen, N. P. E., & van der Gen, A. (1981) *Biochem. Pharmacol. 30*, 2499–2502.
Weisburger, E. K. (1977) *EHP Environ. Health Perspect. 21*, 2804–2814.
White, R. D., Gandolfi, A. J., Bowden, G. T., & Sipes, I. G. (1983) *Toxicol. Appl. Pharmacol. 69*, 170–178.
Wong, L. C. K., Winston, J. M., Hong, C. B., & Plotnick, H. (1982) *Toxicol. Appl. Pharmacol. 63*, 155–162.

Conformation of d(CpG) Modified by the Carcinogen 4-Aminobiphenyl: A Combined Experimental and Theoretical Analysis†

Robert Shapiro,*,‡ Graham R. Underwood,‡ Halina Zawadzka,‡ Suse Broyde,§ and Brian E. Hingerty∥
Chemistry Department and Biology Department, New York University, Washington Square, New York, New York 10003, and Health and Safety Research Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830
Received July 31, 1985

ABSTRACT: The change in DNA conformation produced by the attachment of a reactive substance is likely to be a vital factor in determining the biological consequences of the reaction. We have prepared a deoxydinucleoside monophosphate containing the major adduct derived from the carcinogenic amine 4-aminobiphenyl and analyzed its conformation by theoretical and experimental methods. Reaction of d(CpG) with *N*-acetoxy-*N*-(trifluoroacetyl)-4-aminobiphenyl afforded the product modified at C-8 of guanine with 4-aminobiphenyl. After purification by reverse-phase high-performance liquid chromatography, milligram amounts of product were obtained. It was analyzed by circular dichroism, proton magnetic resonance, and minimized potential-energy calculations. A flexible molecule with a mixture of conformers is indicated. Both carcinogen–base-stacked states and base–base-stacked states, with guanine both syn and anti, contribute to the population mixture on the dimer level. The global minimum-energy conformation has *syn*-guanine and carcinogen–base stacking. Forms of this type are calculated to represent roughly 58% of the conformer population. Because of the twisted nature of the biphenyl moiety, carcinogen–base stacking inherently involves less overlap than that in the planar and rigid three-ringed aminofluorene analogue. This difference might relate to the diminished effectiveness of the aminobiphenyl vs. the aminofluorene adduct as a frameshift mutagen in *Salmonella typhimurium* 1538.

Only a slight structural modification often distinguishes potent carcinogens and mutagens from related compounds that are innocuous. This difference may involve the presence or location of a methyl group, for example, or the location of a ring junction (Dipple et al., 1984; Garner et al., 1984). In the case of the *anti*-benzo[*a*]pyrenediol epoxides (Buening et al., 1978), two enantiomers showed vastly different biological effects. It would be valuable if some simple principle could explain these differences. Carcinogenesis and mutagenesis by chemicals is the result of a series of processes, however, all of which must be considered if the relation of structure to biological effect is to be understood. These steps include entry of a chemical into the cell, metabolic activation, reaction with the critical target (presumably DNA), and response of the modified DNA to the repair and replication systems that subsequently interact with it.

† This work was supported jointly by DOE Contract DE-ACO2-81 ER60015, by USPHS Grant 1R01 CA 28038-05, awarded by the National Cancer Institute, DHHS, by BRSG Grant RR07062, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, by National Science Foundation Grant DMB8416009, and by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract DE-AC05-84OR21400 with Martin-Marietta Energy Systems, Inc. (B.E.H.).
‡ Chemistry Department, New York University.
§ Biology Department, New York University.
∥ Oak Ridge National Laboratory.

0006-2960/86/0425-2198$01.50/0

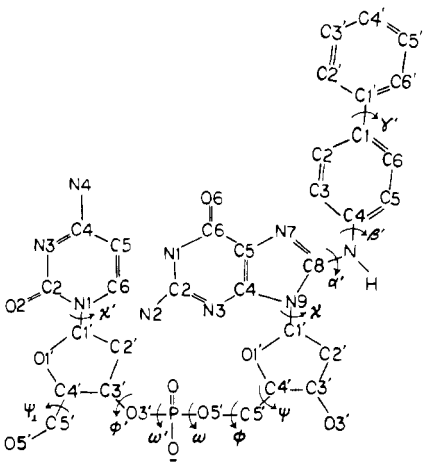

FIGURE 1: Structure, numbering scheme, and variable conformational angle designations for d(CpG)-ABP. The dihedral angles A–B–C–D are defined as follows: χ', O1′–C1′–N1–C6; χ, O1′–C1′–N9–C8; ψ_1 and ψ, C3′–C4′–C5′–O5′; ϕ', P–O3′–C3′–C4′; ϕ, C4′–C5′–O5′–P; ω', O5′–P–O3′–C3′; ω, C5′–O5′–P–O3′; α', N9–C8–N–C4; β', C8–N–C4–C5; γ', C6–C1–C1′–C6′. The angle A–B–C–B is measured by a clockwise rotation of D with respect to A, looking down the B–C bond. A eclipsing D is 0°. In addition, both sugar puckers are flexible. These are defined by the pseudorotation parameter **P** (Altona & Sundaralingam, 1972). $\mathbf{P}_1$ is 5′-linked; $\mathbf{P}_2$ is 3′-linked.

The last-named process is the one that claims our current attention. In particular, we wish to understand the conformational changes that result from chemical modification of DNA, as these provide a basis for an understanding of subsequent events, such as replication and repair. Unfortunately, little information is at hand that relates covalent alterations in DNA to detailed changes in conformation.

Spectroscopic studies upon modified DNA provide one approach to this problem. In the frequent cases where multiple adducts are formed by chemical reaction with DNA, however, the data reflect only their combined effects, with the contribution of each individual alteration somewhat obscured. Shorter oligonucleotides containing a single modification may be prepared by synthesis, but this can be a laborious process.

We feel that a considerable contribution to this area can be made by minimized potential-energy calculations, especially in those cases where experimental data can be obtained only with great difficulty. In particular, we have been carrying out calculations on DNA fragments modified by aromatic amines and certain polynuclear aromatic hydrocarbons [see Broyde & Hingerty (1985) and references cited therein]. These studies have been carried out initially with dinucleoside monophosphates but are currently being extended to larger units, up to the dodecamer level (Hingerty & Broyde, 1985). The potentials and approximations that we employ are subject to continual improvement, however, and for this purpose, a comparison of our calculations with experimental data in specific, well-defined systems is invaluable. We now report the result of such an effort, directed at the dideoxynucleoside monophosphate d(CpG), with modification by 4-aminobiphenyl at the 8-position of guanine (Figure 1). Polymer models suggested from some of the dimer minima have been presented elsewhere (Broyde et al., 1985).

The choice of 4-aminobiphenyl modification was made for a number of reasons. This compound and its *N*-acetyl derivative are closely related in structure to ones in the much-studied 2-aminofluorene series, differing only by the absence of a one carbon bridge. Both series show pronounced carcinogenic properties, yet the organ specificities in experimental animals vary considerably (Beland & Kadlubar, 1985). Usually, multiple adducts are formed that undergo differential rates of repair, producing a situation that is difficult to interpret (Gupta & Dighe, 1984). A much more revealing comparison has been made with *Salmonella typhimurium* 1538 by using the *N*-hydroxy derivatives of 4-aminobiphenyl and 2-aminofluorene. Both *N*-hydroxy amines produced frameshift mutations in this strain, but 2-aminofluorene was 5–6 times more effective when equal amounts of each amine were bound to DNA (Beland et al., 1983). The greater potency of 2-aminofluorene on the basis of dose administered had been demonstrated in an earlier study (Scribner et al., 1979), with the same strain. When strain TA 98, with capacity for error-prone repair, was used, however, the two amines were roughly equal in ability to produce frameshift mutations. In this study, we were interested in seeing whether some conformational difference, perhaps even one detectable at the dimer level, lay under these differences in mutagenesis and repair.

Materials and Methods

Materials. The following materials were obtained from the sources indicated: 2′-deoxyguanosine (P-L Biochemicals); 2′-deoxycytidine, 2′-deoxycytidine 3′-monophosphate, 2′-deoxycytidylyl(3′–5′)-2′-deoxyguanosine, tris(hydroxymethyl)aminomethane hydrochloride, and phosphodiesterase II from bovine spleen (Sigma Chemical Co.); D_2O, $CDCl_3$, and Me_2SO-d_6 (Stohler Isotope Chemicals). All other solvents and chemicals were from the Aldrich Chemical Co.

Chromatographic Methods. TLC[1] was performed with the following substrates: (A) 0.2-mm analytical silica gel (EM Reagents, no. 5775) and (B) 1.0-mm preparative silica gel sheets (Woelm, Analtech), both with fluorescent indicator. The following mobile phases were employed: (a) benzene–95% ethanol (9:1), (b) benzene–95% ethanol (20:1), (c) benzene–ethyl acetate (1:1), and (d) chloroform–ethanol–pyridine (140:60:1). HPLC was performed with the following columns: (A) μBondapak RP C_{18} (Waters Assoc., 3.9 mm × 30 cm) and (B) Nucleogen DEAE 60-7 (Alltech Assoc., 4.0 mm × 12.5 cm). Mobile-phase programs employed were as follows: (a) 20–80% methanol, convex gradient, 30 min; (b) same as (a), 45 min; (c) isocratic, 60% methanol; (d) 20–80% methanol, linear gradient, 25 min; (e) same as (d), 15 min; (f) 0–0.8 M Li_2SO_4 in sodium acetate (0.02 M, pH 6.5), 30% methanol, 40 min. All flow rates were 1 mL min^{-1}.

Spectroscopic Methods. All pH measurements were made at room temperature. The p*K* values were determined by measuring the change in UV absorption in a series of buffers made up of mixtures of 5-aminovaleric acid, K_2HPO_4, and H_3BO_3, containing 20% methanol. Molar extinction coefficients of d(CpG), of modified nucleoside dG-ABP, and modified dinucleoside monophosphate d(CpG)-ABP were determined in methanol and in aqueous potassium phosphate (1 mM, pH 7.05). CD spectra were recorded on a Cary Model 60 spectropolarimeter (Applied Physics Corp., Monrovia, CA). Spectra were recorded in a 1-cm path-length cell at 27 °C.

[1] Abbreviations: TFAAABP, *N*-acetoxy-*N*-(trifluoroacetyl)-4-aminobiphenyl; dG, deoxyguanosine; dG-ABP, *N*-(2′-deoxyguanos-8-yl)-4-aminobiphenyl; d(CpG), 2′-deoxycytidylyl(3′–5′)-2′-deoxyguanosine; d(CpG)-ABP, *N*-[2′-deoxycytidylyl(3′–5′)-2′-deoxyguanos-8-yl]-4-aminobiphenyl; TLC, thin-layer chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; FT, Fourier transform; ppm, parts per million; HPLC, high-performance liquid chromatography; RP, reverse phase; BP, biphenyl; ABP, 4-aminobiphenyl; AAAF, *N*-acetoxy-*N*-acetyl-2-aminofluorene; AF, 2-aminofluorene; Me_2SO, dimethyl sulfoxide.

Extinction coefficients of the substances studied by CD were obtained directly with samples of 0.5–0.6 mg, in the appropriate solvents. They are expressed on a molar basis for both nucleosides and dinucleoside monophosphates. Ellipticities (θ) are given on a molar basis for monomers and on a per residue basis for dimers, to facilitate comparisons.

All samples for NMR were dried under vacuum over P_2O_5 and were dissolved in the best deuterated solvents commercially available. Those in aqueous solvent were buffered to pD 7.05 with potassium phosphate (1 mM). pD measurements were obtained by adding 0.4 to the measured pH values. Spectra were recorded at 27 °C on a General Electric QE-300 300-MHz spectrometer operating in the FT mode. The chemical shifts were all referenced to an internal standard of CH_3OH (3.340 ppm).

Syntheses. All evaporations and concentrations were performed under reduced pressure.

(*A*) *TFAAABP.* The starting material for the syntheses was conveniently prepared from 4-nitrobiphenyl, by reduction with zinc dust–ammonium chloride in ethanol to yield the biphenyl hydroxylamine (Kamm, 1941) followed by treatment of the product with trifluoroacetic anhydride and acetic anhydride to yield the desired *N*-acetoxy-*N*-(trifluoroacetyl)-4-aminobiphenyl (TFAAABP) (Lee & King, 1981). It was purified by recrystallization from hexane–ethyl acetate (5:1) at 0 °C, which produced yellow needles (85% yield). TLC showed one spot, R_f (substrate, solvent), 0.81 (A, a), 0.65 (A, b), and 0.66 (A, c). It decomposes in solution (25 °C) with a half-life of 90 min (75% methanol–25% water), 40 min (50% ethanol–50% water), and 23 h (95% ethanol–5% water).

(*B*) *dG-ABP.* This was prepared with a modification of the published procedure for rG-ABP (Lee & King, 1981). Deoxyguanosine (280 mg, 1.04 mmol), dissolved in sodium citrate (320 mL, 2 mmol, pH 7.0), was brought to 50 °C and stirred under a blanket of argon. TFAAABP (1340 mg, 4.15 mmol) dissolved in 95% ethanol (320 ml) was added dropwise (2 h) and stirred for an additional 1 h. The reaction mixture was brought to room temperature and concentrated to 300 mL. The solution was extracted with ethyl ether (5 × 150 mL). The remaining aqueous layer was extracted with ethyl acetate (6 × 100 mL). The combined ethyl acetate layers were evaporated to dryness, and the remaining oil was redissolved in ethanol (6 mL) and precipitated from ether (140 mL). The precipitate was shown by HPLC (column A, program a) to be a mixture of three compounds: the desired adduct (R_t 11 min) and two contaminants (R_t 12 and 26 min). This crude mixture was further purified by TLC (substrate b, solvent d). The developed plates were dried under reduced pressure, and the UV-active band (366 nm, blue fluorescence) was extracted with ethanol at 50 °C. The solution was concentrated and the product was precipitated with ether. Repetitive purification by HPLC (column A, program b) produced pure dG-ABP in 15% yield. The beige solid was homogeneous on TLC under the above conditions and appeared as one peak by HPLC (column A, program c, R_t 14.0 min). It was stable under anhydrous conditions or in CD_3OD for several months at 0 °C: pK_a = 3.25 and 9.78 (determined at 300 nm); isosbestic point 285 (pH range 1.18–7.86); UV (methanol) λ_{max} (ϵ) 305 nm (31 000) and 250 (14 000), λ_{min} 265 nm (12 300); UV (11% methanol, 89% 1 mM potassium phosphate, pH 7.05) λ_{max} 297 nm (28 800) and 250 (14 000), λ_{min} 263 nm (13 500); CD (methanol) (θ) 320 (-0.12×10^{-4} and 280 nm (0.89×10^{-4}); CD (aqueous 1 mM potassium phosphate, pH 7.05) (θ) 317 (-0.54×10^{-4}) and 282 nm (1.35×10^{-4}); Proton NMR (CD_3OD) δ 7.566 (d), 7.516 (d), 7.706 (d), 7.382 (t), 7.255 (t), 6.456 (t), 4.580 (m), 4.022 (m), 3.9 (m), 2.704 (m), and 2.168 (m).

(*C*) *d(CpG-ABP).* d(CpG) (20 mg, 35 μmol) dissolved in a mixture (5 mL) of sodium citrate (28 mM, pH 7.05) and methanol (1:1) was stirred at 37 °C under argon. TFAAABP (60 mg, 185 μmol) in methanol (5 mL) was added dropwise. The mixture was stirred for 4 h at 37 °C, cooled, and evaporated to dryness. The residual solid was suspended in water (60 mL) and extracted with ether (3 × 50 mL). The combined ether washings were reextracted with water (20 mL), and the combined aqueous layers were evaporated to dryness, redissolved in methanol–water (60:40), and chromatographed. The HPLC (column A) was eluted with 20:80 methanol–water for 10 min and then with a linear gradient (15 min) to 80% methanol. The gradient was stopped (approximately 50% methanol) while the major component was collected. The peak corresponding to d(CpG-ABP) (R_t 23 min) was the major peak at 280 nm. The peaks collected from several runs were evaporated to dryness. The product was washed with ether (4 × 5 mL), the suspension was spun down, and the ether was discarded. The product was a pale yellow solid, yield 4 mg (17%). The material isolated in this manner contained some minor impurities, less polar than the adduct. Repetitive chromatography under the same conditions generated pure material as judged by NMR and HPLC (column A, program c, R_t 11.5 min; column B, program f, R_t 40 min). The substance is stable under anhydrous conditions or in D_2O solution at 0 °C for several months. It gave the following physical data: HPLC R_t (column A, program e) 16.5, (column A, program a) 15.5, and (column B, program f) 18.8 min; pK_a = 3.49 (determined at 275 nm, pH range 1.56–6.95); isosbestic points 285 and 296 nm. Spectra taken over the range pH 6.04–8.37 were essentially identical. The pK_a in the basic range could not be determined accurately due to decomposition of the material in the basic solution. The following were determined: UV (in methanol) λ (ϵ) 290 nm (27 300) and 250 (18 200); UV (potassium phosphate, 0.85 mM, pH 7.05) λ (ϵ) 285 nm (25 100) and 250 (19 700); CD (methanol) (θ) 275 nm (9.5×10^{-5}); CD (potassium phosphate, 1 mM, pH 7.05) (θ) 310 (4.3×10^{-5}) and 270 nm (1.54×10^{-4}); Proton NMR (D_2O) δ 7.608 (d), 7.408 (d), 7.543 (d), 7.456 (t), 7.352 (t), 7.409 (d), 6.163 (t), 6.017 (t), 5.692 (d), 4.408 (m), 4.16 (m), 4.16 (m), 3.995 (m), 3.51 (m), 3.49 (m), 3.006 (m), 2.33 (m), and 1.720 (m). For assignments, see Figure 3.

Enzymatic Hydrolysis of d(CpG)-ABP. The adduct ($\sim 2A_{260}$) dissolved in Tris-HCl (0.6 mL, 5 mM, pH 8.04) was incubated with phosphodiesterase II[2] (0.11 unit) at 37 °C overnight. The digest was subjected to RP HPLC and eluted stepwise, with 10% methanol (10 min) and then with 80% methanol. Three major peaks eluted: R_t 2.3, 4.8, and 18 min. These correspond respectively to dCp, dC, and dG-ABP. No starting material remained.

Theory

The total energy of the molecule, E_{tot}, is partitioned into contributions of nonbonded, E_{nb}, electrostatic, E_{el}, torsional, E_{tor}, deoxyribose strain, E_{st}, and deoxyribose and phosphate anomeric terms, E_{an}, as described previously in detail (Hingerty & Broyde, 1982; Broyde & Hingerty, 1983). These potentials follow the treatment of Olson (1982) and Srinivasan and Olson (1980), as described earlier. Counterion condensation is treated by reduction of the partial charge on the nonlinking phosphate oxygens, and solvent is treated by a distance-de-

[2] In addition to 3′-exonuclease activity, the enzyme also has some 5′-exonuclease activity (indicated by the supplier).

pendent dielectric constant. Modifications to incorporate biphenyl have also been described (Broyde et al., 1985).

All eight DNA backbone torsion angles (Figure 1), plus the two pseudorotation parameters, $\mathbf{P}_1$ and $\mathbf{P}_2$, which define the conformations of the deoxyriboses (Altona & Sundaralingam, 1972), plus the torsions at the carcinogen–base linkage, α' (N9–C8–N–C4) and β' (C8–N–C4–C5), and the torsion linking the two phenyl rings, γ', were simultaneously variable parameters, for a total of 13 degrees of conformational freedom. Minimizations, performed by a modified version of the Powell algorithm (Powell, 1964), were carried to an accuracy of 1° in each parameter at the minimum, with no angle permitted to vary by more than 100° at any step. About 2500 trials, detailed earlier (Broyde et al., 1985), which surveyed the entire ω', ω, ψ conformation space at 120°, intervals were made. While this is a fairly extensive search, the nature of the multiple minimum problem is such that important minima may be missed. What can be said with reasonable confidence is that many important minima have been found by this search.

Results and Discussion

Circular Dichroism Spectra. A dramatic difference in the CD of two related compounds, for example, a large change in amplitude or the inversion of a major peak, is regarded as an indicator of an important conformational difference between the two compounds (Warshaw & Cantor, 1970). The spectra may suggest certain types of conformational change but do not specify them uniquely.

The CD spectra of deoxyguanosine and its aminobiphenyl-modified derivative dG-ABP are compared in Figure 2a. Attachment of the aromatic amine residue to dG shifted its ultraviolet absorption spectrum to longer wavelengths (see Materials and Methods). A negative Cotton effect now appears in this area, while a positive one is displayed at 285 nm. The spectrum of dG-ABP is similar to that of 8-(methylamino)guanosine. The spectrum of this latter compound was analyzed by a group that had made an extensive comparison of the circular dichroism of substituted guanine nucleosides (Miles et al., 1971). In their analysis, the aqueous spectrum of deoxyguanosine was associated with a predominantly anti conformation while the spectrum of 8-(methylamino)guanosine was anomalous, and not of either anti or syn type. They suggested a possible high anti conformation for the latter, which would accomodate the steric requirements of the 8-substituent. More recently, NMR studies of 8-(alkylamino)guanine nucleosides have suggested a flexible syn–anti mixture. The anti conformer, stabilized by an amino to 5'-CH_2OH hydrogen band, predominates (Jordan & Niv, 1977; Lassota et al., 1984).

In Figure 2b, the neutral aqueous CD spectra of the modified dimer d(CpG)-ABP, unmodified d(CpG), and the composite of the monomers dC and dG-ABP are compared. The prominent positive Cotton effect present in all three spectra appears at shorter wavelength in d(CpG)-ABP than in the others, with moderately enhanced intensity. The negative Cotton effect at 310 nm of d(CpG)-ABP has a slightly greater amplitude than that of its component monomers. The spectra of d(CpG)-ABP and d(CpG) were also obtained in 95% methanol, a solvent that weakens stacking interactions (data not shown). Both were similar, with a single prominent Cotton effect displayed above 240 nm. The amplitude of this peak ($\times 10^{-4}$) was 0.95 at 275 nm for d(CpG)-ABP and 0.67 at 275 nm for d(CpG).

Our results can be compared with those of other workers who have studied oligonucleotides containing guanine modified at the 8-position with 2-aminofluorene or *N*-acetyl-2-aminofluorene (Grunberger et al., 1970; Nelson et al., 1971; Santella et al., 1980; Leng et al., 1980; Rio et al., 1983). In the (acetylamino)fluorene case, the amplitude observed in the CD spectrum of r(ApG)-AAF was an order of magnitude greater than that observed with modified monomer or unmodified dimer. This result was interpreted in terms of "strong base stacking between AAF and the base adjacent to the substituted guanosine residue" (Nelson et al., 1971). A similar effect was later observed with d(ApG)-AAF. In the case of d(ApG)-AF, however, the CD change in amplitude produced by modification was "only one-third that of AAF modification indicating less of a stacking interaction" (Santella et al., 1980). In all of the cited cases, an increase in temperature or a change to an organic solvent produced a decrease in the CD spectrum, which was "consistent with disruption of a stacking interaction" (Santella et al., 1980).

In our own study with d(CpG)-ABP, the increase in CD amplitude of the modified dimer over the constituent monomers was only 50%, less than that observed in the above cases. This modest enhancement disappeared when the spectra were run in methanol, which suggested that it was also due to a base-stacking interaction. The weakness of the effect with our modified dimer may be due to a difference in the neighboring base and/or the nature of the biphenyl group. In the AAF

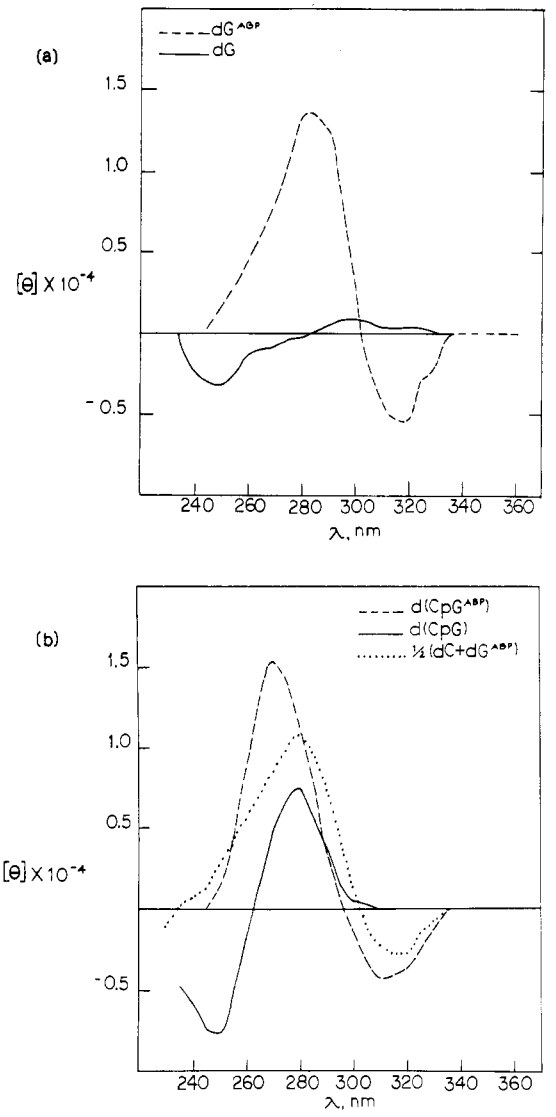

FIGURE 2: (a) Circular dichroism spectra of dG and dG-ABP in phosphate buffer. (b) Circular dichroism spectra of d(CpG), d(CpG)-ABP, and $^1/_2$-(dC + dG-ABP).

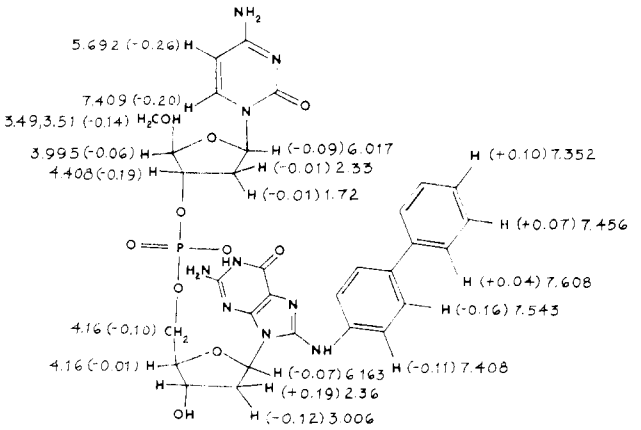

FIGURE 3: NMR chemical shifts of d(CpG)-ABP in D_2O (pH 7.05) relative to CH_3OD (3.340 ppm) are listed adjacent to the relevant protons. Alongside, within parentheses, are given these chemical shifts minus unmodified d(CpG). The values for the biphenyl protons represent modified dimer (in D_2O) and modified monomer (in CD_3OD). The shifts of the BP 2,6- and 3,5-protons are somewhat solvent dependent. This may result in values that are somewhat high for the 2,6-protons and low for the 3,5-protons.

series, observed spectral changes have been greater when a purine, rather than a pyrimidine, was adjacent on the 5′-side of the modified base. In addition, the biphenyl moiety has an inherently smaller stacking capacity than AF and AAF. Not only does it lack the central ring formed by the CH_2 bridge of the fluorenes, but the phenyl rings are twisted with respect to one another in solution (Canselier & Cassoux, 1977).

Proton NMR Spectra. The NMR chemical shifts of the nonexchangeable protons of d(CpG)-ABP in D_2O are shown in Figure 3. The assignments were made with the help of extensive decoupling experiments. Each proton is coupled to all neighboring protons or to phosphorus but not to the protons of the other sugar. Therefore, it is possible to group the proton resonances of a given sugar by sequential decoupling (Cheng & Sarma, 1977) before the actual identity of that sugar is known. Then, if the identity of one of these resonances can be established, the complete assignment of all the sugar resonances is possible. The resonances of the 1′-protons of the deoxyriboses are most readily discernable. The more downfield of these resonances (6.163 ppm) was tentatively assigned to H-1′ of guanosine. On this basis then, a tentative assignment of all the sugar resonances of the adduct could be made. The relative ordering of all the resonances assigned in this manner was identical with the relative order of the resonances in d(CpG) itself.

The aromatic region of the spectrum was made up primarily of a one-proton doublet (cytosine C-6), a one-proton triplet (BP-4′), a two-proton triplet (BP-3′,5′), and a two-proton doublet (BP-2′,6′). These assignments were confirmed by appropriate decouplings. The remaining pair of mutually coupled doublets (BP-3,5 and -2,6) was assigned on the basis of (i) lanthanide-induced shifts of (acetylamino)biphenyl in methanol, (ii) the assignment of the spectrum of the corresponding guanine (C8)–aniline adduct in methanol,[3] and (iii) predictions based on theoretical and experimental studies of substituted anilines (Bennett et al., 1969).

The chemical shift changes produced by aminobiphenyl substitution of d(CpG) are also presented in Figure 3. Downfield shifts (deshielding effects in the modified dimer) are associated with positive numbers; upfield shifts (shielding effects), with negative ones. The largest changes observed are upfield shifts of the cytosine ring protons. These changes are consistent with an increased population of conformers which place these protons in a shielding region of one or more aromatic rings. Similar but larger effects on the ring protons of the base adjacent to a modified one were observed in d(ApG)-AAF (0.6 ppm upfield shift of the adenine protons) and d(ApG)-AF (0.3–0.44 ppm shift of the adenine protons) (Santella et al., 1980; Leng et al., 1980). These changes were interpreted in terms of a stacking interaction between the introduced carcinogen and the neighboring adenine.

Since both the biphenyl and the fluorenyl moieties are made up of identical benzenoid rings, their ring current effects should be directly comparable. Therefore, the smaller change observed upon modification of d(CpG) with the biphenylamino moiety suggests stacking between the biphenyl and cytosine rings, but to a lesser extent than is observed in the cited cases. Another noteworthy upfield shift in d(CpG)-ABP is observed for the 3′-proton of the dC residue; again, this suggests the shielding effect of an aromatic ring.

A downfield shift of 0.19 ppm was exhibited by the dG H-2′ resonance. The change in the chemical shift of this proton upon 8-substitution of a guanine nucleoside has been used as an indication of a change to a greater population of syn conformers. Thus, a shift of 0.58 ppm of H-2′ of 8-bromoguanosine, relative to guanosine, was interpreted in terms of an almost entirely syn conformation for the former (Lassota et al., 1984). In the case of dG-AAF, a shift of 0.41 ppm relative to dG was interpreted similarly, but a shift of only 0.06 ppm for dG-AF was considered as evidence for a higher population of the anti conformer in this adduct (Evans et al., 1980) than in the AAF adduct. The aminobiphenyl-modified monomer showed a shift of the H-2′ resonance (relative to dG) of only 0.02 ppm; the modified dimer had a change of 0.19 in this resonance, however, compared to the unmodified dimer. We interpret this in terms of a modest increase in the syn population after modification at the dimer level but little change at the monomer level.

The chemical shift changes in the biphenyl ring protons of d(CpG)-ABP, relative to those of the modified monomer dG-ABP, are also indicated in Figure 3. An upfield shift is seen for the protons of the ring proximate to the guanine, suggesting some stacking interaction, while a smaller deshielding effect is seen in the other ring.

Theoretical Results and Comparison with Experiment. Tables I–IV in the supplementary material (see paragraph at end of paper regarding supplementary material) present minimum energy conformations to about 3 kcal/mol computed for d(CpG)-ABP. The conformers have been grouped according to stacking type, i.e., carcinogen–base stacking or base–base stacking, and according to guanine glycosidic torsion, i.e., anti or syn. It is important to note that we employ the term "carcinogen–base stacking" to describe conformers that have the carcinogen approximately parallel to the cytidine (as opposed to the guanine being parallel to cytidine), although actual overlap of aromatic moieties may be small. The same is true for the category "base–base stacking"; the degree of overlap between guanine and cytidine may vary from little to very significant, but the bases are close to parallel. Three types of conformers are found in the lowest 1 kcal/mol, namely, carcinogen–base-stacked forms with guanine both syn and anti and base–base-stacked conformers with guanine anti. *syn*-Guanine base–base-stacked conformations are less preferred.

Approximate statistical weights, P, have been computed from the formula $P = e^{-\Delta E/(RT)}/\sum e^{-\Delta E/(RT)}$. ΔE is the relative energy of each state in kcal/mol, R is the universal gas con-

[3] M. D. Jacobson and G. R. Underwood, unpublished results.

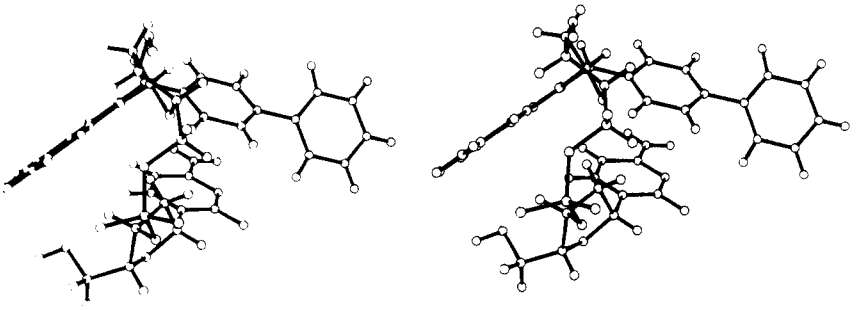

FIGURE 4: Global minimum energy conformation (Table III, no. 1) calculated for d(CpG)-ABP, in stereo view. The guanine is syn, and the biphenyl ring nearest guanine is stacked with cytosine. Torsion angles are $\chi' = 53°$, $\psi_1 = 60°$, $\phi' = 217°$, $\omega' = 314°$, $\omega = 265°$, $\phi = 166°$, $\psi = 57°$, $\chi = 238°$, $\mathbf{P}_1 = 41°$, $\mathbf{P}_2 = 105°$, $\alpha' = 67°$, $\beta' = -14°$, and $\gamma' = 39°$.

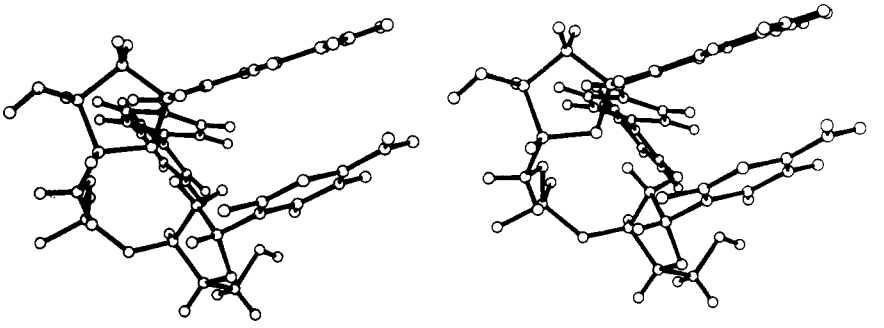

FIGURE 5: Guanine–cytosine stacked conformer (Table I, no. 1) with guanine anti, in stereo view. Torsion angles are $\chi' = 56°$, $\psi_1 = 54°$, $\phi' = 192°$, $\omega' = 274°$, $\omega = 288°$, $\phi = 164°$, $\psi = 62°$, $\chi = 55°$, $\mathbf{P}_1 = 77°$, $\mathbf{P}_2 = 139°$, $\alpha' = 199°$, $\beta' = 118°$, and $\gamma' = 37°$.

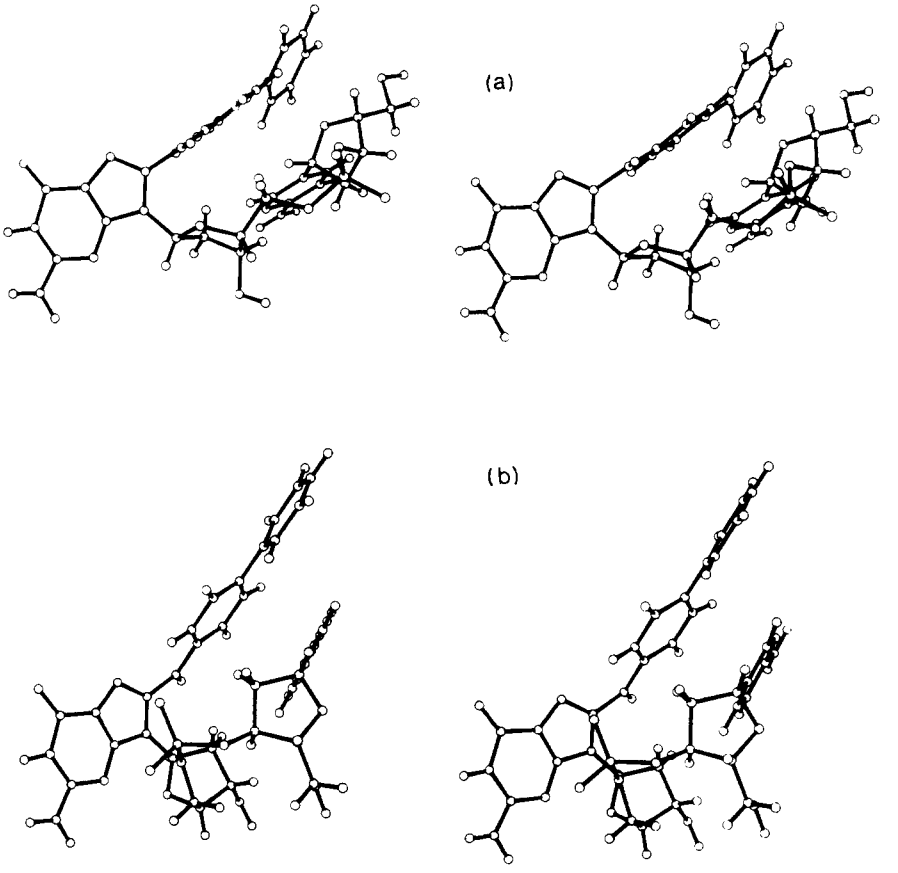

FIGURE 6: ABP–cytosine stacked conformers with guanine anti or high anti, in stereo view; (a) and (b) are no. 1 and 2 in Table II, respectively. Torsion angles are (a) $\chi' = 45°$, $\psi_1 = 178$; $\phi' = 193°$, $\omega' = 263°$, $\omega = 287°$, $\phi = 190°$, $\psi = 290°$, $\chi = 47°$, $\mathbf{P}_1 = 157°$, $\mathbf{P}_2 = 179°$, $\alpha' = 99°$, $\beta' = 175°$, and $\gamma' = -32°$ and (b) $\chi' = 64°$, $\psi_1 = 60°$, $\phi' = 189°$, $\omega' = 46°$, $\omega = 65°$, $\phi = 188°$, $\psi = 66°$, $\chi = 163°$, $\mathbf{P}_1 = 208°$, $\mathbf{P}_2 = 31°$, $\alpha' = 230°$, $\beta' = -1°$, and $\gamma' = 31°$.

stant in kcal deg^{-1} mol^{-1} and T is 300 K. The denominator is summed over all states to 3 kcal/mol. All states are assumed to have equal entropy in this crude approximation. The combined weights for the four categories of conformers are 58% carcinogen–base stacking with guanine syn, 23% carcinogen–base stacking with guanine anti, 17% base–base stacking with guanine anti, and 2% base–base stacking with guanine syn. Figures 4–6 show the most important conformers

relevant to the solution conformation of d(CpG)-ABP.

The global minimum (Figure 4) has *syn*-guanine with carcinogen–base stacking and a twist of 39° between the two phenyl rings. In the minima presented in Tables I–IV, phenyl–phenyl twists ranging from a low of 22° to a high of 54° are computed, with the 30–40° region preferred. Various solution studies of biphenyl have found the twist angle to be in the computed range [Schmid & Brosa, 1972; Barret & Steele, 1972; Canselier & Cassoux (1977) and references cited therein]. Only the phenyl ring proximate to guanine stacks with cytosine, while the *syn*-guanine occupies a position above the deoxycytidine sugar ring, near the 3′-hydrogen of that ring. These features are in good agreement with the conformational picture that emerged from the nuclear magnetic resonance and circular dichroism studies on this compound, discussed above. These studies suggested a stacking interaction between the biphenyl ring system (in particular the ring proximal to guanine) and the neighboring cytosine, some shielding of the H-3′ proton of the dC residue, and enhanced content of guanine syn conformers in the modified dimer, relative to the modified monomer.

Biological Implications. The global minimum for d(CpG)-ABP is conformationally similar to the one calculated for d(CpG)-AF (Broyde & Hingerty, 1982). However, the latter compound differs in that the coplanarity of the three-ring fluorene moiety permits a better stacking interaction than is possible with the twisted two-ring biphenyl system. While the low-energy forms at the dimer level are not necessarily the predominant structures when the carcinogens are bound to a larger polymer, we feel it likely that the difference in stacking ability displayed in these dimer minima will persist in the relevant polymer structures and play some role in defining the differences in mutagenic and carcinogenic effectiveness of the two systems. If, as has been suggested (Broyde et al., 1985), a carcinogen–base stacked state were required to produce a mutation at the replication fork, the better stacking possible for AF might stabilize the inserted state more than for ABP, and thus explain its higher mutagenic potency (Beland et al., 1983).

A more dramatic difference can be observed, even at the dimer level, if we compare the two compounds discussed above with d(CpG)-AAF. The presence of the additional bulky acetyl group in the last named substance greatly destablizes *anti*-guanine conformations and leaves the lowest energy region entirely populated with *syn*-guanine species. When these species are incorporated into models of large polymers, a number of striking conformational effects result, which have been observed in solution studies (Grunberger et al., 1970; Fuchs & Daune, 1971; Sage & Leng, 1980; Santella et al., 1981) and by potential-energy calculations and model building (Hingerty & Broyde, 1982). The mutagenic spectrum produced upon introduction of (acetylamino)fluorene groups into DNA differs greatly, of course, from those produced by aminofluorene or aminobiphenyl modification (Beranek et al., 1982; Scribner et al., 1979; Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985). We feel that the differences in conformational properties contribute to the differences in biological consequences.

Conclusions

The combined techniques of CD, NMR, and minimized semiempirical potential-energy calculations have been employed to investigate the conformation of d(CpG)-ABP. The combined results indicate a population mix with both carcinogen–base-stacked states and base–base-stacked states and with guanines both syn and anti. A predominant conformation with carcinogen–base stacking and guanine syn is computed to contribute roughly 58% to the population distribution. Differences between the AF and the ABP adduct reside in subtle distinctions in the extent of overlap between carcinogen and base: the twisted biphenyl moiety has an inherently diminished capacity for overlap compared to AF. The differences between AAF on the one hand and AF and ABP on the other are much more overt. Both AF (Broyde & Hingerty, 1983) and ABP have significant conformer contributions among the lowest energy states with guanine anti and DNA backbone conformation like the normal "A" or "B" helix, while such states are entirely disfavored for AAF adducts (Hingerty & Broyde, 1982). Differences in biological outcomes resulting from these lesions may be related to their differing conformational influences.

Supplementary Material Available

Four tables presenting minimum energy conformations of d(CpG)-ABP (4 pages). Ordering information is given on any current masthead page.

Registry No. *p*-$NH_2C_6H_4Ph$, 92-67-1; d(CpG), 15178-66-2; *p*-$PhC_6H_4N(COCF_3)(OAc)$, 78281-05-7; d(CpG)-NHC_6H_4Ph-*p*, 97546-15-1.

References

Altona, C., & Sundaralingam, M. (1972) *J. Am. Chem. Soc. 94*, 8205–8212.

Barrett, R., & Steele, D. (1972) *J. Mol. Struct. 11*, 105–125.

Beland, F. A., & Kadlubar, F. F. (1985) *EHP, Environ. Health Perspect. 62*, 19–30.

Beland, F. A., Beranek, D. T., Dooley, K. L., Heflich, R. H., & Kadlubar, F. F. (1983) *EHP, Environ. Health Perspect. 49*, 125–134.

Bennett, J., Delmas, M., & Maire, C. (1969) *Org. Magn. Reson. 1*, 319–327.

Beranek, D., White, G., Helfich, R., & Beland, F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 69*, 5175–5178.

Bichara, M., & Fuchs, R. P. P. (1985) *J. Mol. Biol. 183*, 341–351.

Broyde, S., & Hingerty, B. (1983) *Biopolymers 22*, 2423–2441.

Broyde, S., & Hingerty, B. (1985) in *The Molecular Basis of Cancer: An Interdisciplinary Discussion on Basic and Applied Aspects of Cancer* (Rein, R., Ed.) pp 153–171, Liss, New York.

Broyde, S., Hingerty, B. E., & Srinivasan, A. R. (1985) *Carcinogenesis 6*, 719–725.

Buening, M. K., Wislocki, P. G., Levin, W., Yagi, H., Thakker, D. R., Akagi, H., Koreeda, M., Jerina, D. M., & Conney, A. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5358–5361.

Canselier, J., & Cassoux, P. (1977) *J. Mol. Struct.*, 301–305.

Cheng, D., & Sarma, R. (1977) *J. Am. Chem. Soc. 99*, 7333–7348.

Dipple, A., Moschel, R. C., & Bigger, A. H. (1984) in *Chemical Carcinogens* (Searle, C. E., Ed.) 2nd ed., Vol. 1, pp 41–163, American Chemical Society, Washington, D.C.

Evans, F. E., Miller, D. W., & Beland, F. E. (1980) *Carcinogenesis 1*, 955–959.

Fuchs, R. P. P., & Daune, M. (1971) *FEBS Lett. 14*, 206–208.

Garner, R. C., Martin, C. E., & Clayson, D. B. (1984) in *Chemical Carcinogens* (Searle, C. E., Ed.) 2nd ed., Vol. 1, pp 175–276, American Chemical Society, Washington, D.C.

Grunberger, D., Nelson, J. H., Cantor, C. R., & Weinstein, I. B. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 488–494.

Gupta, R. C., & Dighe, N. R. (1984) *Carcinogenesis 5*, 343–349.

Hingerty, B. E., & Broyde, S. (1982) *Biochemistry 21*, 3243–3252.
Hingerty, B. E., & Broyde, S. (1985) *Biophys. J. 47*, 225a.
Jordan, F., & Niv, H. (1977) *Biochim. Biophys. Acta 476*, 265–271.
Kamm, O. (1941) *Organic Synthesis*, Collect. Vol. I, pp 445–447, Wiley, New York.
Koffel-Schwartz, N., Verdier, J.-M., Bichara, M., Freund, A.-M., Duane, M. P., & Fuchs, R. P. P. (1984) *J. Mol. Biol. 177*, 33–51.
Lassota, P., Stolarski, R., & Shugar, D. (1984) *Z. Naturforsch., C: Biosci. 39C*, 55–63.
Lee, M.-S., & King, C. M. (1981) *Chem.-Biol. Interact. 34*, 239–248.
Leng, M., Ptak, M., & Rio, P. (1980) *Biochem. Biophys. Res. Commun. 96*, 1095–1102.
Miles, D. W., Townsend, L. B., Robins, M. J., Robins, R. K., Inskeep, W. H., & Eyring, H. (1971) *J. Am. Chem. Soc. 93*, 1600–1608.
Nelson, J. H., Grunberger, D., Cantor, C. R., & Weinstein, I. B. (1971) *J. Mol. Biol. 62*, 331–346.
Olson, W. (1982) *J. Am. Chem. Soc. 104*, 278–286.
Powell, M. (1964) *Comput. J. 7*, 155–162.
Rio, P., Malfoy, B., Sage, E., & Leng, M. (1983) *EHP, Environ. Health Perspect. 49*, 117–123.
Sage, E., & Leng, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4597–4601.
Santella, R. M., Kriek, E., & Grunberger, D. (1980) *Carcinogenesis 1*, 897–902.
Santella, R., Grunberger, D., Weinstein, I. B., & Rich, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1451–1455.
Schmid, E., & Brosa, B. (1972) *J. Chem. Phys.*, 6267–6268.
Scribner, J. D., Fisk, S. R., & Scribner, N. K. (1979) *Chem.-Biol. Interact. 26*, 11–25.
Srinivasan, A. R., & Olson, W. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2199.
Warsaw, M. M., & Cantor, C. (1970) *Biopolymers 9*, 1079–1103.

Restriction Endonuclease *Eco*RI Alters the Enantiomeric Preference of Chiral Metallointercalators for DNA: An Illustration of a Protein-Induced DNA Conformational Change†

Jacqueline K. Barton*
Department of Chemistry, Columbia University, New York, New York 10027
Shanthi R. Paranawithana
Department of Biochemistry, City University of New York, New York, New York 10036
Received March 7, 1985; Revised Manuscript Received November 8, 1985

ABSTRACT: A conformational change in the DNA plasmid $ColE_1$ appears to occur upon specific binding of the restriction endonuclease *Eco*RI. Enzyme association alters the chiral discrimination found in binding metallointercalators to DNA sites. The complexes tris(1,10-phenanthroline)ruthenium(II), $Ru(phen)_3^{2+}$, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II), $Ru(DIP)_3^{2+}$, and tris(4,7-diphenyl-1,10-phenanthroline)cobalt(III), $Co(DIP)_3^{3+}$, in general, bind stereoselectively to DNA helices, with enantiomers possessing the Δ configuration bound preferentially by right-handed B-DNA. In the presence of *Eco*RI, however, this enantioselectivity is altered. The chiral intercalators, at micromolar concentrations, inhibit the reaction of *Eco*RI, but for each enantiomeric pair it is the Λ enantiomer, which binds only poorly to a B-DNA helix, that inhibits *Eco*RI preferentially. Kinetic studies in the presence of Λ-$Ru(DIP)_3^{2+}$ indicate that the enzyme inhibition occurs as a result of the Λ enantiomer binding to the enzyme–DNA complex as well as to the free enzyme. Furthermore, photolytic strand cleavage experiments using $Co(DIP)_3^{3+}$ indicate that the metal complex interacts directly at the protein-bound DNA site. Increasing concentrations of bound *Eco*RI stimulate photoactivated cleavage of the DNA helix by Λ-$Co(DIP)_3^{3+}$, until a protein concentration is reached where specific DNA recognition sites are saturated with enzyme. Thus, although Λ-$Co(DIP)_3^{3+}$ does not bind closely to the DNA in the absence of enzyme, specific binding of *Eco*RI appears to alter the DNA structure so as to permit the close association of the Λ isomer to the DNA helix. Mapping experiments demonstrate that this association leads to photocleavage of DNA by the cobalt complex at or very close to the *Eco*RI recognition site. This study provides evidence that in solution, under enzymatic conditions, a DNA-binding protein may distort the DNA helical structure and further illustrates how small molecular probes of DNA conformation might be used in examining the structure of protein-bound DNA sites.

Proteins that recognize specific DNA base sequences play a major role in the regulation of DNA expression, in DNA replication and transcription, and in repair processes. The structural basis for this macromolecular recognition is a subject of considerable interest. The crystal structures of several DNA binding proteins have been reported (Anderson et al., 1981, 1982; Frederick et al., 1984; McKay & Steitz, 1981; Pabo & Lewis, 1982; Steitz et al., 1982; Tanaka et al., 1984). On the basis of the complementarity of these structures to B-form DNA, also characterized crystallographically (Wing et al., 1980), models for protein–DNA recognition sites have been proposed. While several workers have suggested non-B conformations for DNA bound to proteins (Crick & Klug, 1975;

† J.K.B. is a Research Fellow of the Alfred P. Sloan Foundation and an NSF Presidential Young Investigator. This work was also generously supported by Grant GM 32203 from the National Institutes of Health.
* Author to whom correspondence should be addressed.

0006-2960/86/0425-2205$01.50/0