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Sequence-Influenced Interactions of Oligoacridines with DNA Detected by Retarded Gel Electrophoretic Migrations†

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ABSTRACT: We have found that di-, tri-, tetra-, and hexa-9-acridinylamines are so efficiently associated with DNA during electrophoresis in polyacrylamide or agarose gels that they retard its migration. The retardation is roughly proportional to the reagent to base pair ratio, and the magnitude of the retardation indicates that a combined charge neutralization/helix extension mechanism is mainly responsible for the effect. Furthermore, DNA sequence dependent differences are observed. Thus, the pUC 19 restriction fragments (*Hae*III or *Alu*I), which in the native state comigrate upon gel electrophoretic analysis, could be separated in the presence of a diacridine, and specific DNA fragments responded differently to different diacridines. These results suggest that the effect also is due to a contribution from the DNA conformation and that the DNA conformation dynamics are influenced differently upon binding of different diacridines. We foresee three applications of this observation: (1) in analytical gel electrophoretic separation of otherwise comigrating DNA molecules, (2) in studies of polyintercalator–DNA interaction, and (3) in measurements of polyintercalator-induced DNA unwinding.

The development and DNA-binding properties of polyintercalating reagents have attracted much attention (Wakelin, 1986) due to the potentials of such compounds as chemotherapeutics (Atwell et al., 1986) as well as to the general interest in DNA–ligand (not least protein) interactions.

Anomalous electrophoretic migration of DNA fragments in polyacrylamide gels has recently been demonstrated as a sensitive measure of altered DNA conformations such as DNA

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bending (Koo et al., 1986). Similarly, the sequence-specific binding of some proteins to DNA has been suggested to result in DNA bending, and abnormally slow electrophoretic migration of such protein–DNA complexes has been reported (Wu & Crothers, 1984).

We now report that the DNA binding of oligo-9-aminoacridines and presumably other DNA binding ligands with comparable binding affinities may also be studied by gel electrophoresis.

Materials and Methods

Elemental analyses were performed by Preben Hansen, The H. C. Ørsted Institute, University of Copenhagen. NMR spectra were recorded on a JEOL FX 90Q spectrometer in dimethyl sulfoxide-d_6 or methanol-d_4 with tetramethylsilane as internal reference. The syntheses of compounds **1e,h,i,k** and **5a,b** (Hansen et al., 1983), **2** (Hansen & Buchardt, 1983), and **3** (Hansen et al., 1983b) have been reported previously. Compound **4** was prepared analogously to **3**, and **1a,b,d–g,h** were prepared as described previously from the amines and 9-chloroacridine in phenol (Canellakis et al., 1976; Chen et al., 1978; Ledochowski et al., 1959; Stefanowska et al., 1972). Tritiated **1i** was obtained as previously described (Nielsen, 1985). All new compounds gave satisfactory ^{1}H NMR spectra and elemental analyses. The pK_a values were determined from titration curves.

pUC 19 plasmid DNA was isolated by the alkaline extraction method (Birnboim & Doly, 1979) and purified by CsCl gradient ultracentrifugation. Cleavage with restriction enzyme *Alu*I, *Hpa*II, *Hae*III (Boehringer) was done with 0.5 unit of enzyme/μg of DNA at 37 °C for 16 h. The DNA was subsequently purified by phenol extraction and ethanol precipitation.

For binding/migration studies the desired amount of acridine was incubated for 30 min at 37 °C with 2.5 μg of restriction enzyme cleaved pUC 19 or 0.5 μg of intact pUC 19 in 10 μL of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) containing 1 mM ethylenediaminetetracetic acid (EDTA), pH 7.5; 2 μL of 50% glycerol in 5 times concentrated gel buffer was then added, and the samples were analyzed on 6% polyacrylamide gels [0.2% bis(acrylamide)] run in TBE buffer (90 mM Tris–borate, 1 mM EDTA, pH 8.3) or on 1% agarose gels in 0.5 × TBE or TAE buffer (40 mM Tris–acetate, 2 mM EDTA, pH 7.0). The gels were subsequently stained with ethidium bromide and photographed through a red filter.

For examination of tritiated **1i** bound to DNA in the gel, the gel was scanned at 254 nm (Shimadzu CS 930 scanner) and sliced. Determination of ^{3}H radioactivity was done by liquid scintillation counting in Lipo Luma/Luma Solve (Lumac).

Footprinting analyses were performed with the 375 base pair *Eco*RI/*Bam*HI fragment of pBR 322: pBR 322 was cleaved with *Eco*RI and 3′-^{32}P-labeled with [α-^{32}P]dATP by use of the Klenow fragment of *E. coli* DNA polymerase I. The plasmid was subsequently cleaved with *Bam*HI, and the 375 base pair *Eco*RI/*Bam*HI framgent was purified from a 6% polyacrylamide gel.

Incubations for footprinting contained 18 μL of 10 mM Tris-HCl (pH 7.9), 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 0.01 mM dithiothreitol, 154 μM calf thymus DNA, 200 cps of the ^{32}P-labeled pBR 322 fragment, and the desired amount of oligoacridine. Following an equilibration period of 30 min at 37 °C, 0.1 μg of DNase I was added, and the reaction was stopped after 30 s by addition of 2 μL of 0.5 M EDTA. The DNA was precipitated with ethanol, redissolved in loading buffer (80% formamide, TBE buffer), and analyzed by electrophoresis on 8% polyacrylamide/7 M urea gels. The ^{32}P-labeled DNA fragments were detected by autoradiography.

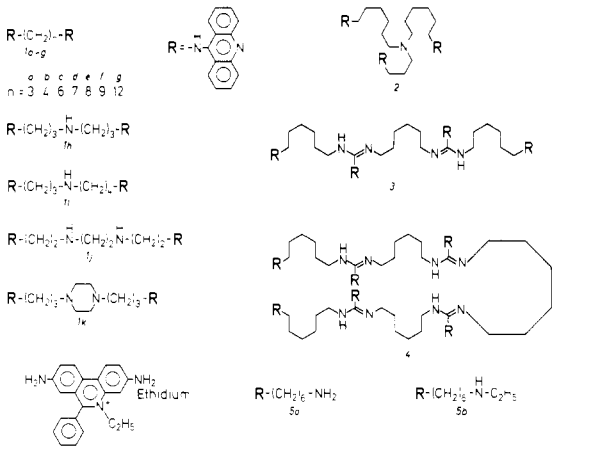

FIGURE 1: Structures of the acridines used.

Table I: Relative Migration of DNA Fragments in Polyacrylamide Gel in the Presence of Reagent **1i** Calculated on the Basis of the Results Presented in Figure 1[a]

	reagent/bp			
	0	0.012	0.04	0.12
theoretical relative migration[b]	1.00	0.96	0.88	0.70
DNA fragment (bp)				
587	1.00	0.97	0.85	0.74
458	0.96	0.93	0.86	0.78
434	1.03	1.00	0.88	0.76
296	1.00	0.96	0.88	0.77
267	1.02	1.02	0.92	0.82
257	1.00	0.97	0.92	0.82
174	1.00	0.99	0.95	0.84
102	1.00	1.00	0.97	0.90
80	1.00	1.00	0.97	0.91

[a] The data are expressed on the basis of a semilogarithmic plot fitted to as many data points as possible, and the migrations relative to this plot are given. The mean values for the DNA migration were used for $0.012 \leq r \leq 0.04$. [b] Based on the length and charge influence.

Results

We have used *Hae*III restriction fragments of pUC 19 DNA to study the effect of the di-, tri-, tetra-, and hexa-9-acridines (Figure 1) on the electrophoretic migration of DNA in polyacrylamide gels. The results presented in Figure 2 show that the DNA migration is retarded in the presence of *N,N′*-di-9-acridinylspermidine (**1i**). The retardation is roughly proportional to the reagent/base pair ratio (r, Table I), and for $0.01 < r < 0.04$ a significant broadening of the bands is observed. Furthermore, a DNA sequence dependence is apparent. This is based on the observation that fragments of 434 and 458 bp comigrate as one sharp band at $r = 0.12$ (Figure 2a, lane 5; Figure 3e), whereas fragments of 257 and 267 bp, which comigrate in the native state (Figure 2a, lane 1; Figure 3a), appear as two distinct bands at $r = 0.12$ (Figure 3e).

Similarly, the 257-, 245-, and 226-bp fragments of pUC 19 restricted with *Alu*I are separated electrophoretically in the presence of reagent **1i** (Figure 3j,k).

Analogous retardation effects were observed with an extensive series of diacridines (Figure 1; **1a–h**) although distinct differences in their effect on the individual DNA fragments were observed (Figure 2c,d and Figure 3f–h).

The tri-, tetra-, and hexaacridines (**2**, **3**, and **4**) also exhibited a DNA retardation effect, but with these compounds broadened DNA bands were always observed (Figure 2a,b), and at

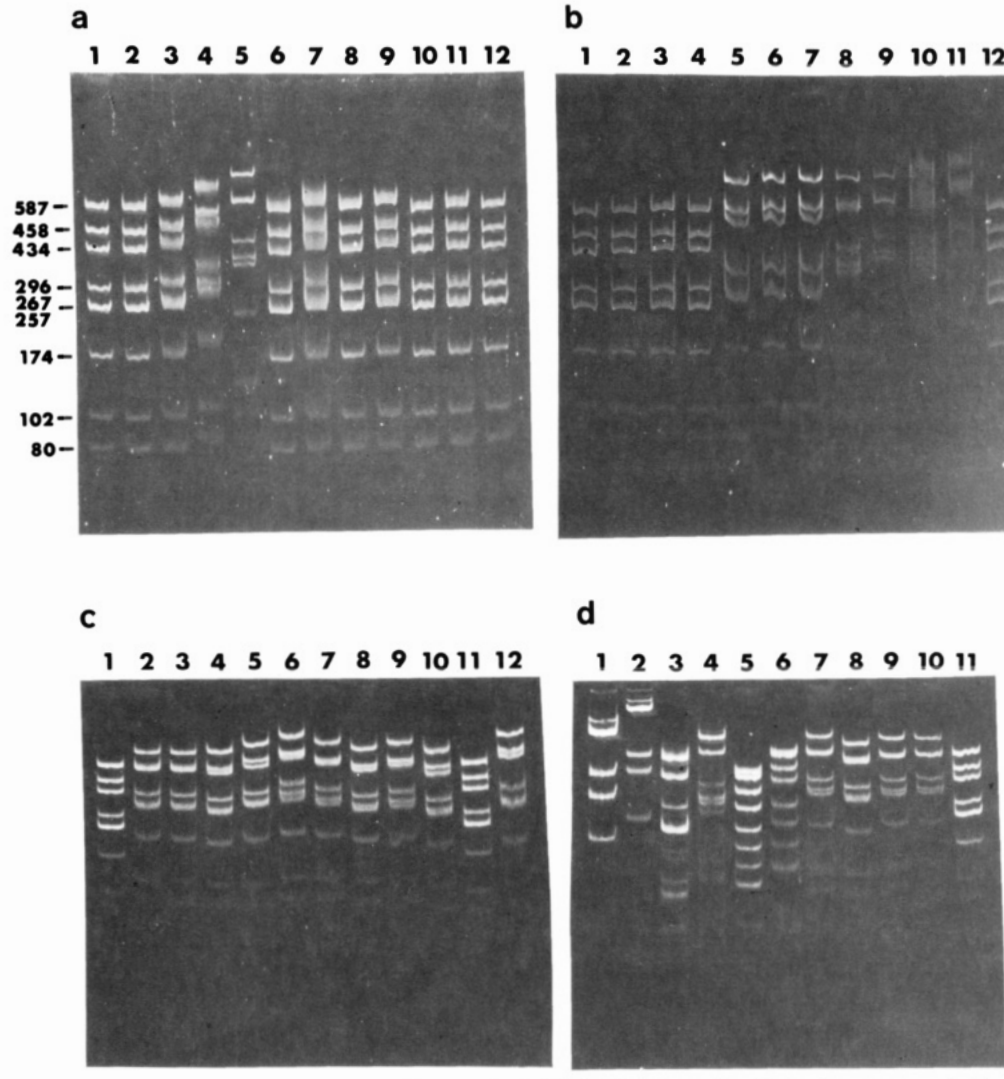

FIGURE 2: Electrophoretic migration of *Hae*III restriction fragments of pUC 19 in polyacrylamide in the presence of oligoacridines. (a) Lanes 1 and 12: control. Lanes 2–5: diacridine **1i** at $r = 0.004$, 0.012, 0.04, and 0.12, respectively. Lanes 6 and 7: triacridine **2** at $r = 0.0027$ and 0.008. Lanes 8 and 9: tetraacridine **3** at $r = 0.002$ and 0.006. Lanes 10 and 11: hexaacridine **4** at $r = 0.0015$ and 0.005. (b) Lanes 1 and 12: control. Lanes 2–4: 9-aminoacridine at $r = 0.03$, 0.1, and 0.3. Lanes 5–7: ethidium bromide at $r = 0.02$, 0.06, and 0.2. Lanes 8 and 9: **1i** at $r = 0.04$ and 0.12. Lanes 10 and 11: **2** at $r = 0.03$ and 0.08. (c) Lanes 1 and 11: control. Lane 2: **5a** at $r = 0.36$. Lane 3: **5b** at $r = 0.35$. Lane 4: **1a** at $r = 0.24$. Lane 5: **1b** at $r = 0.24$. Lane 6: **1c** at $r = 0.20$. Lane 7: **1d** at $r = 0.20$. Lane 8: **1e** at $r = 0.20$. Lane 9: **1f** at $r = 0.20$. Lane 10: **1g** at $r = 0.20$. Lane 12: ethidium bromide at $r = 0.40$. (d) Lane 11: control. Lane 7: **1h** at $r = 0.12$. Lane 8: **1j** at $r = 0.12$. Lane 9: **1i** at $r = 0.12$. Lane 10: **1k** at $r = 0.12$. Lanes 1 and 2: pUC 19 + *Hin*f1. Lanes 3 and 4: pUC 19 × *Alu*I. Lanes 5 and 6: pUC 19 × *Hpa*II. Lanes 1, 3, and 5 are controls while the samples in lanes 2, 4, and 6 contained reagent **1i** ($r = 0.14$). All samples containing 2.5 μg of DNA and the indicated amount of acridine in 10 mM Tris–1 mM EDTA, pH 7.5, were incubated for 30 min at 37 °C prior to analysis on 6% polyacrylamide gels in TBE buffer. The gels were stained with ethidium bromide and photographed. The fragment sizes in base pairs are indicated.

higher r values ($r \sim 0.2$) DNA precipitation occurred, thereby preventing the DNA from entering the gel.

No effect of 9-aminoacridine itself was observed, whereas the presence of ethidium bromide caused some retardation of the DNA migration but no significant change in the band pattern (Figures 2b and 3i).

In order to ascertain that the effect on the DNA migration was due to binding of the reagents during electrophoresis, experiments were undertaken with tritiated *N*,*N*′-di-9-acridinylspermidine (**1i**) (Nielsen, 1985), and the results clearly showed that this was the case (Figure 4). [Recently a similar phenomenon was observed with porphyrins but not explored further (Kelly & Murphy, 1985).]

The migration of DNA molecules in agarose gels is also retarded by the presence of polyintercalators (Figure 5), and this effect is particularly pronounced in the case of supercoiled circular DNA. The specific effect on this type of DNA is undoubtedly due to the conformational changes induced by intercalators caused by DNA unwinding, thereby decreasing the superhelical density of the DNA. A relative measure of unwinding angles may be obtained by this technique (Figure 6 and Discussion). An analogous assay using DNA relaxation by topoisomerase has previously been reported (Keller, 1975).

DNase I footprinting analyses of the binding of acridines **1i**, **2**, and **3** to a 375 base pair fragment of pBR 322 DNA were also performed (Figure 7). The results indicate a complex interplay between the acridines, the DNA, and the action of DNase I. At low ligand/base pair ratios ($r \sim 0.1$), a significant enhancement of DNase cleavage was observed. This enhancement is highly site specific occurring predominantly at GC base pairs followed by TA stretches (Figure 8). Furthermore, slight but distinct differences were observed when comparing the effects of the di-, tri-, and tetraacridines, thereby indicating differences in the binding modes and/or binding-sequence preferences. At higher ligand/base pair ratios ($r >$ 0.5), a pronounced inhibition of the DNase activity was ob-

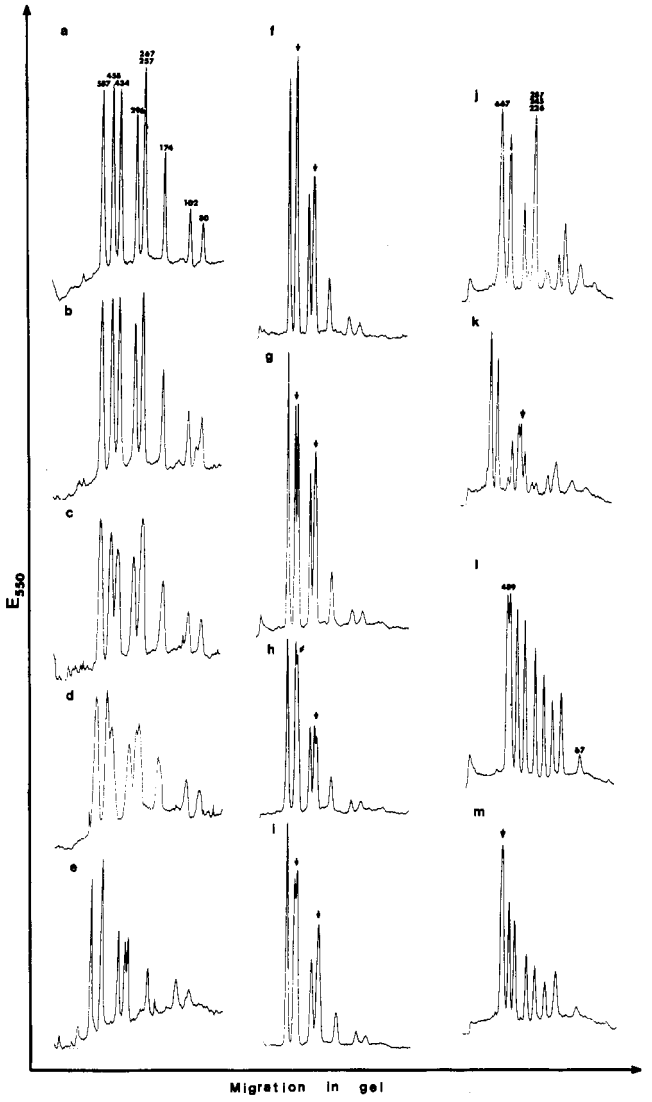

FIGURE 3: Densitometric scans of the photographs shown in Figure 2. Scans a–e correspond to Figure 2a, lanes 1–5. Scans f–i correspond to Figure 2c, lanes 3, 5, 9, and 12, respectively. Scans j–m correspond to Figure 2d, lanes 3–6. The arrows denote peaks of particular interest, and the numbers refer to the fragment sizes in base pairs. (a–i) pUC 19 × *Hae*III: (a) control; (b) **1i**, $r = 0.004$; (c) **1i**, $r = 0.012$; (d) **1i**, $r = 0.04$; (e) **1i**, $r = 0.12$; (f) **5b**, $r = 0.35$; (g) **1b**, $r = 0.14$; (h) **1f**, $r = 0.20$; (i) ethidium, $r = 0.40$. (j) pUC 19 × *Alu*I, control. (k) pUC 19 × *Alu*I, **1i**, R = 0.14. (l) pUC 19 × *Hpa*II, control. (m) pUC 19 × *Hpa*II, **1i**, $r = 0.14$.

served, and the remaining cleavage was confined to G residues. We do not yet draw any firm conclusions concerning the sequence specificity of the binding of oligoacridines to DNA, but the results seem to be in accordance with the finding that acridines exhibit an increased affinity for poly(dAT)·poly(dAT) (Capelle et al., 1979; Denny et al., 1985). The results also indicate that the DNA binding of oligoacridines is sequence dependent.

Discussion

DNA Affinity. The observation that the diacridine (**1i**), and probably all other oligoacridines as well, stays bound to the DNA upon migration in an electric field is quite surprising since the acridine is at least partly protonated at pH 8.3 ($pK_a \sim 8.5$, unpublished) and free acridine would be expected to migrate in a direction opposite to the migration of the DNA. Furthermore, kinetic studies have shown that the off-rate constants of diacridines are in the order of 10^{-2} s^{-1} (Langaa et al., 1985), giving $\tau_{1/2} \sim 100$ s for the diacridine–DNA

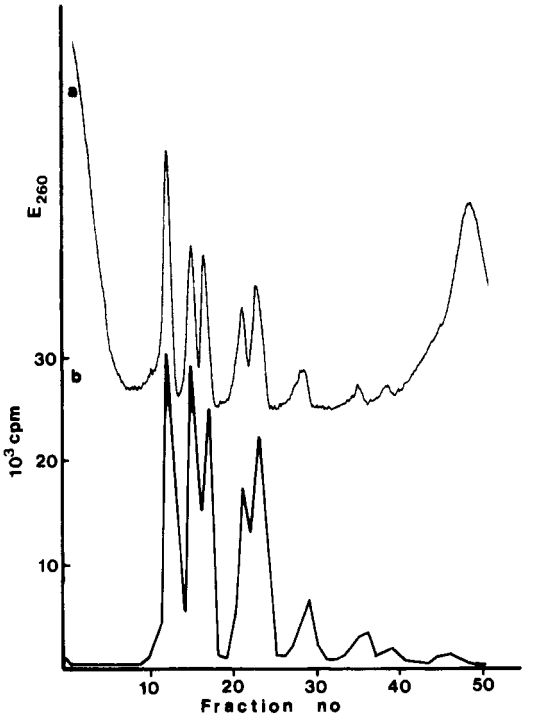

FIGURE 4: Binding of ^{3}H-labeled diacridine **1i** to pUC 19 *Hae*III DNA fragments during electrophoresis in polyacrylamide. A total of 10 μg of pUC 19/*Hae*III was incubated with 1.6×10^5 cpm (~0.25 μg) of [^{3}H]-**1i** and analyzed on a 6% polyacrylamide tube gel. The gel was scanned at 260 nm (a) and subsequently sliced from the top (b). The peak at fraction 13 is the 587-bp fragment while the peak at fraction 28 is the 174-bp fragment.

complex. The observed acridine–DNA comigration thus cannot be explained simply by the DNA affinity constant. Other factors must be involved, and these may be related to the process of electrophoresis itself.

First of all the local DNA concentration in the gel is quite high, and the acridine can therefore only "escape" from the surface of the DNA band in the gel, and any escaped acridine from smaller DNA fragments would be picked up by the slower migrating, larger DNA fragments. Such an effect should result in some tailing of the acridine combined with an increased amount of acridine bound to the larger DNA fragments after electrophoretic separation. The results using the tritiated diacridine, however, strongly indicate that practically no escape is occurring since we observed no tailing and equal distribution of the acridine between the DNA fragments (Figure 4). We believe it more likely that the DNA–acridine is migrating as an ion pair in the gel and that the observed acridine–DNA comigration is closely related to the protein–DNA comigration now commonly used to study the DNA bending induced by sequence-specific binding proteins (Crothers, 1987).

Cause of Retardation Effect. There is little doubt that the decreased DNA mobility in the presence of oligoacridines is mainly caused by two factors. Upon intercalation the DNA helix length is extended 3.4 Å per intercalated ligand. Furthermore, the overall negative charge of the DNA molecule is reduced upon binding of the acridinylamines due to the basicity ($pK_a \sim 7$–9) of the amino groups in these compounds. Assuming that one intercalated ligand results in a helix extension corresponding to that of one base pair and that one amino group neutralizes one DNA–phosphate corresponding to a migration change of one-half base pair, the theoretical migration retardations shown in Table I are obtained. The results show, however, that some fragments migrate differently

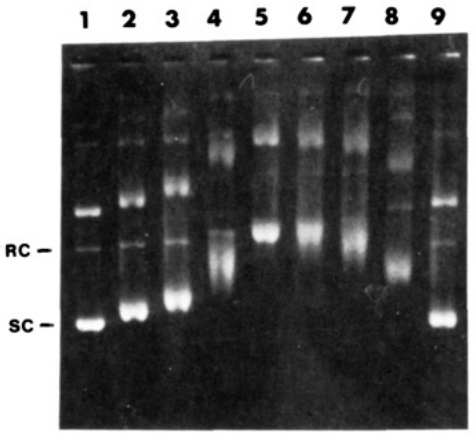

FIGURE 5: Electrophoretic migration of supercoiled circular (sc) and relaxed circular (rc) pUC 19 DNA in 1% agarose gels in the presence of diacridine **1i**. A total of 0.5 μg of pUC 19 plasmid DNA was incubated with the desired amount of acridine for 30 min at 37 °C in 10 mM Tris-HCl–1 mM EDTA, pH 7.4, and then analyzed by gel electrophoresis. Samples 1 and 9 are controls without acridine. Samples 2–8 contained diacridine **1i** (r = 0.036, 0.054, 0.072, 0.090, 0.108, 0.126, and 0.144, respectively). The slower migrating bands in the gel are dimers of pUC 19 plasmid. Note that the effect on the supercoiled DNA (sc) shows a maximum at an intermediate r value ($r \sim 0.09$) corresponding to the total unwinding (relaxation) of the plasmid. At higher r values the DNA becomes positively supercoiled and consequently migrates faster in the gel (lanes 7 and 8).

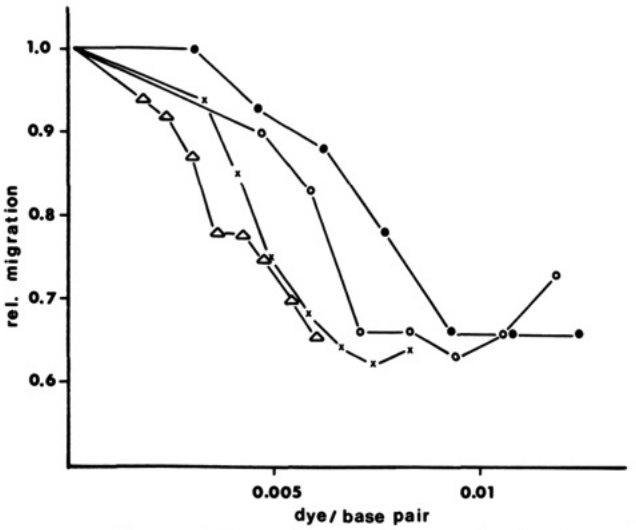

FIGURE 6: Influence of oligoacridines on the electrophoretic migration of supercoiled pUC 19 DNA in agarose gels (1% agarose, 0.5× TBE, 100 mA, 3 h). With reagent **1c** (●) as reference (unwinding angle = 34°; Wakelin, 1986), the unwinding angles for reagent **2** (○) = 41°, reagent **3** (×) = 44°, and reagent **4** (△) = 50° may be obtained. Half-maximum retardation was used for the calculations. Compound **1i** gave 40° (Figure 5 and results not shown).

than predicted on the basis of this model and the discrepancies are more pronounced for the smaller fragments. We ascribe these differences to sequence- (and intercalator-) dependent changes of the DNA conformation and/or dynamics upon intercalation (vide infra).

Sequence Specificity. The results presented here show that DNA fragments differing in sequence are affected differently upon binding of oligoacridines (e.g., the 458/434-bp and the 267/257-bp fragments of pUC 19 × *Hae*III, the 257/246/226-bp fragments of pUC 19 × *Alu*I, and the two 489-bp fragments of pUC 19 × *Hpa*II). However, this effect varies for different acridines (e.g., **1i** compared to **5a** or **1f**) and is not observable for others (including ethidium bromide) even

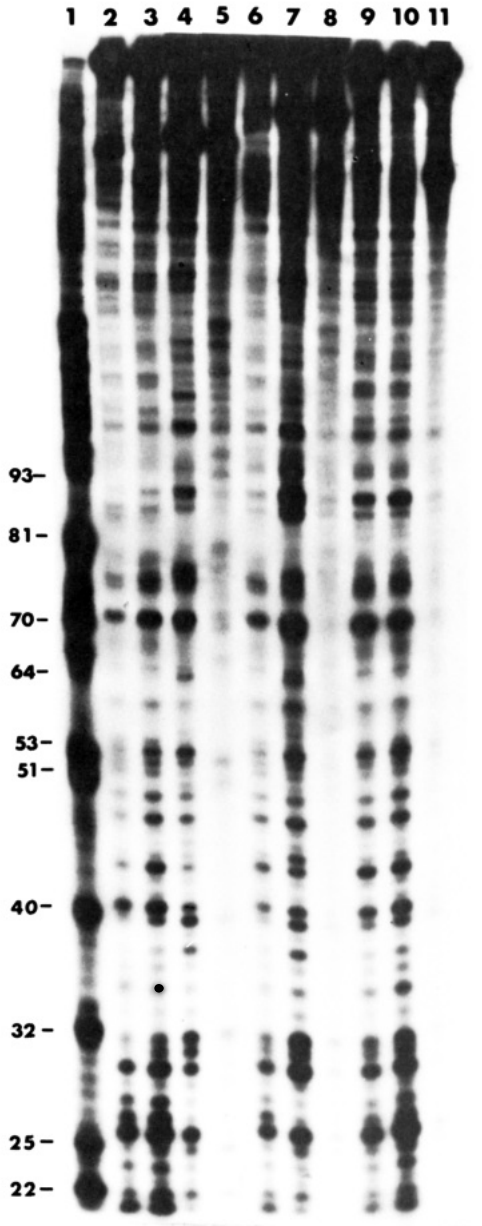

FIGURE 7: DNase I footprinting experiment performed as described under Materials and Methods. Lane 2 is a control without reagent. Lanes 3–5: reagent **3** at r = 0.03, 0.09, and 0.3. Lanes 6–8: reagent **2** at r = 0.04, 0.12, and 0.4. Lanes 9–11: reagent **1i** at r = 0.006, 0.18, and 0.6. Lane 1 is a Maxam–Gilbert G-specific sequencing reaction. The positions of the G residues were determined from an autoradiogram of shorter exposure time. Similar experiments with the monoacridine **5a** only resulted in enhancement of DNase I cleavage (not shown).

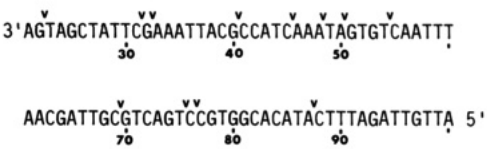

FIGURE 8: DNA sequence of footprinting area, showing the major sites of DNase I cleavage enhancement (v).

though comparable retardation of the gel migration is observed (Figure 3e–i).

These results clearly suggest that some sequence-dependent interaction between the intercalators and the DNA is taking place. Binding studies with synthetic and natural DNA of varying base composition have indicated that acridines have somewhat higher affinity for AT sequences as compared to

GC sequences (Capelle et al., 1979; Denny et al., 1985). Our footprinting results support these findings. On the other hand, it is not simply a function of the AT content of the DNA fragments, since the 257 and 267 base pair pUC 19 fragments have identical AT content (49%) and the 434 base pair fragment which is retarded more than the 458 base pair fragment has the lower AT content of the two (42% versus 57%). None of these fragments contain any obviously peculiar sequences such as phased $(A)_n$ tracts (Koo et al., 1986) either. Therefore, we propose that the sequence-influenced gel migration changes discussed in this paper are due to sequence-dependent differences in the conformation (persistence length, bending, etc.) of the DNA–intercalator complexes. Additionally, the differences could be due to differences in the intercalator site density of the various fragments. Thus a 13% difference in site density of the 458/434-bp pUC 19 × *Hae*III fragments could explain the observed migration difference in the presence of acridine, **1i** (Figure 3e).

We suggest that the difference in DNA migration retardation effect between the diacridines are reflecting differences in their DNA-binding geometries, i.e., in their influence on the DNA base twist angle (unwinding) and base tilt angles.

Intercalation. The present results also relate to the process of intercalator–DNA binding itself. It is characteristic that at diacridine to base pair ratios of 0.01–0.05 (e.g., Figure 3c,d) the DNA bands are significantly broadened, indicating a heterogeneous distribution of binding either between or within the DNA molecules. At $r \sim 0.1$, however, the bands regain their sharpness (Figure 3e–m), indicating that a situation of homogeneous site distribution has now been reached. This observation strongly suggests that this binding ratio corresponds to a saturation level having a density of roughly one binding site per eight base pair, thus obeying the next-neighbor exclusion principle.

It is noteworthy that sharp bands were never obtained in the case of tri-, tetra-, and hexaacridines. Using circular and flow linear dichroism analyses, we have recently shown that these acridines bind to DNA in a way in which not all of the acridinyl groups appear to have identical binding geometry. In particular, compounds **3** and **4** only behave as trisintercalators, and triacridine **2**, although binding to DNA by trisintercalation, only gives rise to an unwinding angle of 41° (Figure 6). Furthermore, on the basis of the induced circular dichroism spectrum it was inferred that the binding geometry of the acridinyl groups is significantly different from that found from, e.g., diacridine **1c** (Wirth et al., 1987). Although we cannot rule interstrand DNA binding of the oligoacridines out, we regard it as a very unlikely possibility, because the optical measurements were performed with very diluted samples ($\sim 10^{-4}$ M; Wirth et al., 1987) and at low ionic strength (10^{-3} M). Combining this information with the finding of complex sequence specificity of the binding of the oligoacridines to DNA, we believe that the DNA binding of triacridine and higher acridines is characterized by a number of equally potent binding modes and that the broad bands observed upon gel electrophoretic polyintercalator–DNA comigration (Figure 2b, lanes 10 and 11) are caused by such site heterogeneity.

Unwinding. The specific effect of the oligoacridines on the migration in agarose gels of supercoiled circular DNA as opposed to relaxed circular DNA is ascribed to the DNA unwinding induced by intercalative binding. Previously, the effect of the superhelical density of circular DNA on its hydrodynamic properties has been exploited in viscometry or sedimentation assays of intercalator unwinding angles (Crawford & Waring, 1967; Revet et al., 1971). The present results suggest that an analogous assay, requiring much less material and being less time consuming than the previously described assays, can be based on gel electrophoretic migration in the presence of intercalators. This assay, however, seems to require binding efficiencies of the intercalator of a certain magnitude. The lower limit of the affinity constant has not yet been precisely determined—but it has to be larger than 10^4–10^5 M^{-1} since we do not observe any effect of 9-aminoacridine and only a slight effect of ethidium. The unwinding angles of moderately binding intercalators therefore cannot be determined by the gel assay.

Furthermore, the unwinding angles obtained by this assay should be regarded as minimum values until it has been ascertained that the intercalator under scrutiny stays bound to the DNA through the electrophoresis. It should also be kept in mind that the ionic conditions of the medium influence the superhelical density of DNA (Andersen & Bauer, 1978).

CONCLUSIONS

The described bisintercalator and higher intercalator influence on the electrophoretic behavior of DNA was unexpected and thus of interest in itself. More important, it should be of some use in analytical biochemistry. Thus it can be useful in gel electrophoretic separation of otherwise comigrating DNA molecules, and it consitutes a very simple way of determining DNA unwinding angles of oligoacridines.

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Microheterogeneity in the Major Phenobarbital-Inducible Forms of Rabbit Liver Microsomal Cytochrome P-450 As Revealed by Nucleotide Sequencing of Cloned cDNAs†

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ABSTRACT: We have isolated one full-length cDNA clone, termed pHP1, and a number of clones of shorter insert lengths, tentatively called b14, b46, etc., all encoding phenobarbital- (PB-) inducible forms of rabbit liver microsomal cytochrome P-450, and determined their nucleotide sequences. The polypeptides encoded by these cDNAs can be classified into five types, represented by HP1, b14, b46, b52, and b54, the deduced amino acid sequences of which are more than 95% similar to one another. Amino acid differences among them total 24 positions, which are distributed over the entire sequence, in contrast to the microheterogeneity observed in two PB-inducible rat liver microsomal cytochromes P-450 (P-450b and P-450e). The primary structure deduced for the HP1 protein is 97% similar to that determined for rabbit P-450 LM2 (form 2), which has been purified by Coon and co-workers [van der Hoeven, T. A., Haugen, D. A., & Coon, M. J. (1974) *Biochem. Biophys. Res. Commun. 60*, 569–675; Haugen, D. A., & Coon, M. J. (1976) *J. Biol. Chem. 251*, 7929–7939] as the major PB-inducible form of rabbit liver microsomal cytochrome P-450. The amino acid sequence of P-450$_1$, which we have purified as the major PB-inducible rabbit liver cytochrome P-450, was partially determined with the sequence reported for P-450 LM2 as a reference. The two sequences are closely similar to each other, but at least two amino acid differences can be detected between them. The primary structure predicted from the nucleotide sequence of b14 cDNA, which covers only the carboxyl-terminal 40% of the entire sequence, is identical with that determined for P-450$_1$. It is concluded that the liver of PB-treated rabbits expresses at least five distinct but closely similar forms of cytochrome P-450, all of which are encoded by genes belonging to the rabbit P450IIB (major PB-inducible) subfamily.

Cytochromes P-450 play a crucial role in the oxidative metabolism of a large variety of xenobiotics as well as a number of endogenous substrates. It is now established that multiple forms of cytochrome P-450 occur in liver microsomes and administration of drugs to animals induces the synthesis of a specific form(s) of the cytochrome depending on the drug administered. The multiplicity and inducibility have been recognized and confirmed by purification of distinct forms of cytochrome P-450 from liver microsomes of untreated and variously drug-treated animals and in recent years also by isolation and structural analysis of cDNA and genomic clones encoding different forms of the cytochrome.

The first successful isolation of homogeneous preparations of hepatic microsomal cytochrome P-450 was reported by two groups. Thus, we purified a form, called P-450$_1$, from liver microsomes of phenobarbital- (PB-)[1] treated rabbits (Imai & Sato, 1974; Imai et al., 1980), whereas Coon and co-workers isolated a preparation, termed P-450 LM2 or form 2 (hereafter will be referred to as LM2), from the same source (van der Hoeven et al., 1974; Haugen & Coon, 1976). Since the molecular, spectral, and catalytic properties of P-450$_1$ and LM2 were hardly distinguishable from each other, we concluded that they are identical with each other and represent the major PB-inducible form in rabbit liver microsomes (Imai et al., 1980). On the other hand, two forms of cytochrome P-450, called P-450b and P-450e, have been purified from liver microsomes of PB-treated rats (Ryan et al., 1979, 1982). These two forms have been shown to be immunochemically indistinguishable and exhibit only minor functional differences (Ryan et al., 1982; Vlasuk & Walz, 1982). Furthermore, their amino acid sequences, deduced from cDNA nucleotide sequences, have been found to be 97% similar to each other (Fujii-Kuriyama et al., 1982; Mizukami et al., 1983a). Despite such close similarities, evidence has been presented that they are actually encoded by different genes (Mizukami et al., 1983a; Suwa et al., 1985).

In this study we examined whether microheterogeneity can also be found in major PB-inducible forms of cytochrome P-450[2] in rabbit liver. For this purpose, we isolated one

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[1] Abbreviations: PB, phenobarbital; LM2, P-450 LM2 or rabbit form 2; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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