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Synthesis of an *N*-Methyl-*N*-nitrosourea Linked to a Methidium Chloride Analogue and Its Reactions with ^{32}P-End-Labeled DNA†

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ABSTRACT: The synthesis and characterization of an *N*-methyl-*N*-nitrosourea (MNU) analogue that is covalently linked to a methidium nucleus (**9**) is described. At 37 °C in pH 8.0 buffer **9** hydrolyzes via pseudo-first-order kinetics, with a calculated $t_{1/2}$ = 77 min. By use of polyacrylamide sequencing gels the formation of piperidine-labile N^7-methylguanine adducts from the reaction of **9** and MNU with 5′-^{32}P-end-labeled DNA restriction fragments is reported. DNA methylation by **9** in 10 mM Tris buffer is enhanced with increasing ionic strength (50–200 mM NaCl), which contrasts to the inhibition of MNU-induced cleavage with increasing salt. In addition, **9** methylates all G sites equally, while MNU shows a clear preference for d(G)$_n$ ($n \geq 3$) runs and an asymmetrical methylation pattern within these G-rich regions. The results are discussed in terms of the delivery of the MNU moiety to the DNA target by a non-sequence-specific intercalation process and the subsequent hydrolytic generation of a nondiffusible alkylating intermediate.

The mutagenic, carcinogenic, and antineoplastic activities of *N*-alkyl-*N*-nitrosoureas are associated with their covalent modification of DNA (Druckrey et al., 1967; Magee, 1976; Lawley, 1984; Preussmann & Stewart, 1984). The conversion of *N*-alkyl-*N*-nitrosoureas to electrophilic alkylating intermediates in aqueous solution at near-neutral pH entails an initial specific-base-catalyzed hydrolysis (Garrett et al., 1965; Synder & Stock, 1980) to a transient alkanediazotic acid (White & Woodcock, 1968; Moss, 1974) followed by ionization to the corresponding alkanediazonium ion (Huisgen & Rüchardt, 1956; Streitweiser & Schaeffer, 1957; Friedman, 1970; Kirmse, 1976; Southam & Whiting, 1982; Gold et al., 1984). Depending on the nature of the alkyl substituent, the diazonium ion directly (McGarrity & Smyth, 1980; Ford, 1986) or via a nitrogen-separated ion pair or triplet (White & Woodcock, 1968; Moss, 1974; Gold et al., 1984) alkylates DNA (Figure 1). The high bond dissociation energy for methanediazonium ion (Ford, 1986) and NMR studies on the protonation of diazomethane (McGarrity & Smith, 1980; McGarrity & Cox, 1983) indicate the involvement of methanediazonium ion in DNA alkylation by MNU.[1] We have previously observed that the MNU-mediated formation of N7-MeG is sequence specific, with d(G)$_3$ runs showing heavy methylation at the central G site (Wurdeman & Gold, 1988). In d(G)$_4$ sequences the asymmetrical methylation pattern is quite striking and distinctly different from that observed for DMS. In addition, DNA methylation was dose-dependently inhibited by the addition of NaCl (50–200 mM) or by the addition of micromolar concentrations of the cationic DNA affinity binders ethidium bromide, distamycin A, or spermine. This inhibition was independent of sequence, i.e., methylation at all G sites is equally reduced. The alkylation pattern was attributed to the electrostatic attraction between d(G) stretches and a positively charged alkylating agent. The inhibition results from decreased electrostatic ion pairing of the cationic methylating agent and the polyanionic DNA molecule (Perahia et al., 1979; Cauchy et al., 1980; Pullman & Pullman, 1981). Accordingly, methylation of DNA by DMS was not affected by increasing ionic strength or by the presence of the cationic DNA affinity binders (Wurdeman & Gold, 1988).

In order to understand the basis for the sequence specificity observed for MNU and to increase the yield of DNA methylation, an MNU analogue was covalently linked to a methidium chloride intercalator. Methylation of sequence-characterized ^{32}P-end-labeled DNA restriction fragments indicates that the preference of MNU for poly-d(G) runs and the inhibition of DNA methylation at high salt are overcome by the sequence- and salt-independent intercalation process.

Materials and Methods

Materials

Solvents. DMF was passed over a column of 4-Å molecular sieves and dried by sequential azeotropic distillation with benzene and reduced-pressure distillation from BaO. Chlorobenzene and nitrobenzene were dried over 3-Å molecular sieves and distilled from P_2O_5. DMSO was freshly distilled from CaH_2 after initial drying over 4-Å molecular sieves. *N*-Ethylmorpholine and ethylenediamine were dried over KOH, and 1,1′-carbonyldiimidazole was recrystallized from anhydrous THF.

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[1] Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; DMS, dimethyl sulfate; bp, base pair(s); DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-pressure liquid chromatography; *N*7-MeG, N^7-methylguanine; THF, tetrahydrofuran; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane.

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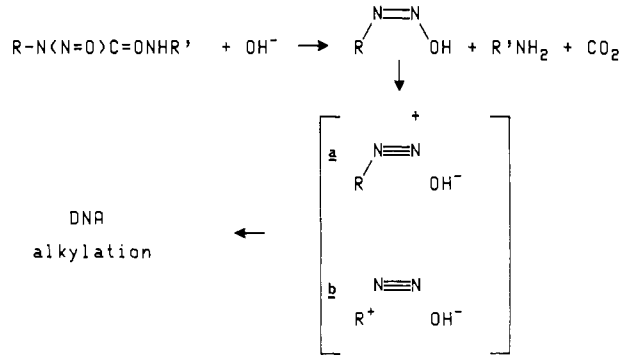

FIGURE 1: Pathway for the hydrolysis of *N*-alkyl-*N*-nitrosoureas to yield potential DNA-alkylating intermediates: (a) alkanediazonium ion; (b) nitrogen-separated ion pair.

Methods

NMR. NMR spectra were run on a Varian XL300, and homonuclear decoupling experiments (COSY) were used to assign peaks in ^{1}H NMR spectra.

Preparation of 2-Amino-4,4'-dinitrobiphenyl (**2**). A solution of 2-aminobiphenyl (**1**) (43.2 g, 0.25 mol) in 95% H_2SO_4 (500 mL, 8.92 mol) was cooled to 0 °C and stirred during the addition of powdered KNO_3 (52 g, 0.514 mol) so that the internal temperature remained <5 °C. After an additional 4.5 h at 0–5 °C, the reaction mixture was poured into 500 mL of ice–water. The solid was collected by filtration, washed with H_2O, dried, and crystallized from acetone to afford orange-yellow crystalline product (**2**). Yield 72%; mp 209.5–210.0 °C (acetone) (lit. mp 209 °C from 2-ethoxyethanol; Cymerman & Short, 1949); TLC (silica, $CHCl_3$) R_f = 0.60; IR (KBr disk) 3467, 3379, 1634, 1598, 1507, 1343 cm^{-1}; ^{1}H NMR (acetone-d_6) δ 7.27–8.41 (m, aryl and NH_2); UV (95% EtOH) λ_{max} (log ϵ) 238 (4.27), 261 (4.30), and 306 nm (4.19).

Preparation of 2-(p-Cyanobenzamido)-4,4'-dinitrobiphenyl (**3**). **2** (30.8 g, 0.119 mol) and *p*-cyanobenzoyl chloride (23.6 g, 0.140 mol) were heated in refluxing chlorobenzene (150 mL) for 1.5 h. After cooling, the reaction mixture was concentrated in vacuo and the resulting crude product crystallized from glacial acetic acid. Yield 93%; mp 246.0–246.5 °C; TLC (silica, $CHCl_3$) R_f = 0.51; IR (KBr disk) 3432, 2230, 1688, 1534, 1515, 1345 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.6–8.5 (m, 11 H, aryl), 10.6 (br s, 1 H, NH); UV (95% EtOH) λ_{max} (log ϵ) 240 (4.53), and 273 nm (4.48).

Preparation of 9-(p-Cyanophenyl)-2,7-dinitrophenanthridine (**4**). **3** (2.0 g, 5.1 mmol) and $POCl_3$ (0.62 mL, 6.8 mmol) in nitrobenzene (12 mL) were refluxed for 2.5 h. The solution was concentrated in vacuo and the residue triturated with boiling EtOH (50 mL) for 10 min. After filtration, the solid product was washed again with hot EtOH (50 mL). The product obtained was pure enough to be used in the next step. Yield 99%; mp 330.5–332.0 °C (2-ethoxyethanol); TLC (silica, $CHCl_3/CCl_4$, 1:1) R_f = 0.26; IR (KBr disk) 3104, 2233, 1615, 1590, 1514, 1343 cm^{-1}.

Preparation of 9-(p-Cyanophenyl)-2,7-dinitro-10-methylphenanthridinium Sulfate (**5**). **4** (1.0 g, 2.70 mmol) and DMS (1.7 g, 13.5 mmol) were heated at 180 °C in nitrobenzene (13 mL) for 1.5 h. The nitrobenzene was removed by steam distillation, and the residual aqueous yellow solution was filtered hot and concentrated in vacuo to yield dark yellow crystalline product (99% yield) which showed an indefinite melting–decomposition point. This material was used without further purification.

Preparation of 2,7-Diamino-9-(p-carbamoylphenyl)-10-methylphenanthridinium Chloride (**6**). A mixture of **5** (1.0 g, 2.36 mmol) and 1.3 M HCl (9 mL) was refluxed for 6 h with vigorous stirring. After cooling, H_2O (3.3 mL), EtOH (18.8 mL), and Fe (1.20 g, 21.5 mmol) were added in the order specified with vigorous stirring, and the resulting mixture was refluxed for 4 h. The hot mixture was filtered and the pH of the filtrate adjusted to 8.8 with concentrated NH_4OH. The colloidal precipitate was removed by filtration and the filtrate strongly acidified with concentrated HCl and then concentrated in vacuo to afford maroon crystalline material. This material was dissolved in a minimum volume of H_2O and applied to an Amberlite XAD-2 resin column (50 g) which was washed with H_2O (400 mL) to remove NH_4Cl. Subsequent elution with 30% aqueous MeOH yielded purple fractions which were concentrated and dried in vacuo to afford purple crystalline product. Yield 76%; mp 210–219 °C (dec); TLC (silica, 0.05% acetic acid in MeOH) R_f = 0.6; IR (KBr disk) 3301, 3185, 1619, 1605, 1492 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.65 (d, 1 H, J = 9.3 Hz, H_5), 8.62 (d, 1 H, J = 9.0 Hz, H_4), 8.27 (d, 2 H, J = 8.1 Hz, $H_{2'}$ and $H_{6'}$), 7.91 (d, 2 H, J = 8.1 Hz, $H_{3'}$ and $H_{5'}$), 7.55 (d, 1 H, J = 9.0 Hz, H_3), 7.37 (d, 1 H, J = 9.3 Hz, H_6), 7.35 (s, 2 H, $CONH_2$), 7.31 (s, 1 H, H_8), 6.25 (s, 1 H, H_1) 3.94 (s, 3 H, CH_3); UV (H_2O) λ_{max} 286, 323, and 490 nm (EtOH), 295, 335 and 540 nm.

Preparation of 2,7-Diamino-9-(p-carboxyphenyl)-10-methylphenanthridinium Chloride (**7**). A solution of **6** (2.3 g, 6.1 mmol) in 75% H_2SO_4 (20 mL) was heated at 130 °C for 2 h. After cooling, the reaction mixture was poured onto crushed ice, adjusted to pH 8.3 with NH_4OH, and then strongly acidified with concentrated HCl, and the volume of the solution was reduced in vacuo. The concentrated solution was then applied to an Amberlite XAD-2 column (110 g) which was washed with H_2O (1.3 L) to remove NH_4Cl and $(NH_4)_2SO_4$. Subsequent elution with 50% aqueous MeOH yielded red fractions which were concentrated and dried in vacuo to afford a purple solid. Yield 59%; mp 245–253 °C; TLC (silica, EtOAc/MeOH, 3:2) R_f = 0.48; IR (KBr disk) 3325, 3209, 3340–2500, 1622 (shifts to 1700 upon acidification), 1619, 1544, 1492, 1375, 1260 cm^{-1}; ^{1}H NMR (D_2O) δ 8.43 (d, 1 H, J = 9 Hz, H_5), 8.38 (d, 1 H, J = 9 Hz, H_4), 8.18 (d, 2 H, J = 8 Hz, $H_{2'}$ and $H_{6'}$), 7.55 (d, 2 H, J = 8 Hz, $H_{3'}$ and $H_{5'}$), 7.52 (d, 1 H, J = 9 Hz, H_3), 7.4 (d, 1 H, J = 9 Hz, H_6), 7.37 (d, 1 H, J = 2 Hz, H_8), 6.66 (s, 1 H, H_1), 4.08 (s, 3 H, CH_3); ^{1}H NMR (DMSO-d_6) δ 8.64 (d, 1 H, J = 9 Hz, H_5) 8.60 (d, 1 H, J = 9 Hz, H_4), 8.15 (d, 2 H, J = 8.4 Hz, $H_{2'}$ and $H_{6'}$), 7.54 (d, 2 H, J = 8.4 Hz, $H_{3'}$ and $H_{5'}$), 7.50 (d, 1 H, J = 9 Hz, H_3), 7.31 (d, 1 H, J = 9 Hz, H_6), 7.27 (s, 1 H, H_8), 6.40 (s, NH_2), 5.96 (s, 1 H, H_1) 3.99 (s, 3 H, CH_3); ^{13}C NMR (D_2O + HCl) δ 171.70 (COOH), 167.14 (C=N$^+$), 138.59, 137.36, 136.88, 135.86, 135.06, 134.58, 133.40, 132.01, 130.18, 129.62, 128.53, 128.45, 128.39, 128.01, 117.55, 46.70 ($^+$NCH$_3$); UV (MeOH) λ_{max} (log ϵ) 292 (4.72), 330 (sh), and 520 nm (3.74).

Preparation of 2,7-Diamino-9-[p-[(2-aminoethyl)carbamoyl]phenyl]-10-methylphenanthridinium Chloride (**8**). **7** (208 mg, 0.548 mmol of the free base) was dissolved in 20 mL of concentrated HCl, and after stirring for 10 min at room temperature, the volume was reduced in vacuo. Benzene and anhydrous EtOH (to aid in removal of H_2O) were added to the residue, and the mixture was concentrated to dryness. The purple solid obtained was thoroughly dried in vacuo (>24 h) over P_2O_5. The HCl salt was dissolved in anhydrous DMSO (10 mL), and *N*-ethylmorpholine (65.9 mg) was added under an argon atmosphere. 1,1'-Carbonyldiimidazole (96.6 mg, 0.596 mmol) in 1.6 mL of dry DMSO was then added at room temperature, and the solution was stirred for 1 h. The contents

of the flask were transferred slowly via a Teflon cannula to a solution of ethylenediamine (400 mg, 6.66 mmol) in 0.4 mL of dry DMSO under argon. Stirring was maintained for 24 h, followed by concentration in vacuo at 50 °C to yield a purple solid which was dissolved in a minimum volume of MeOH. The product was precipitated with dry ether (30 mL), collected by filtration, and washed with ether to remove residual imidazole. This crude product was flash chromatographed on silica gel with MeOH and then with acidic MeOH [0.1% (v/v) acetyl chloride in anhydrous methanol]. The acidic MeOH eluent was concentrated and dried in vacuo at room temperature (>48 h) over P_2O_5 to yield product. Yield 235 mg (97%); indefinite melting–decomposition point >300 °C; paper chromatography (1-BuOH/AcOH/H_2O, 4:1.3:2.5 v/v) R_f = 0.57; IR (KBr disk) 3332, 3209, 3000, 2962, 2500, 2000, 1624, 1538, 1424 cm^{-1} (acidification of the product produced a characteristic strong NH_3^+ band between 3200 and 2100 cm^{-1}); ^1H NMR (D_2O) δ 8.16–6.38 (m, 10 H, aryl), 3.90 (s, 3 H, CH_3), 3.87 (m, 2 H, J = 6.3 Hz, $CONHCH_2$), 3.41 (t, 2 H, J = 6 Hz, NH_2CH_2); ^{13}C NMR (D_2O) δ 172.92 (CONH), 160.54 ($C{=}N^+$), 152.73, 149.36, 138.06, 137.13, 132.04, 131.04, 130.88, 130.45, 126.74, 126.40, 122.92, 120.34, 113.05, 102.04, 102.54, 45.16 (CH_3), 42.06 ($CONHCH_2$), 40.36 (CH_2NH_2); UV (MeOH) λ_{max} (log ϵ) 293 (4.71), 330 (sh), and 525 nm (3.75).

Preparation of 2,7-Diamino-9-[p-[[2-[(N-nitroso-N-methylcarbamoyl)amino]ethyl]carbamoyl]phenyl]-10-methylphenathridinium Chloride (**9**). To a cold solution (−5 °C) of *N*-nitroso-*N*-methylcarbamic acid *N*-succinimidyl ester (Martinez et al., 1982) (64 mg, 0.274 mmol) in anhydrous DMF (17 mL) under an argon atmosphere was slowly added a mixture of **8** (105 mg, 0.229 mmol) and ethyldiisopropylamine (98 mg, 0.744 mmol) in dry DMF (17 mL) via a Teflon cannula. After the solution was stirred for 7 h at ~−5 °C, the reaction mixture was concentrated in vacuo at 35 °C. The residue was dissolved in a minimum of volume of MeOH (~3 mL), and then 35 mL of EtOAc was added with stirring to precipitate the product. The stirring was continued for 30 min at room temperature to completely extract the *N*-hydroxysuccinimide byproduct impurity from the precipitate. The purple product was collected by filtration and dried in vacuo over P_2O_5 at room temperature for >30 h. Yield 79%; indefinite melting–decomposition point at >235 °C; paper chromatography (1-BuOH/AcOH/H_2O, 4:1:2.5 v/v) R_f = 0.71; IR (KBr disk) 3393, 3332, 3209, 3103, 2962, 1717, 1628, 1539, 1492, 1464 cm^{-1}; ^1H NMR (CD_3OD) δ 8.60 (d, 1 H, J = 9.0 Hz, H_5), 8.55 (d, 1 H, J = 9.0 Hz, H_4), 8.18 (d, 2 H, J = 8.1 Hz, $H_{2'}$ and $H_{6'}$), 7.67 (d, 2 H, J = 8.1 Hz, $H_{3'}$ and $H_{5'}$), 7.54 (d–d, 1 H, J = 9.0 and 2.4 Hz, H_3), 7.38 (d–d, 1 H, J = 9.0 and 1.8 Hz, H_6), 7.36 (d, 1 H, J = 1.8 Hz, H_8), 6.50 (d, 1 H, J = 2.4 Hz, H_1), 4.11 (s, 3 H, $^+NCH_3$), 3.71 (s, 4 H, CH_2CH_2), 3.15 (s, 3 H, CH_3); ^{13}C NMR (MeOH-d_4) δ 169.27 (ArCONH), 159.54 ($C{=}N^+$), 155.99 (HNCON), 152.92, 149.52, 138.17, 137.83, 136.70, 130.47, 130.12, 129.59, 129.43, 126.25, 125.74, 123.87, 121.54, 119.30, 110.06, 99.60, 43.18 ($^+NCH_3$), 41.44 and 41.08 ($CONHCH_2$'s), 26.89 (CH_3); UV (MeOH) λ_{max} (log ϵ) 294 (4.68), 330 (sh), and 528 nm (3.76); MS (FAB, Xe) m/z (rel abundances, assignment) 472 (9, M − Cl), 443 (11, M − NOCl + 1), 412 (27, methidium isocyanate).

Stability of **9**. A 2 mM solution of **9** in 10% DMSO–90% 50 mM sodium phosphate buffer (pH 8.1) was maintained at 37 °C. Aliquots were periodically removed, and the amount of the unreacted **9** was determined by HPLC [column, Waters Nova Pak C_{18}, 4 μm; solvent, 23 mM K_2HPO_4/NaH_2PO_4 buffer (pH 6.8) in aqueous MeOH (buffer/MeOH, 64:36 v/v); flow rate, 1 mL/min; detection, 290 nm]. Retention time of **9** is 5.7 min. Good pseudo-first-order rate plots were obtained ($r^2 > 0.998$) with calculated $t_{1/2}$ of 76.8 ± 0.5 min. The $t_{1/2}$'s of MNU and *N*-methyl-*N'*-cyclohexyl-*N*-nitrosourea under identical conditions were determined to be 3.3 and 80 min, respectively.

Preparation of 5'-^{32}P-End-Labeled DNA Restriction Fragments. Two 5'-^{32}P-end-labeled DNA fragments were isolated from a 3220 bp DNA clone from the promotor region of the coat protein gene of the canine parvovirus (Rhode, 1985) by initial restriction with *Nco*I, treatment with calf intestine alkaline phosphatase, phosphorylation with T4 kinase in the presence of [γ-^{32}P]ATP, and *Hin*dIII digestion using methods previously detailed (Maxam & Gilbert, 1980). Two 5'-^{32}P-end-labeled fragments (576 and 85 bp) were isolated by gel purification.

Reactions of MNU and **9** *with ^{32}P-End-Labeled DNA Fragments*. The restriction fragment (80 000–100 000 cpm) was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing the desired concentration of NaCl or cationic DNA affinity binder and then incubated with freshly prepared solutions of MNU or **9** for 2 h at 37 °C. The reactions were terminated and the DNA was purified by precipitation with NaOAc, washing with cold EtOH, and drying in vacuo. The DNA was then treated with 1 M piperidine at 90 °C for 20 min to convert N7-MeG adducts into single-strand breaks (Maxam & Gilbert, 1980; D'Andrea & Haseltine, 1978). The piperidine was then removed in vacuo.

Analysis of DNA for Single-Strand Breaks. The reacted DNA was suspended in loading buffer containing 80% deionized formamide, 50 mM Tris–borate (pH 8.3), 1 mM EDTA, 0.1% xylenecyanol, and 0.1% bromphenol blue, denatured at 90 °C for 1 min, and then cooled in ice. The DNA (30 000 cpm/lane) was loaded onto a 12% polyacrylamide, 7.8 M urea denaturing gel which was run at 65 W.

RESULTS

Synthesis. The synthesis of compounds **2**–**7** was carried out as previously described (Hertzberg & Dervan, 1982; Hertzberg & Dervan, 1984; Basak & Dugas, 1986) with some modifications that are detailed under Materials and Methods (Figure 2). **7**·2HCl, obtained in 41% yield from **2**, was then sequentially treated with *N*-ethylmorpholine, 1,1'-carbonyldiimidazole, and ethylenediamine in anhydrous DMSO. After precipitation of the crude product and chromatographic purification, **8** (free amine) was obtained in 97% yield. The HCl salt of **8** was reacted in DMF with *N*-nitroso-*N*-methylcarbamic acid *N*-succinimidyl ester (Martinez et al., 1982) in the presence of Hunig's base until no **8** could be detected by paper chromatography. Crude **9** was obtained by precipitation, and the contaminating *N*-hydroxysuccinimide was removed by exhaustive EtOAc extraction. The yield of **9** was 79%, and its homogeneity was established by reverse-phase HPLC analysis. The overall yield of **9** from **2** was 31%. The FAB-MS (Xe) of **9** does not show a parent ion, but M^+ − Cl and M^+ − NOCl fragments are observed. Another major ion results from formal loss of a CH_4N_2O (methanediazotic acid) fragment to yield an isocyanate residue.

Stability Studies. The hydrolytic stability of **9** was evaluated at 37 °C in 50 mM sodium phosphate buffer (pH 8.1) by using an HPLC assay to monitor the loss of *N*-nitrosourea starting material. It was observed that as the disappearance of starting material progressed, a peak appeared close to the solvent front that is not amine **8**. Although this peak was not characterized, the production of the more polar isocyanate

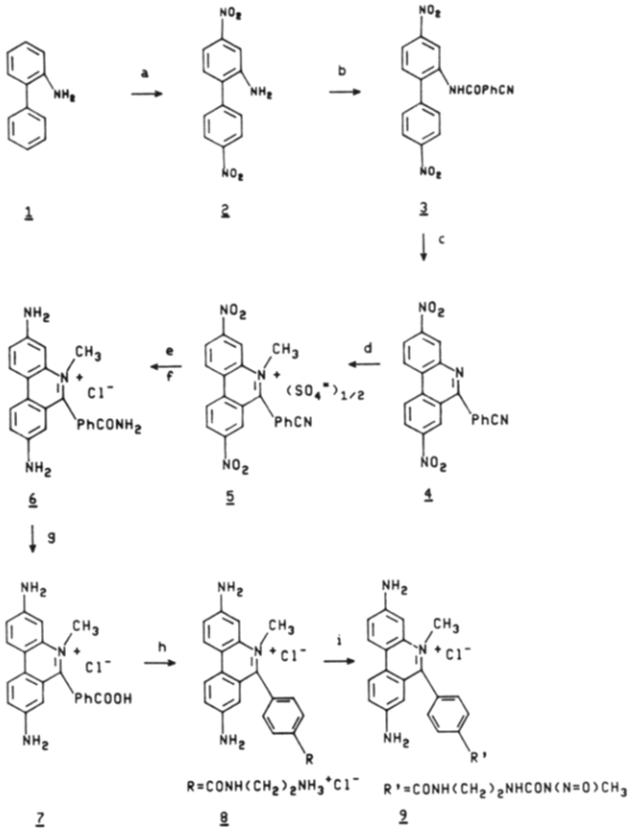

FIGURE 2: Reactions: (a) KNO_3, H_2SO_4; (b) *p*-(N≡C)C_6H_4COCl; (c) $POCl_3$; (d) $(CH_3)_2SO_4$; (e) HCl; (f) Fe, HCl; (g) 75% H_2SO_4; (h) 1,1′-carbonyldiimidazole, *N*-ethylmorpholine, $H_2N(CH_2)_2NH_2$; (i) *N*-nitroso-*N*-methylcarbamic acid *N*-succinimidyl ester, $CH_3CH_2N[CH(CH_3)_2]_2$.

from the base-catalyzed hydrolysis of the **9** is anticipated (Werner, 1919; Boivin & Boivin, 1951; Synder & Stock, 1980). A linear pseudo-first-order plot (correlation coefficient > 0.999) for the hydrolysis of **9** was obtained with a calculated $t_{1/2}$ of 76.8 ± 0.5 min from three determinations. This $t_{1/2}$ is characteristic of similarly substituted *N*-methyl-*N'*-alkyl-*N*-nitrosoureas (Garrett et al., 1965) and is not influenced by the methidium group. The $t_{1/2}$ of *N*-methyl-*N'*-cyclohexyl-*N*-nitrosourea is ~80 min under the same conditions, while MNU has a $t_{1/2}$ of ~3 min.

The strategy employed in the synthesis of **9** allows for easy control of the *N*-alkyl substituent, and the unstable *N*-nitrosourea functionality is introduced in the final step of the synthesis. This is particularly attractive for incorporating radiolabeled R groups into the *N*-alkyl position of **9**.

Analysis of ^{32}P-End-Labeled DNA Methylation Reactions. The DNA alkylation reactions were run in pH 8.0 buffer at 37 °C for 2 h. The measured $t_{1/2}$'s of **9** and MNU under these conditions are 77 and 3 min, respectively. Comparison of the two compounds, in terms of the production of N7-MeG lesions (Figure 3 for fragment sequences), requires a modest correction for differences in the generation of reactive methylating intermediate. Approximately 100% of the MNU will hydrolyze during this time period, but ~28% of **9** will remain when the reaction is terminated. The analysis of the polyacrylamide gels (Figures 4–6) shows a linear inverse relationship between the ionic strength (0–200 mM NaCl) and MNU-mediated strand cleavage. The addition of positively charged DNA affinity binders, including ethidium bromide, distamycin A_2, and spermine, has a similar inhibitory effect, although at much lower concentrations (Wurdeman & Gold, 1988). The cleavage of DNA by **9** was minimal in 10 mM

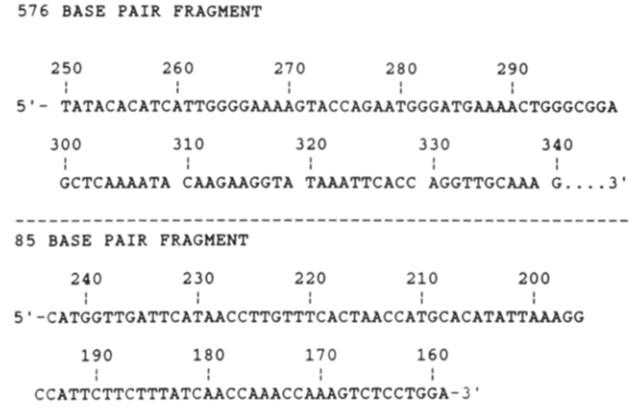

FIGURE 3: DNA sequences of the 576 and 85 bp restriction fragments (Rhode, 1985).

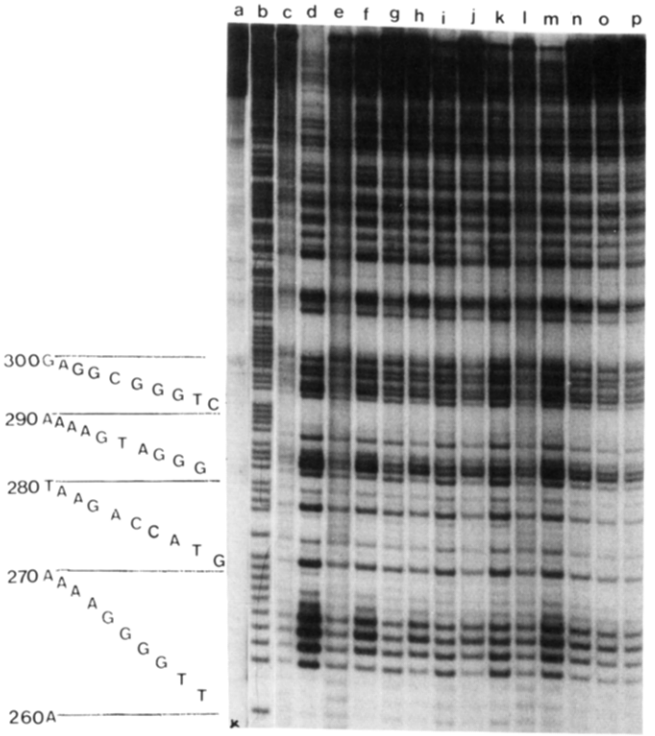

FIGURE 4: Autoradiogram of 12% polyacrylamide gel (65 W) used to map 1 M piperidine induced (heated at 90 °C for 20 min) single-strand breaks in 576 bp fragment caused by N7-guanine methylation by MNU or **9**. The 5′-^{32}P-end-labeled restriction fragment was incubated with MNU or **9** at 37 °C for 2 h in Tris-HCl buffer (pH 8.0); lane a, control; lane b, Maxam–Gilbert G + A; lanes c, e, g, i, and k, 1 mM **9** with 0, 50, 100, 150, and 200 mM NaCl, respectively; lanes d, f, h, j, and l, 1 mM MNU with 0, 50, 100, 150, and 200 mM NaCl, respectively; lanes m, n, o, and p, 1 mM MNU, 1 μM ethidium bromide and 0, 50, 100, and 200 mM NaCl, respectively.

Tris buffer in the absence of added salt (Figure 4, lane c; Figure 5, lane d). However, a salt-related (from 50 to 200 mM NaCl) increase in DNA cleavage by **9** is evident from the sequencing gels (Figure 4, lanes e, g, i, k; Figure 5, lanes f, h, j, l). Comparison of the ratio of MNU- to **9**-mediated cleavage (based on densitometry quantitation) at 0 vs 200 mM NaCl shows >12-fold enhancement. In contrast, attempts to prevent the ethidium bromide inhibition of MNU alkylation by using increasing NaCl were unsuccessful (Figure 4, lanes n–p; Figure 5, lanes o–q). The methylation of DNA by 1 mM **9** in the presence of 200 mM NaCl was inhibited by the addition of ethidium bromide (Figure 6), and the extent of inhibition at equal concentrations suggests that **9** may have

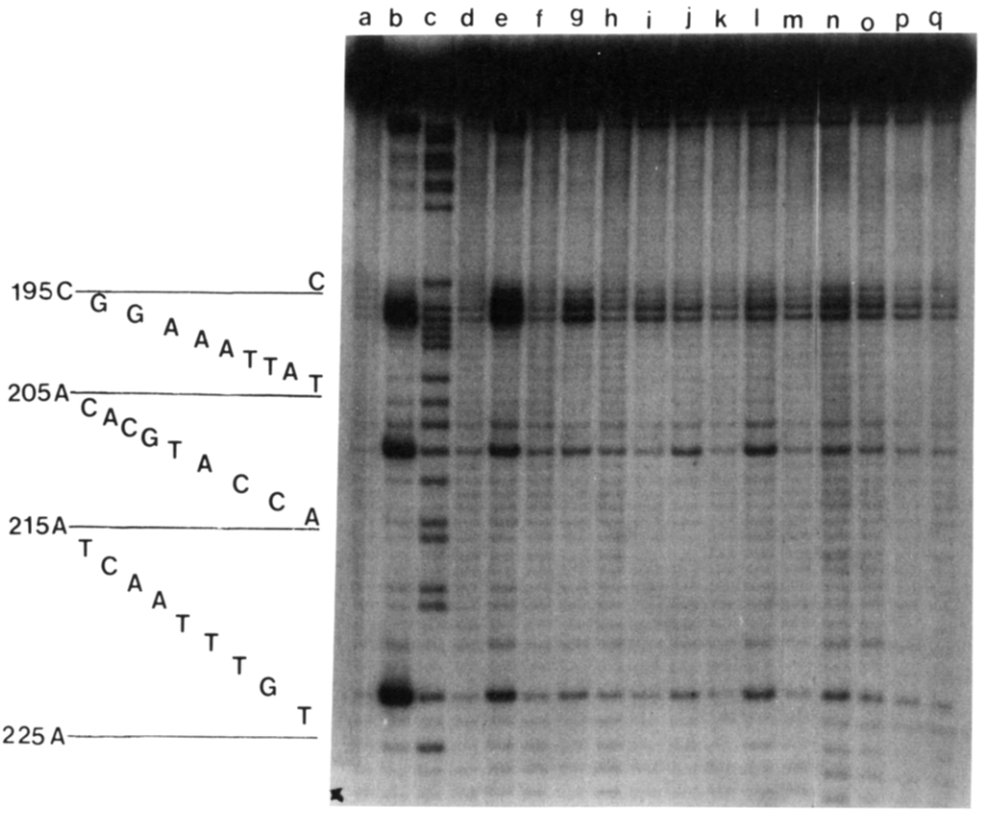

FIGURE 5: Autoradiogram of 12% polyacrylamide gel (65 W) used to map 1 M piperidine induced (heated at 90 °C for 20 min) single-strand breaks in the 85 bp fragment caused by N7-MeG formation from MNU or **9**. The 5′-^{32}P-end-labeled restriction fragment was incubated with MNU or **9** at 37 °C for 2 h in Tris-HCl buffer (pH 8.0): lane a, control; lane b, Maxam–Gilbert G; lane c, Maxam–Gilbert G + A; lanes d, f, h, j, and l, 1 mM **9** with 0, 50, 100, 150, and 200 mM NaCl, respectively; lanes e, g, i, k, and m, 1 mM MNU with 0, 50, 100, 150, and 200 mM NaCl, respectively; lanes n, o, p, and q, 1 mM MNU and 1 μM ethidium bromide with 0, 50, 100, and 200 mM NaCl, respectively.

a similar affinity for DNA as the unmodified intercalator. In all cases the electrophoretic mobility of the G-cleavage bands induced by MNU and **9** line up with those generated in the Maxam–Gilbert G lane, indicating that the cleavage sites bear a 3′-phosphate terminus.

Discussion

Effect of Ionic Strength on DNA Methylation. The sequence-independent inhibitory action of increasing salt concentration on N7-MeG reported here for MNU (Figures 3–5) is consistent with previous observations made by several investigators, which show that the formation of DNA adducts at 3-, 7-, and O^6-G, 3-A, O^2-T, and the phosphate backbone are equally diminished at high ionic strength (Kriek & Emmelot, 1963; McCalla, 1968; Jensen & Reed, 1978; Briscoe & Cotter, 1985). It is important to point out that the ratio of DNA adducts formed is not influenced by salt. However, it is not certain that adduction of bases within different sequences may not be dissimilarly inhibited by changes in ionic strength. Previously, it has been shown that, in 10 mM Tris buffer with no added salt, a 50% inhibition of 2 mM [^{14}C]-MNU methylation of 600 μM rat liver chromatin at O^6- and N7-G and at N3-A was realized by the addition of 25 μM spermine and 100 μM distamycin A (Rajalakshmi et al., 1978). No similar inhibition for the methylation of 5′-deoxyguanylic acid was found.

The role of increased ionic strength on the reduction of N7-MeG from MNU has been reported (Wurdeman & Gold, 1988). On the basis of the inhibitory effect of NaCl and the cationic DNA affinity binders and the failure of the same agents to inhibit the DMS-mediated formation of the same N7-MeG adduct (Wurdeman & Gold, 1988), we have proposed that the salt effect does not result from a change in the MNU-derived alkylating intermediate, or from a conformation-dependent decrease in the accessibility of nucleophilic DNA sites. It would appear that a non-sequence-related modulation of the electrostatic attraction between the positively charged methanediazonium ion and the polyanionic DNA is responsible for the diminution in yield of all N7-MeG lesions at higher salt concentration. A localized increased electrostatic potential in oligo-d(G) runs, as previously proposed (Pullman & Pullman, 1981; Furois-Corbin & Pullman, 1985), may cause the high methylation specificity for such sequences. That these dG runs are not particularly strong targets for DMS is consistent with the above hypothesis.

It is interesting to note the differences in the N7-G methylation profile within these poly-d(G) regions with different alkylating agents. Thus, the 2-chloroethylating agents, *N*-(2-chloroethyl)-*N*′-(*cis*-2-hydroxycyclohexyl)-*N*-nitrosourea and mitozolomide, which are thought to alkylate via a common intermediate, heavily methylate to an even extent the two central G sites in a G_4 run (Hartley et al., 1986). Other 2-chloroethylating agents show the same preference for the oligo-d(G) runs, but the alkylation profiles vary (Gibson et al., 1985; Mattes et al., 1986). DMS, the S_N2 alkylating agent that is routinely used to generate the Maxam–Gilbert G lane, heavily methylates G_{263}–G_{265} (see Figure 3 for sequence), while barely cleaving the G_{266} at the 3′-terminus of the G_4 stretch (Figure 6, lane b). MNU most strongly alkylates G_{265}, with G_{266} and G_{264} being of equal intensity (Figure 4, lane d). In contrast to DMS, the 5′-G in the run is weakly methylated. It has been suggested that the localization of charge on the alkylating intermediate may correlate with the cleavage profile (Gibson et al., 1985; Hartley et al., 1986). While more alkylating substrates need to be studied before the mechanism can be resolved, it is possible that these patterns may eventually

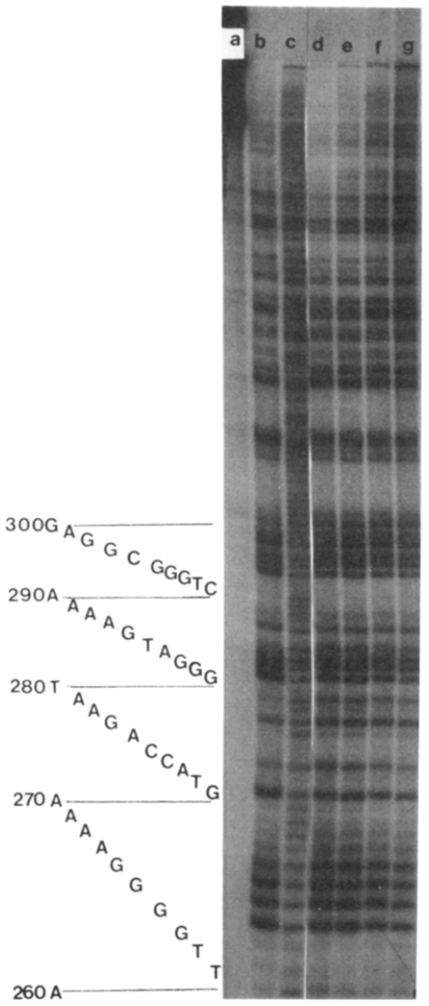

FIGURE 6: Autoradiogram of 12% polyacrylamide gel (65 W) used to map 1 M piperidine induced (heated at 90 °C for 20 min) single-strand breaks caused by N7-guanine methylation by **9**. The 5′-^{32}P-end-labeled 576 bp restriction fragment was incubated with **9** and ethidium bromide at 37 °C for 2 h in Tris-HCl buffer (pH 8.0): lane a, control; lane b, Maxam–Gilbert G; lane c, Maxam–Gilbert G + A; lanes d, e, f, and g, 1 mM **9** and 200 mM NaCl with 0.01, 0.10, 0.50, and 1.00 mM ethidium bromide, respectively.

provide useful fingerprints of the reactive agents responsible for the alkylation reactions, as well as providing some insight into the relationship between DNA sequence and electronic structure of the bases.

Methylation of DNA by **9**. DNA-recognizing molecules that abet specific chemistry on DNA are extensively utilized in nature (Hecht, 1979; Berman & Young, 1981; Feigon et al., 1984; Hurley & Needham-VanDevanter, 1986; Neidle et al., 1987) and are currently of much synthetic interest (Wilson et al., 1985; Barton et al., 1986; Bowler & Lippard, 1986; Dervan, 1986; Kissinger et al., 1987). Accordingly, **9** was synthesized so that the DNA affinity binding properties of the methidium cation could be used to efficiently deliver the *N*-methyl-*N*-nitrosourea moiety to DNA, where it would be converted to methanediazonium ion, the ultimate electrophilic methylating agent. For this strategy to be effective, it is necessary that the initial hydrolysis product of **9**, methanediazotic acid, be converted to the diazonium ion which in turn reacts with DNA at, or near, diffusion-controlled rates. If this criterion is not met, **9** should alkylate akin to MNU. We have previously reported that *N*-alkyl-*N*-nitrosocarbamates are suicide inhibitors of porcine liver esterase because of their hydrolysis to the corresponding alkanediazotic acid in, and rapid reaction with, the active site of the enzyme (Gold & Linder, 1979). A similar result has been reported for the inhibition of chymotrypsin by *N*-alkylnitrosamides (White et al., 1981). Both types of *N*-nitroso compounds hydrolyze to the same alkanediazotic acid (Gold & Linder, 1979). Therefore, it appears that the requirement for extremely rapid rates of alkylation should be met. Another factor that will control the efficiency of DNA methylation is the structure of the **9**–DNA complex, since two potential intercalated orientations are possible. Crystallographic data on ethidium complexes with 5′-iodocytidylyl(3′–5′)guanosine (Jain et al., 1977) and 5′-iodocytidylyl(3′–5′)adenosine (Tsai et al., 1977) define the phenyl and ethyl groups of ethidium as residing in the minor groove of the double helix. If the same orientation holds for **9**, then the *N*-nitrosourea side chain may also protrude into the minor groove. The major site for alkylation by MNU is N7-G, which is the lesion monitored in the sequencing gels, and this site faces into the major groove. Therefore, the delivery of alkylating agent to the major groove from affinity-bound **9** may not be optimized. In spite of this potential shortcoming, the sequencing gels (Figures 4–6) demonstrate the enhanced methylation by **9** at physiological ionic strength. The poor alkylation at low salt is not unexpected on the basis of the different possible competing modes of ethidium binding. Ethidium and related analogues are generally characterized as enthalpy-driven intercalating molecules, although entropy-driven external or preintercalation binding predominates at low salt and high drug concentrations (LePecq & Paoletti, 1967; Zimmermann, 1986; Hopkins & Wilson, 1987). Assuming **9** and ethidium bind by similar mechanisms, at low ionic strength much of **9** will be bound to DNA in a nonintercalated complex. This is a reasonable assumption based on binding constants and DNA unwinding of para-substituted ethidium analogues (LePecq & Paoletti, 1967; Wang, 1974; Dervan & Becker, 1978; Jacquemin-Sablon et al., 1979; Hertzberg & Dervan, 1982, 1984). The decreased DNA alkylation at low salt suggests that the geometry of nonintercalated DNA-affinity-bound **9** is inefficient at delivering the alkylating intermediate to the N7-G site. On the basis of studies with ethidium, the affinity binding of **9** assumes a classical intercalation geometry at high salt. The alkylation of DNA at high salt is, therefore, consistent with the delivery of alkylating agent by predominantly intercalated **9**. It is of interest that although the cleavage by 1 mM **9** at 200 mM NaCl is >4-fold higher than that observed for 1 mM MNU, after correction for hydrolysis rates, the DNA cleavage by 1 mM MNU at low ionic strength (0 NaCl) is still higher than that for **9** at high salt (200 mM NaCl). Therefore, the electrostatic ion pairing between free alkylating intermediate and DNA at low salt directs higher concentrations of reactive alkylating agent to DNA than is possible by an intercalation process.

The cleavage pattern induced by **9** is unique from that for MNU, DMS, and other DNA alkylating carcinogens (Muench et al., 1983; Sage & Haseltine, 1984; Gibson et al., 1985; Mattes et al., 1986; Hartley et al., 1986) in that the intensity of the individual G-cleavage sites is uniform throughout the fragments (Figures 4–6); there is no preference for poly-d(G) stretches. Qualitatively, the alkylation pattern for MNU is not changed by the addition of ethidium, which implies that the sequence-independent methylation pattern shown by **9** is not a consequence of conformational changes resulting from DNA unwinding. These observations further support the central role in DNA methylation by **9** for a non-sequence-specific intercalation of the methidium nucleus at physiological ionic strength. Accordingly, ethidium inhibits DNA meth-

ylation by **9** at 200 mM NaCl (Figure 6). The nonspecific methylation pattern also substantiates the nondiffusible nature of alkylating intermediate generated from **9** (Gold & Linder, 1979; White et al., 1981), since a diffusible species would undoubtedly be able to migrate within the confines of the major groove of the $d(G)_4$ run to give a pattern more similar to that of MNU. This raises a question about the predicted positioning of the nitrosourea functionality of **9** in the minor groove during intercalation, since this mode of binding would require the diffusion of the alkanediazonium to the major groove from its site of formation in the minor groove.

Finally, it is worth noting that **9** does make an excellent G-specific cleavage reagent that provides, unlike DMS, an unequivocal G lane.

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Registry No. **1**, 90-41-5; **2**, 51787-75-8; **3**, 102387-17-7; **4**, 66442-93-1; **5**, 116375-32-7; **6**, 116375-33-8; **7**, 52671-19-9; **8**, 116375-34-9; **9**, 116405-70-0; MNU, 684-93-5; N7-MeG, 578-76-7.

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Kinetics of Adenosine 5′-Triphosphate and Adenosine 5′-Diphosphate Interaction with G-Actin

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ABSTRACT: Double mixing experiments using a three-syringe stopped-flow apparatus have given values of the second-order rate constants for association of ϵATP, ATP, and ADP to G-actin of 6.8×10^6 M^{-1} s^{-1}, 6.1×10^6 M^{-1} s^{-1}, and 6.3×10^6 M^{-1} s^{-1}, respectively, at pH 7.6, 20 °C, and 0.65 mM free Ca^{2+}. The previously established ca. 100-fold weaker binding of ADP than ATP to G-actin is due to a much faster dissociation rate of ADP than ATP, rather than to a slower association rate as was previously reported. This difference between ADP and ATP largely disappears under more nearly physiological conditions (0.8 mM Mg^{2+} and 100 mM KCl). Association rate constants for the three nucleotides under these conditions are 2.13×10^6 M^{-1} s^{-1}, 1.1×10^6 M^{-1} s^{-1}, and 1.2×10^6 M^{-1} s^{-1}, respectively, for ϵATP, ATP, and ADP. The rate constant for association of ϵATP is only slightly affected by reducing the Mg^{2+} concentration from 0.8 to 0.2 mM, whereas that for ADP association is reduced by a factor of ca. 3. This, together with the observed increase in the apparent association rate constant of ϵATP on increasing the Ca^{2+} concentration in the 10–100 μM range, suggests that G-actin recognizes and binds the metal–nucleotide complexes.

The rate constants for the interaction of ATP and ADP with G-actin are important determining factors in the behavior of the G-actin–F-actin system, particularly with respect to the treadmilling properties of actin filaments. This phenomenon has received much attention in recent years, but it appeared to us that some of the kinetic parameters had not been well characterized. We have therefore undertaken a more detailed investigation of the association and dissociation kinetics of of the G-actin–ATP and G-actin–ADP complexes. The results obtained indicate that previously reported values for the association and dissociation rate constants for ADP at low ionic strength are incorrect and that divalent metal ion complexes of nucleotides are recognized by actin in the binding reaction.

Materials and Methods

Protein Preparations. Actin preparations from rabbit skeletal muscle were obtained as described earlier (Drabikowski & Nowak, 1973). Final ATP–G-actin solutions in 0.2 mM ATP, 0.2 mM $CaCl_2$, and 2 mM Hepes buffer, pH 7.6, were kept at 0 °C for no longer than 1 week.

To obtain ϵATP–G-actin,[1] the ATP–G-actin was polymerized with 0.1 M KCl; F-actin was collected by ultracentrifugation and then depolymerized by homogenization in a Teflon/glass homogenizer in a solution of 0.2 mM ϵATP, 0.2 mM $CaCl_2$, and 2 mM Hepes buffer, pH 7.6, followed by a 24-h dialysis against the same buffer solution.

Fluorescence Measurements. These were carried out essentially as previously described (Nowak et al., 1988) with either an SLM 8000 fluorescence spectrophotometer or, where high time resolution was required, a Durrum D-132 three-syringe rapid-mixing apparatus equipped with a D-137 dual detector unit.

Other Procedures. Actin concentration was determined spectrophotometrically at 290 nm with an absorption coefficient of 0.63 $mg \cdot mL^{-1} \cdot cm^{-1}$ (Houk & Ue, 1974). Molar actin concentrations were calculated with the value of M_r 42 000 for G-actin (Elzinga, 1973).

The concentration of ϵATP was determined at 265 nm with an absorption coefficient of 5700 $M^{-1} \cdot cm^{-1}$ (Secrist et al., 1972).

Reagents. ϵATP was prepared by the method of Secrist et al. (1972) and separated from unreacted ATP by chromatography on a QEAE-Sephadex G-50 column. ATP (disodium salt) was purchased from Pharma-Waldhof (Düsseldorf). ADP (Pharma-Waldhof) was purified prior to use if necessary as previously described (Nowak et al., 1988). Hepes was a

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[1] Abbreviation: ϵATP, $1,N^6$-ethenoadenosine 5′-triphosphate.

0006-2960/88/0427-8613$01.50/0