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Synthesis and Properties of Acrylamide-Substituted Base Pair Specific Dyes for Deoxyribonucleic Acid Template Mediated Synthesis of Dye Polymers†

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ABSTRACT: We have tried to construct synthetic polymers for sequence-specific recognition and complexation of longer deoxynucleotide sequences. For this purpose, we developed a method of template-directed polymerization of base pair specific DNA ligands such as basic dyes. The template-directed polymerization consists in a copolymerization of various dyes of different specificities staying simultaneously in a binding equilibrium with DNA. In the present paper, we describe the synthesis and the properties of base pair specific monomers especially designed for performance of radical chain polymerization reactions in aqueous medium at room temperature. Different acrylamide derivatives of well-known dyes, such as AT-specific malachite green and GC-specific phenyl neutral red, were synthesized and studied for their ability of base pair specific complex formation with DNA of different base composition. Partition equilibrium dialysis and dye titration agarose gel electrophoresis were used to ensure for several dyes that they may be incorporated into different polymers via copolymerization of their acrylamido derivatives with various small base-unspecific monomers without substantial change of their binding parameters. Furthermore, we demonstrate that acrylamide and other small acrylamide derivatives can be used as building blocks for the synthesis of polymeric links between base pair specific monomers. The results and their consequences for template polymerization reactions are discussed.

In the past two decades, a large number of antibiotics, dyes, and other substances have been isolated and studied intensively to elaborate the molecular structure of their complexes with nucleic acids, especially with double-stranded DNA. The main emphasis of these investigations has been to elucidate the mechanisms involved in the binding specificity of naturally occurring complexes between biological macromolecules, e.g., proteins and DNA. Despite these efforts, no generally accepted model for the recognition process between proteins and DNA could be derived, however. We tried to use our knowledge about base pair specific dyes of sufficient DNA affinity for an alternative, abiotic approach toward the synthesis of oligomeric compounds with "repressor-like" properties. Recent publications similar to our approach deal with attempts to improve the binding affinity of DNA-specific ligands by their di- and oligomerization (Le Pecq et al., 1975; Wakelin et al., 1976; Le Bret et al., 1977; Kuhlmann et al., 1978). These experiments mainly revealed that multiplication of the original binding parameters of the monomers after incorporation into a polymer is only observed if the dyes in their polymeric state are allowed to interact with the DNA without any constrictions caused by the polymer backbone. We reached the same conclusion when we studied polypeptide-linked dimers of phenoxazon, the dye chromophor of actinomycin, some years ago (Bünemann, 1971).

At that time, we decided to circumvent the time-consuming conventional synthesis of long dye polymers of a defined sequence in favor of structurally similar polymers which could be obtained in a much simpler way by polymerization reactions. The advantages of each polymerization procedure are founded in its versatility and velocity. Within a short time, different polymers from various combinations of monomers can be obtained and tested for the influence of different monomers on the properties of the resulting polymers. It is clear that the polymerization procedures yield polymers in which the strictly defined distances and sequences of the effective subunits along the chain as in a conventionally synthesized linear macromolecule are replaced by average values and

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random distribution. This major disadvantage is compensated for by the fact that the polymerization reaction can be performed in aqueous medium, in the presence of solved biological macromolecules, and under nearly physiological conditions.

Our concept was to combine all these advantages by performing the polymerization reaction with DNA-bound dye molecules in a similar way as polymerases copy their nucleic acid templates. Experiments of this kind should be of larger interest with respect to macromolecular chemistry, because only biological molecules offer template polymers of a limited number of monomers and defined sequences.

Two different DNA molecules of the same base composition, e.g., may have completely different, but well-defined, sequences. They are therefore specially suited for experiments to answer the question if matrix or template polymerization in its classical sense can be performed by the way of nonenzymatic abiotic polymerization reactions.

At the beginning of our experiments toward template polymerization, we had a number of dyes in our disposition which were synthesized from DNA-binding studies (Müller & Crothers, 1968, 1975; Müller et al., 1973, 1975; Müller & Gautier, 1975). From this collection of dyes, we chose the GC-specific phenyl neutral red (PNR)[1] and the AT-specific malachite green (MG) as primary products for the synthesis of base pair specific monomers for the following reasons. Both monomers form complexes of similar stability with double-stranded DNA. The maxima of their electron spectra are distant from each other more than 50 nm, which allows the simultaneous quantitative determination of both monomers by photometry. Both dyes are supposed to form different types of DNA complexes. PNR binds to DNA via intercalation (Müller et al., 1975) whereas MG sticks to the surface of DNA helix (Müller & Gautier, 1975). These differences in their DNA complex structures should reduce steric hindrance between both ligands when bound simultaneously to DNA. Finally, we knew what kind of substituents could be introduced at which positions of both dye molecules without changing their binding parameters appreciably. The choice of a suitable polymer backbone was constricted by the demand of good water solubility on one hand and the requirement of its synthesis in aqueous salt medium on the other. Since only linear polyacrylamide met these specifications, all monomers were designed as N derivatives of acrylamide in order to enable their incorporation in linear polymers by radical chain polymerization with peroxydisulfate or AIBA as initiator.

In this paper, we report experimental studies of the synthesis and the properties of different acrylamide derivatives especially designed for polymerization reactions with DNA templates in aqueous medium. We are able to show that (1) different acrylamide derivatives of GC-specific phenyl neutral red and AT-specific malachite green can be synthesized that possess binding parameters nearly identical with those of their parent compounds, (2) base pair specific dyes may be attached to polymer backbones of acrylamide or acrylamine via suitable acrylamide derivatives and succeeding radical chain polymerization, and (3) polyacrylamide or polyacrylamine backbones exert no or only minor influences on the specificity of DNA complex formation of their base pair specific dye substituents.

In summary, the results support the idea of abiotic synthetic polymers complementary to certain DNA sequences that can be synthesized by the method of template polymerization. In a succeeding paper, the progress of template polymerizations for a variety of parameters is studied, and the resulting polymers are tested for template specificity.

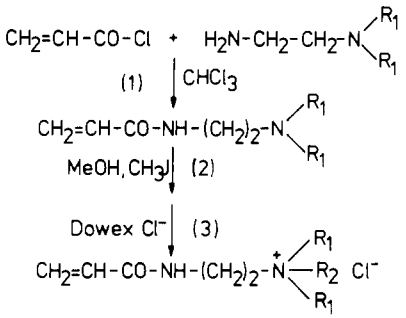
Experimental Procedures

Preparation of DNAs. DNA from *Micrococcus luteus*, *Escherichia coli*, *Bacillus subtilis*, and *Clostridium perfringens* was prepared from frozen (wet) cells (Merck, Darmstadt) by a variation of the procedure described by Marmur (1961) and Thomas et al. (1966). All bacterial DNAs were obtained in a molecular weight range from 4 000 000 to 8 000 000 as determined from sedimentation velocity (Eigener & Doty, 1965). The DNA preparations for equilibrium dialysis and precipitation tests were sheared to a molecular weight of 500 000–1 000 000 by treatment in a Virtis 45 homogenizer at 20 000 rpm at 0.5 °C for 4 h, extensively dialyzed against BPES buffer, and precipitated with 0.66 volume of 2-propanol. The DNA was collected by centrifugation, redissolved in an appropriate buffer, and dialyzed against the same buffer. The DNA of plasmid pML 21 (Lovett & Helinski, 1976) was isolated by cleared lysate–dye cesium chloride density gradient procedures (Clewell & Helinski, 1969, 1970), extracted with *sec*-butyl alcohol, dialyzed extensively against an appropriate buffer, and stored at −20 °C.

Partition Equilibrium Dialysis. Partition equilibrium dialysis experiments were performed as described previously by Müller & Crothers (1975). The affinity parameter σ is defined by $\sigma = \sum_i K_i B_i$ in which B_i is the number of binding sites per base pair, each with an intrinsic stability constant K_i (Crothers, 1968); σ is equal to the intercept of a Scatchard plot of r/m vs. r on the r/m axis. If binding involves only one kind of site and each base pair is a potential binding site, then σ is equal to the binding constant. The specificity parameter α is defined as the ratio of σ(DNA I)/σ(DNA II) (Müller & Crothers, 1975) where DNA I refers to *Micrococcus luteus* DNA (*M. luteus*) and DNA II to *Bacillus subtilis* DNA (*B. subtilis*) if not stated otherwise. The α values were determined by the partition dialysis method (Müller & Crothers, 1975). Determination of the concentration of bound ligands was performed by absorbance measurements after dissociating the complexes by addition of sodium dodecyl sulfate to a final concentration of 2.5% (w/v).

Precipitation Test for Base-Pair Specificity. One milliliter of DNA solution in BPE buffer and 0.2 M NaCl containing 50 μg of an equimolar mixture of *Micrococcus luteus* (*M. luteus*) and ^{3}H-labeled *Escherichia coli* DNA or ^{3}H-labeled *Escherichia coli* (*E. coli*) and *Clostridium perfringens* (*C. perfringens*) DNA was mixed under vigorous stirring with amounts of polymer stock solution. The mixtures were kept

[1] AIBA, azoisobutyroamidine hydrochloride; TLC, thin-layer chromatography; F_3AcOH, trifluoroacetic acid; LSC, liquid scintillation counting; PEG, poly(ethylene glycol); ccc-DNA, covalently closed circular DNA; EDTA, disodium salt of ethylenediaminetetraacetic acid; AA, acrylamide; MA, *N,N*-dimethyl-2-(acryloylamido)ethylamine; EA, *N,N*-diethyl-2-(acryloylamido)ethylamine; QMA, *N,N,N*-trimethyl-2-(acryloylamido)ethylammonium chloride; QEA, *N,N*-diethyl-*N*-methyl-2-(acryloylamido)ethylammonium chloride; MG, phenylbis[4-(dimethylamino)phenyl]carbenium chloride; AMG, [4-(acryloylamino)-phenyl]bis[4-(dimethylamino)phenyl]carbenium chloride; AAMG, [4-((*N*-acryloyl-β-alanyl)amino)phenyl]bis[4-(dimethylamino)phenyl]carbenium chloride; PNR, 5-phenyl-3-amino-7-(dimethylamino)-2-methylphenazinium chloride; APNR, 5-[4-(acryloylamino)phenyl]-3-amino-7-(dimethylamino)-2-methylphenazinium chloride; 1-APNR, 1-(acryloylamino)-3-amino-7-(dimethylamino)-2-methyl-5-phenylphenazinium chloride; AMPNR, 1-[(acryloylamino)methyl]-3-amino-7-(dimethylamino)-2-methyl-5-phenylphenazinium chloride; AEPNR, 1-[(acryloylamino)ethyl]-3-amino-7-(dimethylamino)-2-methyl-5-phenylphenazinium chloride; Boc, *tert*-butyloxycarbonyl; solvent system A, acetic acid/methanol/chloroform (1:1:8 v/v); BPE buffer, 2 mM Na_2HPO_4, 6 mM NaH_2PO_4, and 1 mM EDTA, pH 7.0; BPES buffer, BPE buffer and 180 mM NaCl; electrophoresis buffer, 36 mM tris(hydroxymethyl)aminomethane, 30 mM NaH_2PO_4, and 1 mM Na_2EDTA; r, molar ratio of bound dye molecules/base pair; poly(AA), polyacrylamide.



	R_1	R_2
MA	$-CH_3$	—
QMA	$-CH_3$	$-CH_3$
EA	$-CH_2-CH_3$	—
QEA	$-CH_2-CH_3$	$-CH_3$

FIGURE 1: Scheme of preparation of acrylamine monomers. (1) Acylation of *N,N*-dialkylethanediamine by acryloyl chloride. (2) Quaterization of dialkylamine derivatives by methyl iodide. (3) Conversion of iodide into chloride salts.

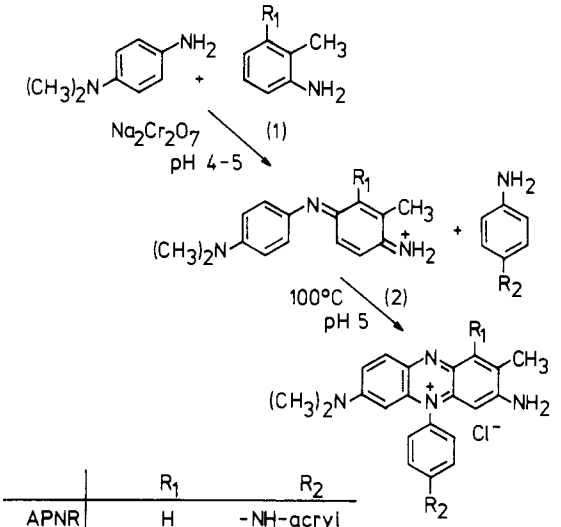
at room temperature for 15 min and centrifuged at 10000 rpm for 15 min. The supernatants were analyzed for their DNA composition by comparing the measured absorbance at 258 nm of a particular sample with the amount of labeled *E. coli* DNA in it, determined by LSC.

Synthesis of Monomers. In the course of our experiments various methods of analysis depended on the availability of labeled monomers. For reasons of economy, the synthesis of all different monomers was directed in such a way that labeling with commerically available [^{14}C]acryloyl chloride (NEN) could be done easily if necessary.

(*a*) *Preparation of Acrylamine Monomers* (*Figure 1*). *N,N*-Dimethyl-2-(acryloylamido)ethylamine (MA) or *N,N*-diethyl-2-(acryloylamido)ethylamine (EA) was synthesized by dropwise addition of a solution of 1 mL of acryloyl chloride (Fluka) in 50 mL of $CHCl_3$ to a stirred solution of 10 mmol of *N,N*-dimethylethylenediamine or *N,N*-diethylethylenediamine, respectively, in 50 mL of $CHCl_3$. After 1 h at room temperature, the chloroform solution was extracted twice with 1 volume of 1 M Na_2CO_3, washed with water, passed through phase separation paper (PS 1 Whatman), and evaporated in vacuo. The remaining syrup, about 0.8 g, was estimated to be pure, giving a single spot on TLC analysis in acetic acid/methanol/chloroform (1:1:8 v/v; solvent system A) when visualized with ninhydrin spray (Merck) by heating the TLC plate to 200 °C. The viscous MA or EA was stored under refrigeration and used for polymerization or methylation without further purification. The precise concentration of these substances was determined by titration of the double bond of the acryloyl residue by permanganate immediately prior to their use for polymerization reaction.

N,N,N-Trimethyl-2-(acryloylamido)ethylammonium chloride (QMA) and *N,N*-diethyl-*N*-methyl-2-(acryloylamido)-ethylammonium chloride (QEA) were prepared by methylation of MA and EA with methyl iodide in methanol according to Seela (1971). The aqueous eluates from the resin were concentrated in a rotary evaporator. TLC of the colorless syrup showed the substances to be pure in solvent system A. Since crystallization could not be obtained, the syrup was dissolved in water and stored under refrigeration. The monomer concentration of aqueous stock solutions was determined, immediately before use, by chloride titration according to Volhard.

(*b*) *Synthesis of Phenazinium Monomers.* The synthesis of phenazinium monomers, schematically shown in Figure 2,



	R_1	R_2
APNR	H	-NH-acryl
1-APNR	-NH-acryl	H
AMPNR	$-CH_2$-NH-acryl	H
AEPNR	$-(CH_2)_2$-NH-acryl	H

FIGURE 2: Scheme of reactions for synthesis of phenazinium monomers. (1) Condensation of *p*-amino-*N,N*-dimethylaniline and R_1-substituted *o*-toluidine with dichromate in slightly acidic medium. (2) Synthesis of the phenazinium dye chromophore by reaction of the condensation product of (1) with R_2-substituted aniline at 100 °C in aqueous medium.

will be described only for the mainly used APNR as an example. The other dyes were synthesized generally by the same procedure with the difference that in the first condensation step *o*-toluidine was replaced by 3-(acryloylamino)-2-methylaniline for the synthesis of 1-APNR, 3-[(acryloylamino)methyl]-2-methylaniline for AMPNR, and 3-[(acryloylamino)ethyl]-2-methylaniline for AEPNR. (The detailed synthesis of the cited derivatives of aniline will be published elsewhere.) In the second condensation step for synthesis of 1-APNR, AMPNR, and AEPNR, aniline was used instead of 4-(acryloylamino)aniline.

Synthesis of 5-[4-(Acryloylamino)phenyl]-3-amino-7-(dimethylamino)-2-methylphenazinium Chloride (APNR). *N,N*-Dimethyl-*p*-phenylenediammonium chloride (2.1 g, 10 mmol) (Merck) and *o*-toluidinium chloride (1.44 g, 10 mmol) (Merck) were dissolved in 200 mL of H_2O. Under magnetic stirring, a solution of 20 mmol of $Na_2Cr_2O_7$ (6 g) in 50 mL of H_2O was added slowly. Within 5 min, a green spongy precipitate was formed, which was immediately sucked off on a large Buchner funnel, extensively washed with H_2O, and resuspended in 50 mL of H_2O in a 2-L Erlenmeyer flask. The homogeneous suspension was diluted with H_2O to 800 mL before a solution of 11.5 mmol of 4-(acryloylamino)aniline (preparation below) in 50 mL of H_2O was added. The solution was adjusted to pH 5 by addition of 40 mL of 3 M sodium acetate and heated slowly on a hot plate. (Immediately before boiling, the blue solution tends to foam!) Within about 5 min, the boiling solution changed its color from blue to violet. After another 5 min, the reaction was complete, and the hot solution was sucked through a large Buchner funnel, adjusted to 2 M with solid NaCl, and kept under refrigeration overnight. The greenish, glittering crystals of crude APNR were collected on a filter and dried in vacuo. The yield of crude product was about 1.7 g. For further purification, about 500 mg of crude product was dissolved in a mixture of 50 mL of methanol and 50 mL of 0.1 M NaCl and chromatographed on a Sephadex LH 20 (Pharmacia) column (60 × 5 cm), equilibrated, and

operated with the same solvent. The progress of the column was monitored by TLC on silica gel 60 in solvent system A. The fractions containing APNR and its 4-methyl isomer (small amounts) were pooled and evaporated in vacuo. After recrystallization from 1 M NaCl solution, about 260 mg of APNR was obtained: ^{1}H NMR (Me_2SO-d_6) δ 2.28 (s, 3 H), 3.06 (s, 6 H), 5.72 (d, J = 4 Hz, 1 H), 5.84 (dd, J = 8.0, 4 Hz, 1 H), 6.22–6.52 (m, 2 H), 7.40–8.18 (m, 7 H). Anal. Calcd for $C_{24}H_{24}N_5O_5Cl$ (perchlorate): C, 57.89; H, 4.86; N, 14.06; Cl, 7.12; O, 16.06. Found: C, 57.55; H, 5.26; N, 14.04; Cl, 7.09; O, 16.06.

4-(Acryloylamino)aniline. A cold solution of 2.4 mL (30 mmol) of acryloyl chloride in dried dioxane was added to a stirred ice-cold solution of 8.4 g (60 mmol) of *p*-nitroaniline in 150 mL of dry dioxane. As soon as the formation of precipitate finished, the reaction mixture was placed at room temperature for about 1 h. Then 2 volumes of H_2O was added, and the precipitate of *N*-acryloyl-*p*-nitroaniline was collected on a filter and recrystallized from methanol. A total of 2.2 g (11.5 mmol) of the recrystallized product was dissolved in 50 mL of boiling acetic acid and poured quickly into a freshly prepared mixture of 20 g of zinc powder in 20 mL of acetic acid. Reduction of the nitro compound was followed by a quick color change from yellow to faint red. When the color change was complete, the hot solution was filtered to remove excess zinc powder. The residue in the filter was washed with small volumes of acetic acid. The filtered solutions were pooled and evaporated in vacuo, leaving a reddish syrup. The syrup containing 4-(acryloylamino)aniline and aqueous zinc acetate was dissolved, in general, in 50 mL of H_2O and used for synthesis of APNR without further purification. For analytical purposes, it was purified by repeated recrystallization of ethyl acetate/cyclohexane: ^{1}H NMR (Me_2SO-d_6) δ 4.03 (s, 2 H), 4.69 (dd, J = 8.0, 4 Hz, 1 H), 5.18 (d, J = 4 Hz, 1 H), 5.27 (d, J = 8 Hz, 1 H), 5.44 (d, J = 9 Hz, 2 H), 6.13 (d, J = 9 Hz, 2 H), 8.11 (s, 1 H). Anal. Calcd for $C_7H_{10}NO$: C, 60.85; H, 7.29; N, 20.27; O, 11.57. Found: C, 60.13; H, 7.59; N, 19.86; O, 12.32.

(c) Synthesis of Malachite Green Monomers (Figure 3). The synthesis of these dyes is shown schematically in Figure 3. Leuco-*p*-nitro-malachite green is a common intermediate for synthesis of AMG and AAMG. For its preparation, a mixture of 1.51 g (10 mmol) of 4-nitrobenzaldehyde and 4.05 g (30 mmol) of $ZnCl_2$ in 3.8 mL (30 mmol) of *N,N*-dimethylanailine was kept at 100 °C for about 5 h until solidification of the green solution was complete. The product was cooled to room temperature and dissolved in 150 mL of acetone. The undissolved zinc salt was sucked off on a Buchner funnel and washed with acetone. The pooled filtrates were diluted with H_2O until crystallization occurred. Crystallization was completed overnight in a refrigerator before crystals were collected by filtration, recrystallized from butanol, and dried in vacuo. A total of 3.09 g (82.4%) of leuco-*p*-nitro-malachite green was obtained: ^{1}H NMR ($CHCl_3$-d) δ 2.94 (s, 12 H), 5.46 (s, 1 H), 6.67 (d, J = 8 Hz, 2 H), 6.96 (d, J = 8 Hz, 2 H), 7.30 (d, J = 8 Hz, 2 H), 8.11 (d, J = 8 Hz, 2 H). Anal. Calcd for $C_{23}H_{25}N_3O_2$: C, 73.57; H, 6.71; N, 11.19; O, 8.52. Found: C, 73.18; H, 7.03; N, 11.12; O, 8.67.

Synthesis of [4-((N-Acryloyl)amino)phenyl]bis[4-(dimethylamino)phenyl]carbenium Chloride (AMG). A total of 1.5 g (4.0 mmol) of leuco-*p*-nitro-malachite green was dissolved in 80 mL of glacial acetic acid and 8 mL of H_2O. The solution was stirred, and 8 g of zinc powder was added. After 25 min, the reduction was complete, and residual zinc was removed by filtration. The filtrate was concentrated under

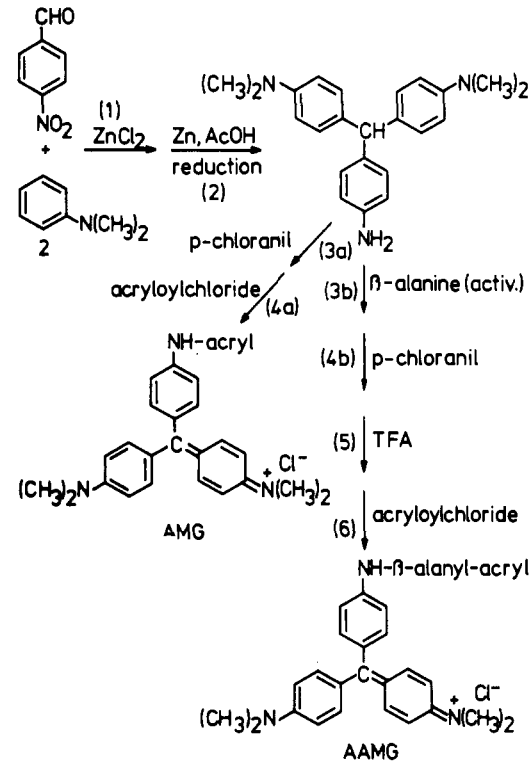


FIGURE 3: Scheme for preparation of malachite green derivatives. (1) Condensation of *N,N*-dimethylaniline and *p*-nitrobenzaldehyde in molten $ZnCl_2$ at 100 °C. (2) Reduction of the resulting leuco-*p*-nitro-malachite green by Zn/acetic acid. (3a) Oxidation by *p*-chloranil yields *p*-amino-malachite green. (4a) Acylation by acryloyl chloride yields AMG. (3b) Coupling of ß-alanyl residue with leuco-*p*-amino-malachite green via the *p*-nitrophenyl ester of (*t*-Boc)-ß-alanine. (4b) Oxidation by *p*-chloranil. (5) Cleavage of the *t*-Boc protective group from the ß-alanyl residue. (6) Acylation by acryloyl chloride yields AAMG.

reduced pressure at 60 °C to yield a reddish syrup. The syrup was dissolved in 20 mL of $CHCl_3$ and washed successively with 200 mL of 1 M Na_2CO_3 and 200 mL of H_2O. The $CHCl_3$ phase, containing leuco-*p*-amino-malachite green, was filtered through a phase separation filter (PS 1, Whatman), stored in the dark, and used for following reactions without further purification. For oxidation of leuco-*p*-amino-malachite green, 4 mL of glacial acetic acid and 880 mg of *p*-chloranil were added, and the $CHCl_3$ solution was shaken at room temperature for about 2 h. After addition of 440 mL of $CHCl_3$ and 60 mL of 1-butanol, the solution was extracted with 700 mL of H_2O and the aqueous phase reextracted twice with 120 mL of $CHCl_3$. The pooled $CHCl_3$ solutions were mixed with an equal volume of CCl_4 before the dye was extracted with 1.3 L of H_2O. The extraction of the organic phase was completed with 800 mL of H_2O. The combined aqueous extracts were filtered, and 60 mL of 5 M sodium perchlorate was added. After crystallization at room temperature overnight, 4-amino-malchite green perchlorate was collected on a Buchner funnel, successively washed with 0.1 M sodium perchlorate and H_2O and then dried in vacuo to yield 1.5 g (85%): ^{1}H NMR (Me_2SO-d_6) δ 3.46 (s, 12 H), 7.10 (d, 2 H), 7.22 (d, 4 H), 7.45 (d, 2 H), 7.55 (d, 4 H), 7.78 (s, 2 H).

For acylation, 450 mg (about 1 mmol) of 4-amino-malachite green perchlorate was dissolved in 200 mL of chloroform and shaken with 100 mL of 1 M NaOH until the aqueous phase became colorless. The orange $CHCl_3$ phase was separated in a separation funnel and filtered through phase separation paper into a 500-mL Erlenmeyer flask containing 5 g of solid Na_2CO_3. Under vigorous stirring, the solution was immediately mixed with 0.3 mL of acryloyl chloride (Merck). One

hour later, the green solution was filtered, and 2 mL of glacial acetic acid was added. The solvent was evaporated in vacuo at 60 °C, and the residue was dissolved in 50 mL of H_2O. The solution was extracted with 50 mL of ethyl acetate and filtered. Solid NaCl was added, and crystallization was completed overnight. Crystals of AMG were collected, washed with water, and dried to yield 272 mg (67%): ^1H NMR (Me_2SO-d_6) δ 3.3 (s, 12 H), 5.7 (q, 1 H), 6.3 (m, 2 H), 6.5–7.5 (m, 12 H). Anal. Calcd for $C_{26}H_{28}NO_3Cl$: C, 71.96; H, 6.50; N, 9.68; O, 3.70; Cl, 8.16. Found: C, 71.80; H 6.31; N, 9.57; O, 4.00; Cl, 8.02.

Synthesis of [4-((N-Acryloyl-β-alanyl)amino)phenyl]bis-[4-(dimethylamino)phenyl]carbenium Chloride (AAMG). From Figure 3, it is obvious that AAMG is an analogue of AMG with an elongated chain between malachite green chromophore and acrylamide residue. The elongation is achieved by β-alanine, which is introduced in the synthesis in the form of its amino-protected *p*-nitrophenyl ester. This activated ester, (*t*-Boc)-β-alanine *p*-nitrophenyl ester, was synthesized according to Stewart & Young (1969), recrystallized from methanol/water, dried in vacuo, and tested for purity by TLC, silica gel 60 F_{254}, in $CHCl_3$/ethyl acetate (8:2 v/v), when visualized with ninhydrin spray (Merck) and heated to 200 °C: ^1H NMR ($CHCl_3$-*d*) δ 1.56 (s, 9 H), 2.85 (t, 2 H). Anal. Calcd for $C_{14}H_{18}N_2O_6$: C, 54.19; H, 5.84; N, 9.03; O, 30.96. Found: C, 54.03; H, 6.02; N, 9.14; O, 30.81. (*t*-Boc)-β-alanine *p*-nitrophenyl ester (1.5 g, 5.1 mmol) was dissolved in a solution of 5 mmol of leuco-*p*-amino-malachite green (preparation see above) in 250 mL of $CHCl_3$. Imidazole (3.5 g, 50.3 mmol) was added, and the solution was stirred at room temperature for 24 h. The progress of coupling reaction was followed by TLC, silica gel 60, in solvent system A. The slower moving spot of unsubstituted amino-malachite green was replaced in time by the quicker moving spot of reaction product. (Color resulted from oxidation of the leuco compounds.) When the coupling reaction was complete (sometimes only after addition of further amounts of active ester), the reaction mixture was mixed with a suspension of 1.2 g (4.9 mmol) of *p*-chloranil in 250 mL of acetic acid and stirred for about 24 h. The solvents were evaporated in vacuo, and the residue was dissolved in 500 mL of $CHCl_3$, extracted several times with equal volumes of 1 M Na_2CO_3 for removal of *p*-nitrophenol, washed with 1 volume of H_2O, and filtered through a phase separation filter. The filtrate was evaporated to dryness in vacuo. The green residue was dissolved in 50 mL of methanol/1% acetic acid and purified by column chromatography on LH 20 (60 × 5 cm) in the same solvent. The green colored fractions were pooled and evaporated to dryness before 10 mL of TFA was added for removing the *t*-Boc protective group from the amino group of the β-alanyl residue. Excess TFA was removed by evaporation in vacuo, and the residue was dissolved in 200 mL of $CHCl_3$. The $CHCl_3$ phase was shaken with 200 mL of 1 M Na_2CO_3 to convert the green dye to its colorless carbinol base. The $CHCl_3$ solution was passed through a phase separation filter and immediately mixed with 0.5 mL of acryloyl chloride. Two grams of solid Na_2CO_3 was added to the stirred solution for neutralization of the HCl liberated by the acylation reaction. About 15 min later, the solution was filtered through a phase separation filter and evaporated to dryness in vacuo. The residue, consisting mainly of AAMG, was dissolved in 50 mL of methanol/0.1% acetic acid and passed through a column of LH 20, operated in the same solvent system. AAMG-containing fractions were pooled, evaporated to dryness in vacuo, and dissolved in 100 mL of H_2O. For polymerization purposes, the dye was precipitated by addition of 5 M NaCl, collected by filtration, and dried in vacuo. For analytical purposes, AAMG was precipitated from the aqueous solution by addition of 1 M sodium perchlorate. The precipitate was washed by water and dried in vacuo. Anal. Calcd for $C_{29}H_{32}N_4O_6Cl$: C, 61.32; H, 5.68; N, 9.86; O, 16.89; Cl, 6.24. Found: C, 61.54; H, 5.72; N, 9.70; O, 16.61; Cl, 6.43.

Polymerization of Dye Short-Chain Polymers. A mixture of 710 mg (10 mmol) of acrylamide (Serva) and about 3 mg (about 6 μmol) of dye chloride was dissolved in 5 mL of H_2O. The solution was kept in a 10-mL test tube and mixed with 0.1 mL of freshly prepared aqueous AIBA solution (20 mg/mL) and 50 μL of mercaptoethanol (Merck). For initiation of the polymerization reaction, nitrogen was bubbled through the solution for 2 min before the test tube was closed and kept at 60 °C for 45 min. Precipitation of the polymers was effected by pouring the reaction mixture into 50 mL of methanol under vigorous stirring. Precipitation was completed by addition of one drop of 1.8 mL of NaCl. The precipitate was pelleted by centrifugation at 5000 rpm and dissolved again in 5 mL of H_2O prior to a second precipitation by methanol. Finally, dye polymers were separated from the excess of unsubstituted acrylamide polymers by absorption on a column of CM-Sephadex (Pharmacia). During elution with H_2O, only the dye polymers were retained on the top of the column. They were eluted by 50% acetic acid and isolated by evaporation in vacuo. The residue was dissolved in H_2O to yield a solution which contained a certain percentage of the initial dye monomer. If ^{14}C-labeled acrylamide of a known specific activity was used, the average chain length of the dye polymer could be calculated directly from the cpm/dye ratio.

Chain-Length Determination of Unlabeled Dye Polymers. The average chain length of unlabeled dye short-chain polymers was calculated from their partition coefficients in a two-phase system of poly(ethylene glycol) and dextran in the following way. A small volume of aqueous dye polymer solution was added to 4 mL of a two-phase system composed of 5.3 g of poly(ethylene glycol) (PEG 6000, Serva), 10 g of dextran (T 40, Pharmacia), and 100 mL of BPE buffer. The mixture was shaken for 15 min at room temperature, and phase separation was achieved by short centrifugation at 1500 rpm. For measurements of dye concentrations in the upper PEG phase and the lower dextran phase 0.5 mL of each phase was removed and mixed with 0.5 mL of BPE buffer/2% $NaDodSO_4$. The partition coefficient was determined as the ratio of dye concentrations of the upper to the lower phase. For evaluation of chain length, the partition coefficients were compared to a calibration curve which was obtained from a series of ^{14}C-labeled polymers of different but known chain length.

When dye polymers of QEA were synthesized, the same experimental procedures for their polymerization were used with the following exceptions: polymers were precipitated by acetone, and CM-Sephadex was replaced by Sephadex LH 20 column and operated with H_2O/1% acetic acid. The slower moving colored band containing the dye polymers was collected and evaporated to dryness in vacuo. The residue was dissolved in a small volume of water, stored in the dark, and used for experiments without further purifications.

Agarose Gel Electrophoresis for Titration of Supercoiled DNA with Intercalating Agents. Electrophoresis was performed in cylindrical gel (0.4 × 12 cm) of 0.6% agarose (Seakem) in electrophoresis buffer. Gels containing different concentrations of dye or dye polymer were prepared by adding aliquots (about 1–50 μL) of a stock dye solution to 5 mL of

Table I: Spectral Data and Affinity and Specificity Parameters of Acrylamide Derivatives[a]

compound	$\lambda_{1,max}$, $\lambda_{2,max}$ (nm)	ϵ_1, ϵ_2 (M^{-1} cm^{-1})	$\sigma_{B.\ subtilis}$ (M^{-1})	$\alpha = \sigma_{M.\ luteus}/\sigma_{B.\ subtilis}$	buffer
APNR	550, 276	54 500, 63 100	8.2×10^3	2.04	BPES
1-APNR	560, 280	45 100, 40 100	3.2×10^2, 4.2×10^3	1.20, 1.73	BPE
AMPNR	556, 278	59 200, 46 700	8.3×10^3	2.0	BPES
AEPNR	555	49 700	1.6×10^3	1.8–2.0	BPES
PNR[b]			5.3×10^3	2.23	BPES
AMG	620, 304	85 900, 14 300	1.7×10^4	0.29	BPES
AAMG	620, 304	85 600, 14 500	1.3×10^4	0.30	BPES
MG[c]			1.85×10^4	0.24	BPES
[^{14}C]QEA			<50	1.0	BPE
[^{14}C]AA			<10		BPE

[a] The α values refer to the ratio of σ for DNAs from *Micrococcus luteus* and *Bacillus subtilis* as described under Experimental Procedures. [b] Data from Müller et al. (1975). [c] Data from Müller & Gautier (1975).

molten agarose in electrophoresis buffer at 50 °C. Samples consisting of 1 μg of DNA in 25 μL of electrophoresis buffer containing 5% sucrose were run at 70 V for about 180 min at room temperature. Since all phenazinium dyes show a red fluorescence under conditions used for the visualization of DNA by ethidium bromide, an alternate staining procedure was necessary. The gels, containing different amounts of dye or dye polymers, were stained for 30 min in freshly prepared electrophoresis buffer containing 1 μg/mL ethidium bromide. The stained gels were transferred into a flat tray filled with 0.5% $Na_2S_2O_3$ (w/v) in H_2O. Selective destaining of phenazinium dyes occurred within 15–30 min. The residual ethidium bromide stained DNA bands were visualized by quickly placing the gels onto a long-wave ultraviolet light source (C 62 Transilluminator). Photographs were taken by Polaroid Land pack or MP 4 camera with Polaroid Type 55 P/N film and UV and orange filter (Schott).

Results and Discussion

Binding Parameters of Different Acrylamide Derivatives. Before starting the polymerization experiments, we determined the binding parameters for most of the newly synthesized monomers, except for APNR whose binding to DNA had been characterized by Müller et al. (1975). The other monomers were investigated for their base-pair specificity and affinity by the partition dialysis method in cylindrical three-chamber dialysis cells made of plexiglass, described in detail by Müller et al. (1973) and Müller & Crothers (1975). For analysis of the binding equilibria, defined volumes were removed from different chambers of the dialysis cell assembly and mixed with $^1/_{10}$ volume of 25% (w/w) sodium dodecyl sulfate (NaDodSO$_4$) in water. Under this condition, DNA–dye complexes are dissociated completely, and dye concentrations can be calculated from extinction coefficients listed in Table I. Colorless monomers, e.g., acrylamide or acrylamine (QEA), were used as ^{14}C-labeled compounds, and concentrations were measured by liquid scintillation counting of 1 volume of monomer solution dissolved in 20 volumes of Aquasol (NEN). The results of partition equilibrium dialysis experiments performed with pairs of sheared bacterial DNAs of different base composition, e.g., *M. luteus* (72.5% G + C) and *B. subtilis* (44.5% G + C) or *E. coli* (51% G + C), respectively, are displayed in Table I.

For an interpretation of specificity (α) and affinity (σ) values of Table I, one has to remember that α values of 1.63 and 2.65 are equivalent to specific binding of a ligand to a single GC base pair and to two adjacent GC base pairs, respectively, when *M. luteus* and *B. subtilis* DNA are used (Müller & Crothers, 1975). The theoretical α values for corresponding AT specifities are 0.50 and 0.25, respectively, for the same DNA pair (Müller & Gautier, 1975). The comparison of the α and σ values of the various PNR and MG derivatives with the corresponding values of the parent compounds PNR and MG reveals that the influence of the acrylamide residue on the binding parameters of APNR, AMPNR, and AMG is small. In general, these results confirm the intercalation model which was derived by Müller et al. (1975) for the structure of DNA complexes of GC-specific phenazinium dyes. From this model, the deviating behavior of 1-APNR may easily be predicted. Corey–Pauling–Koltun models of the complex show that the rigid carboxamido group in the 1 position of the intercalated chromophore prevents optimal stacking between the planar dye chromophore and its neighboring GC base pairs, necessary for GC-specific binding. Insertion of one methylene group between the aromatic ring system and the carboxamido group, as in the case of AMPNR, renders the chain more flexible and yields binding parameters nearly identical with those of APNR. Both PNR derivatives were synthesized to answer the question if template polymerization proceeds preferentially in one of the two grooves of the DNA helix since the acrylamide residues are located in different grooves when APNR and AMPNR are used as complexed monomers.

The binding parameters of AMG and AAMG, which are nearly identical with those of malachite green, confirm the outside binding model postulated for other triphenylmethane dyes by Müller & Gautier (1975). According to their results, two dimethylamino groups are essential for the AT specificity of these dyes. Since these groups are not affected by the attached acrylamide residue, the binding parameters of AMG and AAMG are not altered considerably compared to those of MG. The two different MG derivatives were synthesized for investigating if there is any sterical effect of the polymer backbone on the binding behavior of the triphenylmethane chromophore. The elongated chain of AAMG should enable us to overcome sterical limitations which might occur with AMG.

Although some of the newly synthesized GC- and AT-specific monomers largely fulfilled all of the criteria demanded of monomers suitable for template polymerizations, some additional problems were to be encountered concerning their polymeric connection. It is well-known from dyes that their binding to DNA is limited by several factors, especially by neighbor exclusion (Crothers, 1968). According to this model, one DNA-bound dye prevents binding of further dyes in its neighborhood; it depends upon the dye how many base pairs are blocked. Values in the range of 2–7 base pairs were observed (Müller & Crothers, 1968; Müller et al., 1973). Because of these observations and from experimental studies on dye dimers cited above, it was necessary to connect the different dyes by a polymer backbone which should function

Table II: Partition Coefficients and Affinity and Specificity Parameters of Dye Short-Chain Polymers[a]

	AMG	AMG poly(AA)	AMG poly(QEA)		
k	1.0	0.67	1.0		
$r_{(B.\ subtilis)}$	0.021				
$r_{(E.\ coli)}$		0.0098	0.0071	0.021	0.022
$\alpha'_{(M.\ luteus/B.\ subtilis)}$	0.31				
$\alpha'_{(M.\ luteus/E.\ coli)}$		0.37	0.57	0.52	0.59
$\sigma'_{(B.\ subtilis)}$	11 000				
$\sigma'_{(E.\ coli)}$		6320	4100	74 000	190 000
buffer	BPES	BPES	BPE	BPE	BPE
NaCl (M)			1.0	0.31	0.19
	APNR	APNR poly(AA)	APNR poly(QEA)		
k	1.0	0.65	1.0		
$r_{(B.\ subtilis)}$	0.036	0.025			
$r_{(E.\ coli)}$			0.0016	0.0021	0.0082
$\alpha'_{(M.\ luteus/B.\ subtilis)}$	1.55	1.62			
$\alpha'_{(M.\ luteus/E.\ coli)}$			1.54	1.55	1.65
$\sigma'_{(B.\ subtilis)}$	69 000	3390			
$\sigma'_{(E.\ coli)}$			570	1210	10 700
buffer	electrophoresis	electrophoresis	BPE	BPE	BPE
NaCl (M)			1	0.62	0.39
	1-APNR poly(AA)	AMPNR poly(AA)	AEPNR poly(AA)		
k	0.63	0.64	0.65		

[a] Partition coefficients, k, refer to the ratio of dye concentrations (upper phase/lower phase) in a two-phase system of poly(ethylene glycol)/dextran. $k = 0.64$ corresponds to an average chain length of about 20 acrylamide residues. α' and σ' values are the specificity and affinity parameters corresponding to a particular measurement at $r > 0$ for equimolar DNA mixtures of *M. luteus* DNA (72.5% G + C) and *B. subtilis* DNA (44.5% G + C) or *M. luteus* DNA and *E. coli* DNA (51% G + C), respectively. From the base composition of different DNA pairs, one can calculate that $\alpha' = 1.63$ is equivalent to 1.42 and $\alpha' = 0.50$ corresponds to 0.57 if values of *M. luteus/B. subtilis* samples are compared to those of *M. luteus/E. coli* samples. If, instead of one AT or GC base pair, two adjacent AT or GC base pairs represent optimal binding points, the corresponding values are 2,65 and 2.02 for GC specificity or 0.25 and 0.31 for AT specificity for *M. luteus/B. subtilis* and *M. luteus/E. coli* samples, respectively (Müller & Crothers, 1975).

merely as a spacer without any base-pair specificity of its own. Furthermore, its affinity for DNA should not change the corresponding parameters of the attached dyes. For these reasons, ^{14}C-labeled acrylamide was tested for its ability to bind to DNA. As expected from the widespread utilization of polyacrylamide gels for DNA fractionation, no binding and certainly no base-pair specificity was observed by equilibrium dialysis even when DNA concentration in the range of milligrams per milliliter was used. Nevertheless, from the point of view of template polymerization, acrylamide was no optimal building block for the polymer backbone, because a small affinity to DNA should be helpful to keep the polymerization process in the proximity of the DNA template. Therefore, we synthesized acrylamide derivatives of small tertiary amines and their quaternized analogues and tried to determine their binding to DNA. Of special interest was the binding specificity of QMA or QEA (Figure 1), because their molecular structures were very similar to tetramethyl- and tetraethylammonium salts, which had been shown to bind specifically to AT-rich double-stranded DNAs (Shapiro et al., 1969a,b; Melchior & von Hippel, 1973). Only a small affinity ($\sigma <$ 50), but no AT specificity, was observed in partition equilibrium dialysis experiments for QEA using *M. luteus* and *C. perfringens* DNA (Table I).

Although substituted tertiary amines seemed to be suitable building blocks for the polymer backbone, polymerization experiments had to show if the binding parameters of the base pair specific dyes would become affected after their attachment to a polymer backbone formed from such monomers.

Influence of the Polymer Backbone on the Binding Parameters of Base Pair Specific Dyes. In general, two different types of dye polymers could be expected from template polymerization experiments with acrylamide or acrylamines: linear polyacrylamides with cationic dye substituents along a neutral chain if acrylamide was used as the only spacer monomer or cationic linear polyacrylamines with cationic dye substituents along the chain if acrylamines were used as backbone forming units. For determination of the influence of both types of polymer backbone on the binding parameters of our dyes, suitable tests had to be developed, although it was known for phenyl neutral red and malachite green that their base-pair specificity was not drastically altered after fixing them in a suitable way to a backbone of polyacrylamide. Polymers of that kind, e.g., engrafted on particles of cross-linked bis(acrylamide), were successfully used for base pair specific fractionation of DNA (Bünemann & Müller, 1978). We tried to ascertain these qualitative results by quantitative measurements on dye short-chain polymers, which were designed as very short polymer chains containing only one dye residue per chain.

This sort of dye polymers was synthesized by radical chain copolymerization of dye monomers with a more than 1000-fold excess of AA or QEA in the presence of high concentrations of mercaptoethanol for chain termination. The dye-substituted polymers were separated from the excess of their unsubstituted colorless analogue by various methods of column chromatography (see Experimental Procedures). Their average chain length was calculated from the partition coefficient k in a two-phase system of poly(ethylene glycol)/dextran as described under Experimental Procedures. From typical polymerization reactions, polymers with partition coefficients of about 0.65 were obtained, according to an average chain length of about 20 acrylamide residues. The k values of different polymers, listed in Table II, show that all AA polymers had nearly the same chain length. In contrast to AA polymers, QEA polymers show no asymmetric distribution in the poly(ethylene glycol)/dextran system. Since they were polymerized under identical conditions, we assumed that they had a chain length similar to that of their poly(AA) analogue.

Partition Equilibrium Dialysis of Dye Short-Chain Polymers. So that the polymers were indeed able to cross the dialysis membrane during our equilibrium measurements,

dialysis bags were filled with colored polymer solution and dialyzed extensively against BPE buffer. Since the bags became completely colorless within 2–3 days, we were sure that our dialysis experiments were not falsified by incomplete distribution of the polymers inside the dialysis cell assembly. Whereas partition equilibrium dialysis experiments with dye poly(AA) could be done in the normal way in BPE or BPES buffer, dye poly(QEA) measurements demanded special conditions, because of their tendency to precipitate DNA at NaCl concentrations below 0.3 M.

For these reasons, dialysis experiments with dye poly(QEA) were started in BPE/1 M NaCl. Every 3 days, samples were removed from the dialysis chambers, and dye concentrations were determined without addition of $NaDodSO_4$. Afterward, the DNA solutions were returned to their chambers. The buffer solution from the middle chamber was used for determination of NaCl concentration by conductivity measurement before the dialysis chamber was refilled with fresh BPE buffer. By this procedure, the salt concentration in the dialysis cell assembly could be lowered in a well-controlled step by step manner until precipitation of DNA–polymer complex was observed.

Some results of partition equilibrium dialysis experiments for both types of polymer backbone are summarized in Table II. The data for AMG poly(QEA) and APNR poly(QEA) show that the base pair specificity parameters, α', of a polymer-lined dye is remarkably constant, even if the affinity parameter, σ', is increased about 100-fold according to decreasing NaCl concentration. It is important for our later specificity tests that the binding specificities of our polymer-bound dyes do not show remarkable changes when salt concentration is lowered step by step until precipitation occurs. α' and σ' are values which correspond to give r values (molar ratio of dye to base pair) as defined for a single measurement. Therefore, they are not comparable to the values listed in Table I, which were obtained by extrapolation to $r = 0$ from a set of different measurements. A comparison of both values becomes possible if α' and σ' values for similar r values for APNR and AMG monomers were derived from binding isotherms obtained from dialysis experiments (Müller et al., 1975; Müller & Gautier, 1975). If comparable values of measurements in BPES buffer are taken, it can be seen from the data in Table II that the specificity α'(*M. luteus*/*B. subtilis*) of APNR monomer at r (*B. subtilis*) ~0.02 is similar to corresponding values of APNR poly(AA) and APNR poly(QEA). Therefore, the formation of GC-specific intercalation complexes seems to be relatively insensitive to the composition of the polymer backbone.

For AMG, the situation is different. The AT specificity is obviously diminished if the dye is attached to the QEA polymer. Since this decrease in specificity is not observed for AMG poly(AA), several plausible explanations are possible. (1) The positively charged QEA residues compete with the structural similar dimethylamino groups of AMG for the same binding sites on DNA molecules. (2) The bulky QEA residues hamper complex formation of AMG by sterical effects. (3) A combination of both effects causes the observed loss of base pair specificity.

When the affinities of APNR poly(AA) and AMG poly(AA) are compared to those of APNR and AMG monomers in the same buffer, a general decrease is observed. Since similar affinity changes are found when PNR or MG are covalently attached to poly(ethylene glycol) (C. Eigel and W. Müller, unpublished results), the decrease is obviously due to the motion of the polymer chain. But even if the different r' values and buffers of both experiments are taken into account, the decrease of σ' for APNR poly(AA) is an order of magnitude higher than that of AMG poly(AA), although both polymers should have the same chain length as proved by their identical k values. Therefore, one has to conclude that the formation of the intercalation complex of APNR is much more sensitive to sterical hindrance by the polyacrylamide backbone than the outside binding of AMG. In contrast to the dye poly(AA) species, the dye poly(QEA) analogues show an increased affinity at comparable salt concentrations. This general increase is caused by electrostatic interactions between polyanionic DNA and polycationic polymer.

Although the dialysis measurements could confirm that poly(AA) and poly(QEA) show only little influence on the base-pair specificity of attached dyes, we tried to use the short-chain polymers to answer another basic question regarding the structural details of DNA complexes of different PNR derivatives.

Titration of Supercoiled DNA by Intercalating Agents. We synthesized two different types of phenazinium dyes, APNR, which has an acrylamide residue in the 4′ position of the dye molecule, and 1-APNR, AMPNR, and AEPNR, which have the substitution in the 1 position (Figure 2). Both types of monomer were designed according to a detailed model for the structure of the intercalation complex for phenazinium dyes (Müller et al., 1975). From this model, we could predict for intercalated phenazinium dyes that the acrylamide residue should protrude into the larger groove of the DNA helix in the case of APNR and into the minor one in the cases of 1-APNR, AMPNR, and AEPNR. There is strong evidence to date that the GC specific binding of heterocyclic dye molecules to DNA is due to the formation of intercalation complexes. Therefore, we were tempted to believe that our monomers were able to form intercalation complexes with DNA because of their more or less pronounced GC specificities (Table I).

To prove this assumption directly, we used the rapid and elegant methods to detect intercalation binding of DeLeys & Jackson (1976) and of Lee & Morgan (1978). This method is based on the observation that the formation of intercalation complexes is accompanied by the partial unwinding of the DNA helix (Waring, 1970).

When we used this method for titration of covalently closed supercoiled PML 21 plasmid DNA with our different PNR dyes (for details, see Experimental Procedures), all monomers caused unwinding. The examples in Figure 4 (1a–3a) show that the migration rates of covalently closed circular (ccc) DNAs, the fluorescent leading bands, are reduced from left to right in the gels according to increasing dye concentrations in the gels. For a defined concentration (c'), the critical free dye concentration (DeLeys & Jackson, 1976), comigration of nicked circular and ccc-DNA occurs. If the dye concentration is further increased, then the migration rate of ccc-DNA increases again due to the formation of positive supercoils (Figure 4, 1a). c' for different dyes can be determined by plotting the differences, ΔS, between migration distances of ccc-DNA and open circular DNA against the free dye concentrations c_F in a series of electrophoresis tubes. The results (filled circles in Figure 5) demonstrate that the c' values ($\Delta S = 0$) for our different phenazinium dyes increase in the following sequence: APNR < AMPNR < 1-APNR < AEPNR. If this sequence is compared to the corresponding one for affinity values, $\sigma_{B.\,subtilis}$ in Table I, a general trend is observed which supports the assumption that different c' values are mainly caused by different DNA affinities of the dyes.

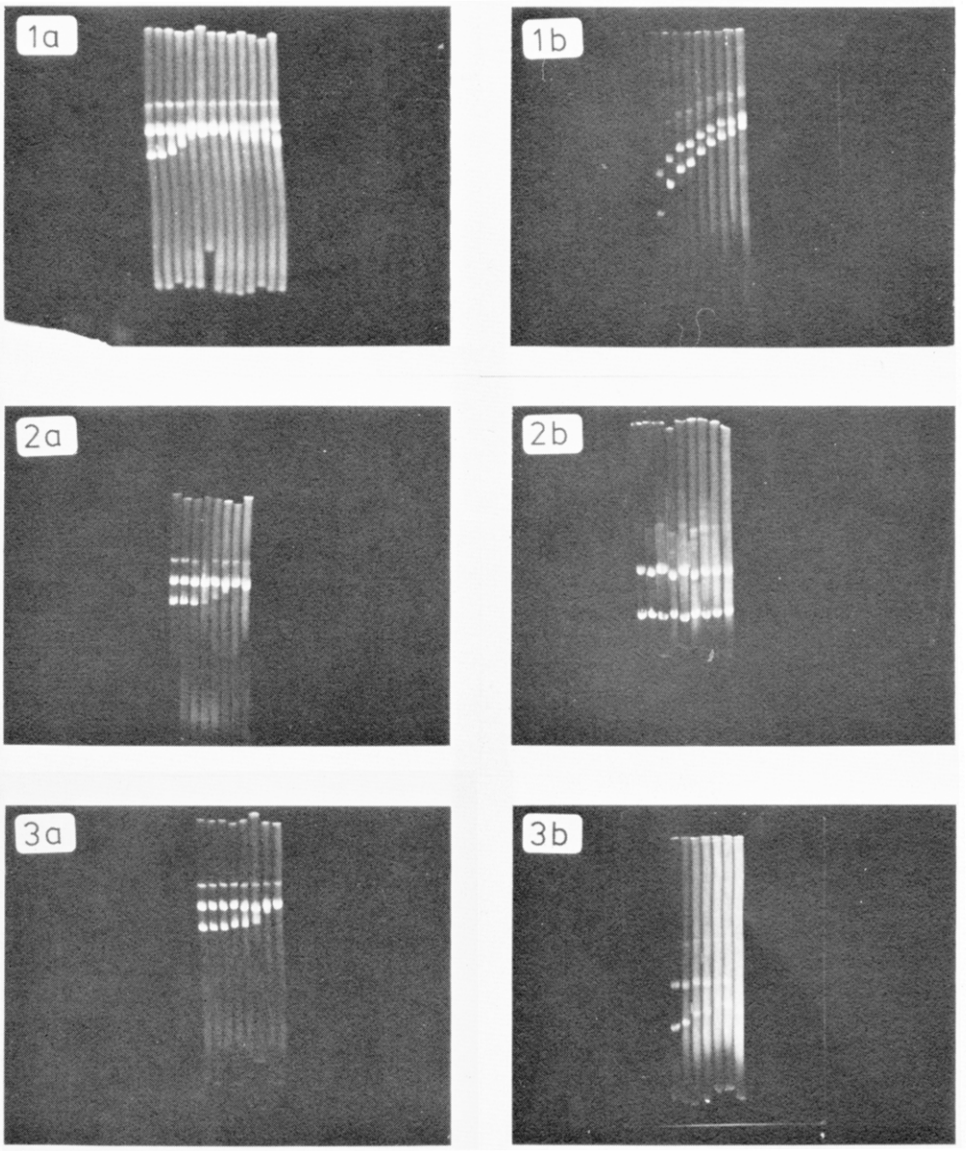


FIGURE 4: Electrophoretic titration of supercoiled plasmid DNA by intercalating agents. Parts 1a–3a show the results of agarose gel electrophoresis experiments of ccc-DNA and open circular DNA of plasmid pML 21 in the presence of increasing concentrations (from left to right) of different PNR acrylamide derivatives. (1a) APNR, 0–3.5 μg/mL; (2a) 1-APNR, 0–20 μg/mL; (3a) AEPNR, 0–20 μg/mL. Parts 1b–3b show gels for the corresponding dye short-chain polymers. (1b) APNR poly(AA), 0–6.5 μg of dye/mL; (2b) 1-APNR poly(AA), 0–14 μg of dye/mL; (3b) AEPNR poly(AA), 0–22 μg of dye/mL. The gels were differentially stained as described under Experimental Procedures. The DNA bands in the gels are arranged from bottom to top in the order ccc- and oc-plasmid DNA and linear chromosomal DNA.

From our partition equilibrium dialysis experiments of APNR and APNR poly(AA) or AMG and AMG poly(AA), we knew that the attachment of these dyes to poly(AA) caused a decrease of affinity but no remarkable loss of specificity. For electrophoretic dye titration experiments with dye short-chain polymers, we could expect an increase of c' values due to the lower affinity of polymer-bound dyes compared to their monomers. The fluorescent bands in Figure 4 (1b–3b) show the results of titration experiments with short-chain polymers of different PNR derivatives. A comparison of (1b) and (1a) reveals that a quantitative analysis of the gels is complicated by additional influences of the poly(AA) residue of DNA-bound dyes on the migration rate of the DNA–dye complex. For a rough normalization, the distances between the bands for ccc-DNA and open circular DNA were corrected by a retardation factor. This factor was calculated for each gel from the ratio of the relative mobilities of open circular DNA in a particular dye–gel to a corresponding gel without any dye. When this crude normalization procedure is applied, the results in Figure 5 (open circles) agree with an unwinding of supercoiled DNA by dye short-chain polymers, although in no gel c' conditions could be reached. The different slopes of the curves for monomer (filled circles) and polymer (open circles) reflect the predicted affinity differences between monomer and polymer. Again, APNR and AMPNR give very similar results, in good agreement with other similarities for both dyes observed before by different measurements. The results for 1-APNR and AEPNR polymers may be understood as a consequence of the drastically reduced DNA affinities of their corresponding monomers.

Binding Properties of Acrylamide and Acrylamine Polymers. Polyacrylamide gels are widely used for permeation chromatography and gel electrophoresis of nucleic acids. Therefore, we were not surprised that our attempts to detect an affinity of linear acrylamide polymers for DNA failed completely. Even when about 1 mL of sheared calf thymus DNA in BPE buffer (1 mg/mL) was passed through a column of Sephadex G-200 (1 × 20 cm) equilibrated by a solution of 0.1% ^{14}C-labeled polyacrylamide in BPE buffer, no binding of polymer to the DNA peak in the excluded volume could

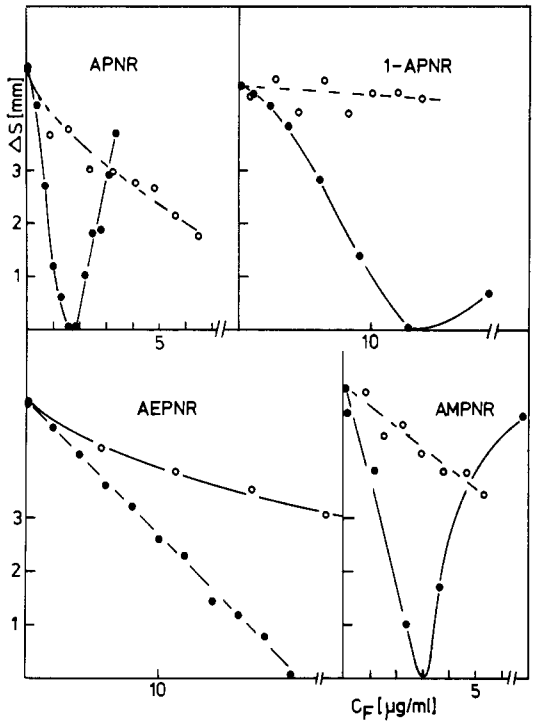


FIGURE 5: Analysis of dye titration experiments for intercalating dyes and their corresponding short-chain polymers (Figure 4). S_{ccc}^x and S_{oc}^x are the migration distances of covalently closed circular and open circular DNA, respectively, in a single gel tube. The difference of migration, ΔS, between the leading band for ccc-DNA and the succeeding one for oc-DNA is diminished if the degree of supercoiling of ccc-DNA is lowered by unwinding due to intercalation of the complexing agent. ΔS is defined as $\Delta S = (S_{ccc} - S_{oc})/R_f$. Since the polymer chain of dye polymers exerts an influence on the migration rates of ccc-DNA as well as oc-DNA, all measurements are normalized by use of a retardation factor R_f, which is defined as $R_f = S_{oc}^x/S_{oc}^0$; S_{oc}^x and S_{oc}^0 are the migration distances of open circular DNAs in particular gels of dye concentration $c_f = x$ and dye concentration $c_f = 0$, respectively. Dye or dye polymer concentrations c_f in the gel tubes are calculated from absorbance measurements of stock solutions used for gel preparation.

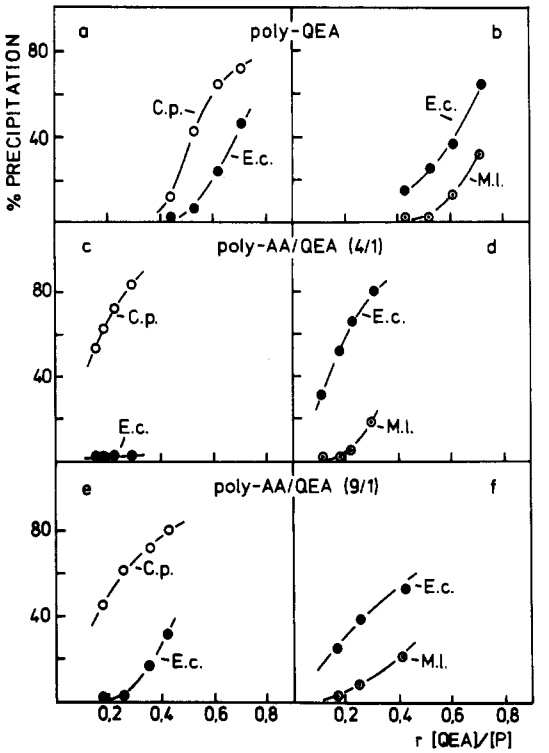


FIGURE 6: Base pair specific precipitation of DNA by QEA-containing polymers. Mixtures of *M. luteus* DNA (72.5% G + C) and ^{3}H-labeled *E. coli* DNA (51% G + C) or *C. perfringens* DNA (31.5% G + C) and ^{3}H-labeled *E. coli* DNA were mixed with increasing amounts of different QEA polymer. Poly(AA)/QEA 4:1 or 9:1 are copolymers of acrylamide and QEA whose average molar ratio in the polymer chain is given by the values in parentheses.

be observed. The lack of affinity of poly(AA) for DNA agrees with our previous observation that the poly(AA) backbone has no influence on the base-pair specificities of our dyes.

In contrast to poly(AA), polymers of QEA form strong complexes with DNA. So we regularly observed the precipitation of DNA during our partition equilibrium dialysis experiments with dye short-chain polymers at low salt concentrations. Similar precipitation of DNA was also obtained if other polycations, such as poly(lysine) or poly(arginine), were added to DNA solutions under appropriate salt concentrations (Leng & Felsenfeld, 1966; Shapiro et al., 1969a,b). Since these polymers show base pair specific binding not detectable for the corresponding monomers, we synthesized QEA polymers for specificity tests with mixtures of DNA of different base composition. These tests should answer the question of whether the loss of AT base pair specificity, which was measured for AMG after its attachment to poly(QEA) (Table II), could be caused by unknown binding specificities of the QEA polymer backbone. Precipitation tests were performed with two different mixtures of sheared bacterial DNAs of different base composition as described under Experimental Procedures. The results shown in Figure 6a,b indicate a preferential precipitation of the AT-rich DNA species from the mixtures in both cases. Obviously the triethylmethylammonium residues of QEA polymers exert a pronounced AT specificity when reacting with DNA in analogy to the AT-specific interaction which was observed with tetramethyl- and tetraethylammonium salts (Melchior & von Hippel, 1973). To ensure whether this AT specificity was really the result of a special secondary structure of the polymer, we synthesized copolymers containing AA and QEA in molar ratios of 4:1 and 9:1. Both copolymers precipitate AT-rich DNA with even stronger preference than poly(QEA) (Figure 6c–f). We tend to interpret this effect as being due to AT specific binding of single independent quaternary groups since the complex formation of QEA residues becomes more AT specific if other bulky groups in their neighborhood are replaced by the small acrylamide as in the case of the 1:4 copolymer. For copolymers with an AA/QEA ratio of 9:1, the specificity of binding seems to be lowered by the motion of the longer poly(AA) loops between neighboring QEA residues. The different QEA/phosphate ratios necessary for precipitation of DNA by different polymers support the model of optimal fitting between a linear array of cations and phosphate anions of DNA. Comparisons of Corey–Pauling–Koltun models of our three different polymers with a model of double-helical DNA clearly favor the 4:1 copolymer for an optimal complex formation between its cationic residues and the anionic pattern of DNA phosphates. The results also favor the idea that the decrease in AT specificity observed for AMG when it is attached to poly(QEA) is caused by sterical influences of the bulky QEA residues on complex formation between the AMG residue and DNA.

Conclusions

From the very beginning of our experiments, we were convinced that it would be necessary to fulfill some basic requirements before DNA template mediated, abiotic polymerization processes could be expected to yield polymers complementary to sequences of the DNA template. (1) Monomers of different base-pair specificity must be available whose

binding specificities are not influenced by their attachment to a polymer backbone. (2) Monomers of opposite base-pair specificity should form complexes of similar stability to warrant their simultaneous binding to DNA. (3) All monomers should contain activated groups suitably located to enable the formation of covalent links between monomers without causing disturbance of their momentary DNA complexes. (4) The polymerization procedure should take place at room temperature for reasons of DNA stability and should proceed as quickly as possible to minimize spatial displacements of bound monomers during the polymerization process.

We were able to realize most of these requirements by synthesis of monomers on the basis of acrylamide derivatives. Throughout our presented experiments, the AT-specific malachite green and the GC-specific phenyl neutral red have been used as primary precursors for the synthesis of base-pair specific monomers. Some of them retained their base-pair specificity not only after modification by an acrylamide residue but also after their attachment to a polymer backbone of acrylamide or acrylamine (QEA). Furthermore, a set of monomers with structural differences but comparable binding parameters was synthesized since no definite predictions could be made for the spatial requirements of polymerization reactions of DNA-bound monomers.

APNR and AMPNR, for example, possess nearly identical α' and σ' values. Both monomers form intercalation complexes with DNA and unwind the DNA helix to a similar extent in their monomeric as well as in their polymeric state. This fact seemed important to us since the acrylamide residues of APNR and AMPNR should protrude into different grooves of the helix when bound to DNA. This steric difference should have consequences on the process of template polymerization. Since the specific binding of malachite green derivatives most probably will require a stereospecific binding of the dye in one of the two grooves, their copolymerization with APNR or AMPNR should give information about details of the polymerization process with respect to the optimal arrangement of these monomers on the surface of the DNA template. In the cases that AT- and GC-specific monomers are both bound in the same groove, they should give rise to a much faster polymerization process as compared to the situation in which the monomers are bound separately to different grooves. In a similar way, a series of different template polymerizations for a variety of monomer combinations and concentrations should help to elucidate the process of DNA template mediated polymerization processes on a molecular level.

Recently, template polymerizations have been done with APNR, AMG, and MA on DNA from either λ or T7 bacteriophage (Kosturko et al., 1979). By a special competition test, the polymers were shown to inhibit preferentially transcription directed by the DNA species used as the template for the synthesis of the polymer.

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