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THE REACTION OF THE CARCINOGEN *N*-ACETOXY-2-ACETYL-AMINOFLUORENE WITH DNA AND OTHER POLYNUCLEOTIDES AND ITS STEREOCHEMICAL IMPLICATIONS

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SUMMARY

The reaction of the carcinogen, *N*-acetoxy-2-acetylaminofluorene (AAAF) with guanosine to give 8-(*N*-2-fluorenylacetomido)guanosine has been used to test the stereochemical hypothesis that purine nucleoside residues in base-paired double helices are sterically hindered at C-8. Native DNA reacts rapidly with AAAF and with a velocity considerably greater than that of synthetic ribo- and deoxyribopolynucleotide complexes. Thus it appears that the *anti* conformation of the nucleosides in duplex DNA does not prevent reaction at C-8. The results suggest that considerable deformation of the DNA duplex is possible, particularly of the tortional angle about the glycosyl bond. This dynamic property may be unique to DNA. The reaction of AAAF with polynucleotides cannot be used as a measure of strandedness and the reaction does not serve to test the stereochemical hypothesis properly.

The reaction of AAAF with adenosine residues is described, as are several novel photochemical properties of AAAF-conjugated nucleosides.

INTRODUCTION

A major stereochemical constraint of base-paired double helices is the maintenance of individual nucleotide residues in the *anti* conformation[1-4]. Replacement of the C-8 proton by bromine in GMP residues of poly G acid gives a polymer, poly-8-bromoguanylic acid, that cannot form duplex structures with complementary polymers, even though the H-bonding sites are unobstructed[5]. Spectral evidence obtained with the polymer in conjunction with crystallographic data on the nucleoside[6,7] and enzymological studies with the corresponding 5'-triphosphate[8,9] suggest that both monomer and polymer residues have the *syn* conformation at the glycosyl bond. Such studies of conformationally restricted analogs emphasise the steric constraints generated at C-8 of purines (and C-6 of pyrimidine) in natural duplex

Abbreviations: AAAF, *N*-acetoxy-2-acetylaminofluorene; rGMP-AAF, 8-(*N*-fluorenylacetamido)guanosine 5'-monophosphate; dGMP-AAF, 8-(*N*-2-fluorenylacetomido)-2-deoxyguanosine 5'-monophosphate; rGMP-AF, 8-(*N*-2-fluorenylamino)guanosine 5'-monophosphate; G-AAF, 8-(*N*-2-fluorenylacetamido)guanine; G-AF, 8-(*N*-2-fluorenylamino)guanine.

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polynucleotides. Conjecturally, these sites should be unavailable to chemical reaction when they are held in a double helical structure. Comparable residues in single-stranded polymers, with free rotation between the *syn* and *anti* conformation should be reactive to an appropriate reagent.

MILLER AND MILLER[10], MILLER *et al.*[11] and KRIEK *et al.*[12] have defined the site of reaction of the carcinogen, *N*-acetoxy-2-acetylaminofluorene (AAAF) as the 8 position of GMP in monomers and polynucleotides, including DNA. In this paper we test the hypothesis that only single-stranded polynucleotides should react with AAAF. Unexpectedly, double-helical DNA reacts rapidly with AAAF at temperatures 40–50° below the T_m. Synthetic polynucleotides, on the other hand, are 5–10-fold less reactive than DNA and appear to be limited by the steric relationships essential for helical conformations. The results can be interpreted as evidence for a dynamic aspect of DNA structure[13–15], unique to DNA, which implies the continual deformation of the static, H-bonded, *anti* conformation double helix.

FINK *et al.*[16] have pursued an independent inquiry into this problem and proposed, in an analysis comparable to ours, that GMP residues in helical polynucleotides maintain the *syn* conformation when conjugated with *N*-Acetyl-2-aminofluorene.

MATERIALS AND METHODS

DNA and polymers

Micrococcus lysodeikticus DNA was isolated as described previously and was the generous gift of Dr. F. Pochon. Poly rG, poly rI, poly rA and poly rC were synthesized by polynucleotide phosphorylase. Poly dG · poly dC, poly dA · poly dT and poly d(A-T) were prepared in primed synthesis with *Escherichia coli* DNA polymerase[17]; the poly dG and poly dA were isolated by centrifugation in alkaline Cs_2SO_4 gradients[18] and supplied by Dr. D. Ward.

N-Acetoxy-2-acetylaminofluorene (AAAF)

Dr. J. Miller, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisc., generously supplied us with 500 mg of AAAF. AAAF is moderately soluble in propylene glycol and solutions of 0.5–10 mg/ml are stable in the dark at −20° for at least a month. Stock solutions of 200 $A^{1\,cm}_{302\,nm}$ units/cm (10 mg/ml) were prepared and used for chemical reactions with polynucleotides. The ratio of carcinogen to nucleotide in these reactions was based on an ε at 320 nm of 20 000 for AAAF, assuming that the band at 302 nm is unperturbed in the guanosine conjugates.

8-(N-2-Fluorenylacetamido)guanosine 5'-monophosphate (rGMP-AAF)

To a solution of 15 mg rGMP in 10 ml 0.15 M sodium citrate (pH 7.4) was added 20 mg AAAF dissolved in 10 ml propylene glycol. After incubation of the reaction mixture in the dark at 37° for 6 h, it was extracted 4 times with 50-ml portions of ether and the aqueous phase adsorbed onto a column (1 cm × 12 cm) of DEAE-cellulose (Whatman DE 22), carbonate form. rGMP followed by rGMP-AAF were eluted with a gradient of 0–0.5 M triethylammonium bicarbonate at approx.

0.10 and 0.20 M, respectively. The product was homogeneous in three chromatographic systems but following removal of the triethylammonium bicarbonate, two blue fluorescent impurities with R_F 0.09 and 0.21 in System II (see Table I) were observed. They were eliminated by preparative paper chromatography in System II. The yield was 30 %.

The ε(P) of rGMP-AAF and dGMP-AAF was determined in the following way. Known quantities of rGMP and dGMP (ε(P) at 253 nm = 13 700 at pH 7.0) and measured absorbance amounts of rGMP-AAF and dGMP-AAF were incubated at 37° in 0.001 M citrate buffer (pH 7.4) for 2 h with 4 μg of bacterial alkaline phosphatase (BAPC, Worthington Biochemical Corp.). The amount of P_i released was determined by the procedure of AMES AND DUBIN[19]. Standard curves relating P_i released and rGMP (or dGMP) were prepared and used to provide a quantiatative assay of phosphate in rGMP-AAF and dGMP-AAF. Hydrolysis of dGMP-AAF and rGMP-AAF by phosphatase was more than 95 % complete as determined by chromatography of the nucleoside and monophosphate in isopropanol–water (70 : 30, by vol.). ε(P) at 302 nm rGMP-AAF = 22 000 at pH 7.0 and 21 000 at pH 1.0; ratio $A_{275\text{ nm}}/A_{302\text{ nm}}$ at pH 7.0 and 1.0 = 1.68 and 1.69, respectively.

8-(N-2-Fluorenylacetamido)-2'-deoxyguanosine 5'-monophosphate (dGMP-AAF)

To a solution of 15 mg dGMP in 15 ml 0.15 M sodium citrate (pH 7.4) were added 20 mg AAAF dissolved in 8.0 ml propylene glycol. Following 8 h incubation in the dark at 37°, the dGMP-AAF product was isolated using procedures described for rGMP-AAF and judged to be homogeneous after chromatography in Systems I, II, III and IV (see Table I). The yield was 42 %. ε(P) at 302 nm at pH 7.0 = 20 800 and at pH 1.0 = 20 300. The $A_{275\text{ nm}}/A_{302\text{ nm}}$ ratio at pH 7.0 and 1.0 = 1.70 and 1.68, respectively.

Descending chromatography was performed in four different solvent systems. R_F values for reference compounds and the fluorene conjugates described in this paper are given in Table I.

Micrococcus lysodeikticus DNA–AAF conjugates

5.2 μmoles of native *Micrococcus lysodeikticus* DNA dissolved in 2.4 ml 0.001 M citrate (pH 7.4), 0.01 M NaCl was divided into two equal portions, one of which was heated at 100° for 5 min and then cooled in ice. To each portion were added 1.2 μmoles of AAAF dissolved in propylene glycol (final concentration 20 %) and both were incubated at 35° for 2 h. Both fractions were then passed over columns of Sephadex G-25 (1.2 cm × 18 cm) in 0.001 M sodium citrate (pH 7.4) 0.01 M NaCl. For analysis 1.3, 2.4 and 2.2 $A^{1\text{ cm}}_{260\text{ nm}}$ units of native DNA-AAF, denatured DNA-AAF and native DNA respectively were depurinated in 1.2 M HCl for 60 min at 100°. Ultraviolet absorption spectra of each sample were recorded and the products then chromatographed in System III. The guanine and adenine for each sample was eluted with 0.1 M HCl and estimated spectrophotometrically. For the native DNA, native DNA-AAF and denatured DNA-AAF, the amounts of adenine and guanine found (in nmoles), respectively, were 34 and 86, 13.3 and 32, 25.6 and 44.

Polyribonucleotide-AAF conjugates

To 660 nmoles of polymer in 1.0 ml of 0.001 M sodium citrate (pH 7.4) in 0.01 M NaCl, 20 % propylene glycol were added 1.1 μmoles of AAAF (solution at

10 mg/ml in propylene glycol). After 2 h at room temperature and in the dark, the solutions were extracted 3 times with ether and dialysed extensively against water. Aliquots of 0.1 ml were removed and analyzed spectrophotometrically (after alkaline hydrolysis) for the amount of AAF covalently bound to ribonucleotides.

Polydeoxynucleotide-AAF conjugates

The poly dG conjugated to AAAF was prepared by incubating 330 nmoles of poly dG with 10 μmoles of AAAF in 50 % aqueous propylene glycol (1 ml) for 2 h at room temperature. The solution was diluted to 2 ml with water, extracted 3 times with ether and dialyzed extensively against water. The composition was determined by depurinating an aliquot in 1.2 M HCl at 100° for 60 min and spectrophotometrically estimating the 8-(*N*-2-fluorenylamino)guanine (G-AF) formed as described for DNA.

The copolymer (dA, dA-AAF) (92 : 8) was prepared by incubating 0.8 μmole of poly dA with 10.0 μmoles of AAAF in 90 % propylene glycol (1 ml) for 4 h at 37°. The solution was diluted with water to 30 % propylene glycol and extracted 3 times with ether then dialyzed against water and then 0.01 M sodium cacodylate (pH 7.0). The composition was estimated as described in the legend to Table III for poly rA conjugated to AAAF.

In a second comparable reaction conducted at 48° with a 5 : 1 ratio of AAAF to poly dA, the product contained 6.5 % dAMP-AAF residues.

Poly dAT-AAF conjugates

Four solutions of poly d(A–T) were prepared each containing 250 nmoles of poly d(A-T) such that the final reaction composition was water–ethanol–propylene glycol (35 : 15 : 50, by vol.). Increasing quantities of AAAF were added to the reaction mixtures to give ratios of AAAF to dAMP of 0, 4.0, 16.0 and 40.0. The reaction mixtures were incubated for 2 h at room temperature then extracted 3 times with ether and dialyzed against water and then against 0.01 M NaCl, 0.001 M sodium cacodylate (pH 7.0). The spectra were recorded in the latter buffer. No reaction was observed if the ethanol was omitted.

RESULTS AND DISCUSSION

Reaction of AAAF with dGMP, rGMP and other nucleotides

The reaction of dGMP with AAAF to yield dGMP-AAF has been described in detail by MILLER *et al.*[11] and KRIEK *et al.*[12]. Using a modified procedure, we have also prepared this compound and its homolog in the ribose series 8-(*N*-2-fluorenylacetamido) guanosine 5'-monophosphate (rGMP-AAF). The ultraviolet optical properties (Figs. 1a and 1b) of both molecules are similar and distinctive from those of common nucleotides in having a sharp peak at 320 nm characteristic of AAF.

dGMP-AAF, rGMP-AAF, polynucleotides containing these residues and cleavage products formed in acid or alkali from these residues absorb light beyond 300 nm. This property provides a convenient quantitative spectrophotometric assay for these compounds in the presence of natural nucleotides. Consequently, we have characterised the reaction of dGMP-AAF in acid and rGMP-AAF in alkali to provide a

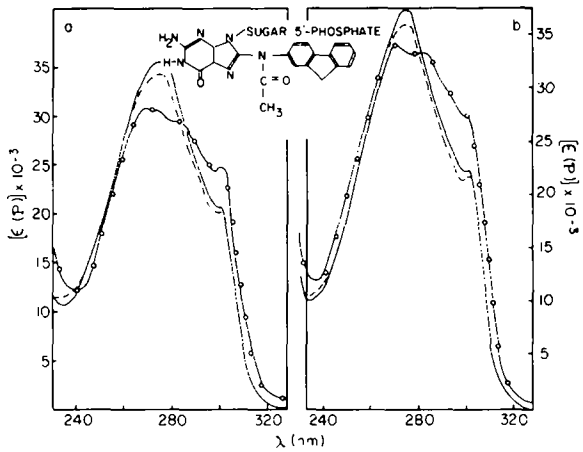


Fig. 1. Ultraviolet absorption spectra of dGMP-AAF at pH 1.0 (- - -), pH 7.0 (———) and pH 13.0 (○–○). b. Ultraviolet absorption spectra of rGMP-AAF. Spectra at pH 1.0 (- - -), pH 7.0 (· —) and pH 13.0 (○–○).

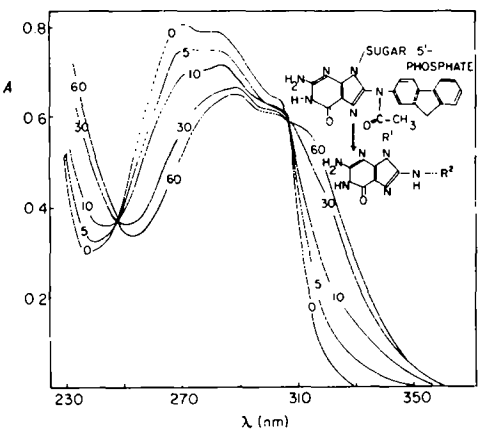


Fig. 2. Conversion of rGMP-AAF to rGMP-AF in alkali. An aqueous solution of rGMP-AAF in 0.01 M NaOH was incubated at 60° in a thermostated 1-cm cell in a Cary 15 spectrophotometer. Spectra were recorded at the time intervals noted (in min). The isosbestic points are at 247 nm and 306 nm. R^1 = ribose 5′-phosphate, R^2 = 2-fluorenyl.

basis for the quantitative estimation of these residues in polynucleotides. As shown in Fig. 2, rGMP-AAF is converted by dilute base to 8-(*N*-2-fluorenylamino)guanosine 5′-monophosphate (rGMP-AF) in the absence of interfering side reactions. The assay of rGMP-AAF is facilitated by the light absorption beyond 310 nm in the alkaline product rGMP-AF.

Similarly, dGMP-AAF is converted to G-AF in acid. The reaction proceeds in two steps, first the glycosyl bond is cleaved to give 8-(*N*-2-fluorenylacetamido)-guanine (G-AAF) (Fig. 3a) then the acetyl group is removed under more stringent hydrolytic conditions (Fig. 3b) to produce G-AF. Both reactions procede quantitatively. G-AF is comparable to rGMP-AF in that both absorb light beyond 310 nm.

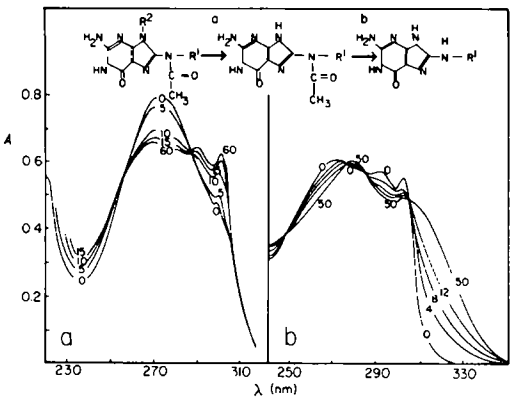
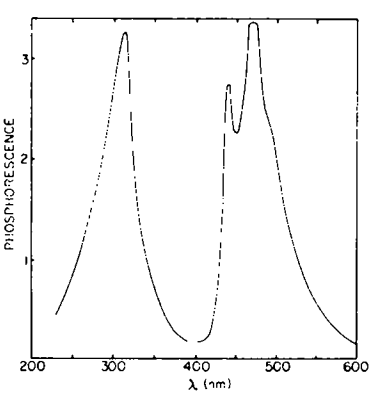


Fig. 3. a. Conversion of dGMP-AAF to G-AAF in dilute acid. dGMP-AAF in 0.12 M HCl was incubated at 40° and spectra recorded at times (in min) indicated in the figure. Isosbestic points are at 253 nm, 287 nm, 307 nm and 313 nm. b. Conversion of G-AAF to G-AF. Following the incubation described in a, the solution was made 1.2 M HCl and incubated at 85°. Spectra were recorded at intervals (in min) indicated in the figure. Isosbestic points are at 228 nm, 242 nm, 274 nm, 282 nm and 304 nm. Chromatography of the product in System III revealed one spot, R_F = 0.41. R^1 = 2-fluorenyl, R^2 = deoxyribose 5'-phosphate.

Hence both can be assayed in the presence of an excess of natural nucleotides following acidic or alkaline hydrolysis of deoxy- or ribopolynucleotides, respectively.

Fluorescence studies of rGMP-AAF, dGMP-AAF using a Zeiss fluorimeter show that the compounds are not luminescent at room temperature. In rigid media at 77°K two phosphorescent bands at 440 and 470 nm with a decay time of 5 sec (Fig. 4) can be seen.



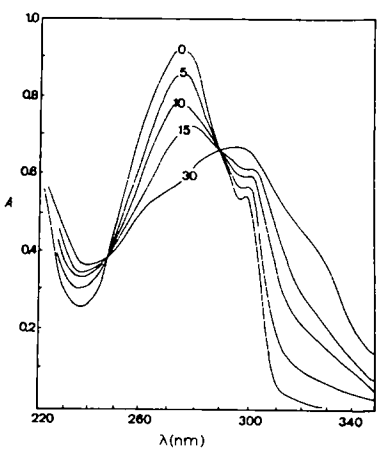


Fig. 4. Phosphorescence spectra at 77°K of rGMP-AAF in water–propylene glycol mixture (1 : 1, by vol.) at a concentration of 0.1 μmole/ml. The spectra were recorded in an Aminco Bowman phosphorimeter (uncorrected). λ_{max} (excitation) = 310 nm, λ_{max} (emission) = 440 nm and 470 nm. Phosphorescence in arbitrary units.

Fig. 5. Photolysis of rGMP-AAF in water. 1-ml of an aqueous solution of rGMP-AAF was irradiated at 302 nm, at 25°, with a Bausch and Lomb monochromater (4-mm slits) *plus* an MTO J280 cutoff filter. Spectra were recorded at intervals indicated in the figure (in min). The isosbestic points are at 247 nm and 292 nm.

In connection with other studies, we measured the photolability of rGMP-AAF and dGMP-AAF following irradiation at 302 nm. Both compounds are rapidly converted to new products in single-step reactions (Fig. 5). Their extreme sensitivity to light makes it advisable to keep them shielded during preparation and study. Under the same conditions of irradiation, copolymer (rG, rG-AAF) (3 : 1) was at least 20 times more photo-resistant than the above compound. The photosensitivity of the monomer fluorene conjugates is in marked contrast to the photostability of purine nucleotides. Their long-wavelength absorption makes selective destruction of residues in polymers possible.

These photoproducts are luminescent at room temperature (Fig. 6). The appearance of fluorescence emission at 410 nm follows exactly the kinetics of transformation by irradiation.

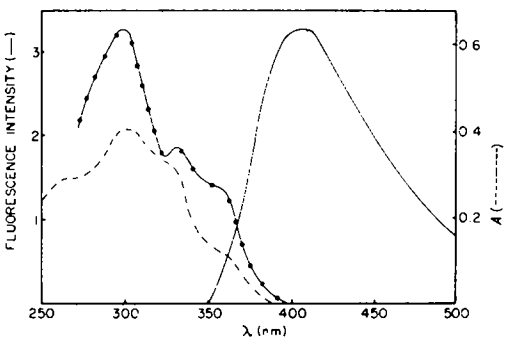


Fig. 6. Fluorescence excitation (●–●) and emission (— · · ·) spectra (corrected) of rGMP-AAF photoproduct in 50 % aqueous ethanol. Fluorescence in arbitrary units. (- - -) Absorption spectrum.

Reactions were performed with rAMP, dAMP, and rIMP under the conditions described for dGMP. At 12-h intervals aliquots were removed and conversion to the respective AAF conjugate assayed by chromatography in System II (Table I). During 45 h of incubation at 37°, dAMP and rAMP gave no evidence of reaction. However, a conjugate of rAMP and AAAF has recently been isolated by column chromatography (L. FINK, personal communication, 1970).

Reaction of DNA with AAAF

Several preliminary experiments confirmed previous observations that native DNA reacts rapidly with AAAF[11,12]. However, we have modified the experimental conditions to ensure that under the conditions of reaction, minimal denaturation of the molecules takes place. Thus *Micrococcus lysodeikticus* DNA was used in all experiments since it has a high G-C content (72 % G+C) and consequently a high T_m, even in 0.01 M Na^+ (T_m = 81.5°). Ethanol was replaced by propylene glycol as solvent for AAAF since the latter does not affect the thermal stability of DNA when present at 20 % (ref. 20). The reaction temperature was 25–35°, some 40–50° below the T_m of the DNA under the salt conditions employed. Finally, characterization was performed initially with DNA isolated from reactions by chromatography on Sephadex G-25.

TABLE I

R_F VALUES

System I, propanol–conc. NH_4OH–water (6 : 3 : 1, by vol.); System II, 1 M ammonium acetate–ethanol (3 : 7, by vol.); System III, formic acid (98 %)–*n*-butanol–water (10 : 77 : 13, by vol.); System IV, acetone–water (3 : 1, by vol.). (A) yellow fluorescence; (B) brilliant blue-white fluorescence; (C) dark blue fluorescence; (D) green fluorescence develops with exposure to short wavelength ultraviolet light.

Compound	R_F			
	System I	*System II*	*System III*	*System IV*
5′-dGMP	0.25	0.41	0.04	0.19
5′-rGMP	0.26	0.37	0.02	—
5′-dAMP	—	0.44	—	—
5′-rAMP	—	0.39	—	—
5′-rIMP	—	0.42	—	—
Guanine	0.45	0.55	0.12	0.48
Adenine	0.58	0.68	0.23	0.61
N-Acetoxy-2- acetylamino fluorene (AAAF)*	0.84	0.93	0.91	—
8-(*N*-2-fluorenylacetamido)guanosine 5′-monophosphate (rGMP-AAF)	0.68	0.62	0.15 (D)	—
8-(*N*-2-Fluorenylacetamido)-2′-deoxy-guanosine 5′-monophosphate (dGMP-AAF)	0.62 (C)	0.74 (D)	0.40 (D)	0.46 (C)
8-(*N*-2-Fluorenylamino)guanosine 5′-monophosphate (rGMP-AF)	—	0.43	—	—
8-(*N*-2-Fluorenylacetamido)guanine (G-AAF)	0.81	0.79	0.72	0.87
8-(*N*-2-Fluorenylamino)guanine (G-AF)	0.16 (A)	0.07 (B)	0.42 (B)	0.25 (B)

* Treatment of AAAF with acid gave rise to ultraviolet-absorbing and fluorescent compounds with $R_F \geqslant 0.84$ in Solvents II and III.

The ultraviolet absorption spectra of native and heat-denatured DNA after reaction with AAAF followed by separation from organic solvent and unreacted AAAF on Sephadex G-25 is shown in Fig. 7. For native and denatured DNA-AAF, λ_{max} shifts from 258 to 261 and 263 nm, respectively. Furthermore, there are two new absorption peaks, one at 300 nm and the other at 336–340 nm. The 340-nm peak can be eliminated in several ways. As illustrated in Fig. 7, addition of ethanol to 50 % (by vol.) is sufficient to remove the absorption at 340 nm. Similarly, extraction of these DNA preparations with ethyl ether or addition of 20 % propylene glycol and heating to 40° has the same effect. The absorption at 340 nm could also be eliminated by heating the DNA in non-alcoholic solutions. Both native and denatured DNA-AAF are smoothly converted by heat to molecules having the same optical properties as in 50 % ethanol. In the case of native DNA-AAF a large fraction of the absorption at 340 nm disappears before the optical transition indicative of collapse of the helix. The absorption at 340 nm is not regained by cooling. Pending further characterisation the 340-nm band can be attributed either to light scattering by organized aggregates (colloidal suspension) of DNA-AAF or to a charge-transfer

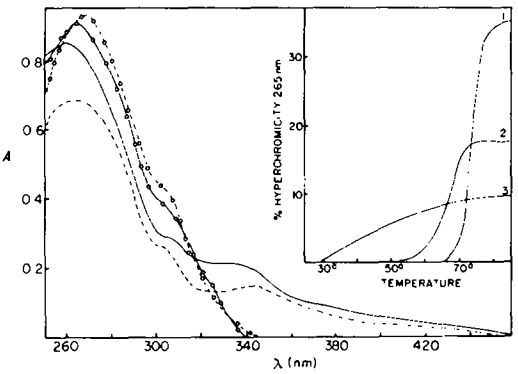


Fig. 7. Ultraviolet absorption spectra of native and denatured DNA-AAF. Native DNA-AAF in (a) 0.001 M citrate (pH 7.4), 0.01 M NaCl (- - -); and (b) 0.001 M citrate (pH 7.4), 0.01 M NaCl *plus* 50 % ethanol (○–○). Denatured DNA-AAF in Solvent a (—— —) and in Solvent b (○- - -○). Insert: optical transition at 265 nm of native Micrococcus DNA (1) in 0.0005 M citrate (pH 7.4), 0.005 M NaCl, 50 % ethanol; (2) native DNA-AAF in the same solvent; and (3) denatured DNA-AAF. The respective T_m's are 75, 67 and 45°. The native DNA-AAF was prepared in a reaction of 900 nmoles native Micrococcus DNA with 720 nmoles AAAF in 20 % propylene glycol containing 0.001 M citrate (pH 7.4), 0.01 M NaCl at 37°. The incubation was for 7 h following which the DNA was isolated by passage through Sephades G-25 equilibrated with the reaction buffer. The denatured DNA-AAF was heated at 100° for 5 min and cooled in ice, prior to reaction under the conditions described above.

complex within the DNA-AAF. This property of DNA-AAF may be relevant to the changes of DNA after reaction with AAAF *in vivo*.

The thermal transitions of native DNA-AAF, denatured DNA-AAF and native DNA in the same solvent are illustrated in the insert to Fig. 7. The denatured DNA-AAF preparation has the characteristic non-co-operative transition of denatured DNA. The transition of the native DNA-AAF is co-operative, but occurs a lower temperature and is broader than that of native DNA. Correction for absorption of the carcinogen gives a percentage hyperchromicity for native DNA-AAF and native DNA of 36 and 35 %, respectively.

Products of the reaction of AAAF with DNA

Following reports in the literature, we assumed that the sole reaction product in DNA was dGMP-AAF[10–12] and established a spectrophotometric assay for this product. The acidic cleavage product, G-AF, of dGMP-AAF absorbs at wavelengths further to the red than any products of de-purinated DNA. A standard curve of nmoles de-purinated *Micrococcus lysodeikticus* DNA *versus* absorption at 270 nm was established experimentally. Samples of DNA which had reacted with AAAF were then de-purinated under the same conditions (100°, 1h, 1.2 M HCl) and the ultraviolet spectrum measured. Absorption at 315 nm gave a quantitative estimate of G-AF (see Fig. 3b) since for de-purinated *Micrococcus lysodeikticus* DNA the ratio of absorption $A_{315\ nm}/A_{270\ nm}$ is less than 0.001. The absorption of de-purinated DNA-AAF samples at 270 nm was corrected to account for the amount of G-AF and reference to the de-purinated DNA standard curve permitted an accurate estimate of the amount of DNA that was hydrolyzed. Thus the percent dGMP residues which had reacted with the carcinogen could be estimated. We confirmed that dGMP-

AAF was a product of the reaction of AAAF with DNA by chromatographing the products of de-purinated DNA-AAF with standards of de-purinated dGMP-AAF.

However, an independent procedure of analysis showed discrepancies. Two preparations of DNA-AAF were made, one from native DNA and the second from heat-denatured DNA. Aliquots of both were de-purinated as was native DNA as a control and the products chromatographed in System III. Guanine and adenine were eluted and estimated spectrophotometrically. These values were compared with the estimates of dGMP-AAF (and hence dGMP and dAMP) obtained directly on the DNA after de-purination using the spectrophotometric procedure described above. As shown in Table II, there is a contradiction between the two determinations. It

TABLE II

TREATMENT OF NATIVE AND DENATURED DNA WITH AAAF

	% *dGMP converted to dGMP-AAF*		
	Spectrophotometric assay on depurinated samples	*Chromatographic assay based on ratio of guanine to adenine*	*Ratio G/A*
Native DNA	–	–	2.52*
Native DNA-AAF	26	5.3	2.41
Denatured DNA-AAF	45	32	1.71

* The literature value for *Micrococcus lysodeikticus* DNA is 2.58.

is most striking for native DNA-AAF where the ratio of guanine to adenine has not changed in a manner consistent with the amount of G-AF (and hence dGMP-AAF) produced in the reaction. The results can be best understood by assuming that a second reaction takes place between AAAF and dAMP residues. If both sets of values are correct, we can calculate that in native DNA-AAF 26 % of the dGMP and 23 % of the dAMP residues had reacted whereas in denatured DNA-AAF 45 % of the dGMP and 19.5 % of the dAMP residues were conjugated to AAF. Further evidence that the reaction of AAAF with dAMP residues in native DNA is quantitatively important, follows from studies with poly d(A–T), poly dA and poly rA.

Effect of salt on the reaction of AAAF with native DNA

The reaction of AAAF with native DNA was measured as a function of NaCl concentration (Fig. 8). Even at 1.0 M NaCl, a significant portion of the dGMP residues reacted. In 0.01 and 0.001 M NaCl, the reaction was rapid and extensive. The composition of the DNA product from these reactions was analyzed by the de-purination procedure. With increasing extent of reaction, the T_m's decreased linearly and the breadth of the optical transition (σ) increased (for example, see Fig. 7, insert). The sample estimated to have 31 % dGMP-AAF hand a σ of 16° whereas native DNA had a σ of 9° under identical conditions.

Reaction of AAAF with homopolynucleotides

Double-helical polynucleotide complexes react less readily with AAAF than single-stranded polymers. As shown in Table III, only poly rG and poly rA react

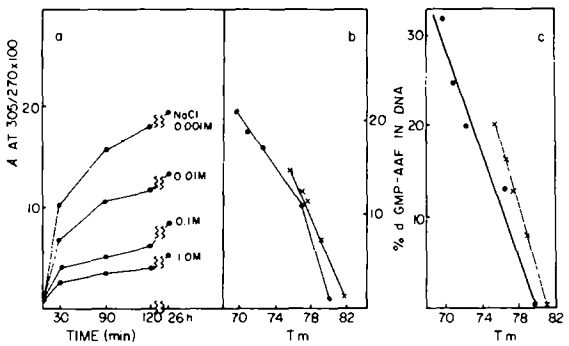


Fig. 8. Effect of salt on the rate of reaction of AAAF with native DNA. a. Four reactions containing 270 nmoles Micrococcus DNA and 1.1 μmoles of AAAF, in 0.001 M citrate buffer (pH7.4) were prepared, each in a final volume of 1.0 ml, 20 % (by vol.) in propylene glycol. The reactions were, respectively 0, 0.01, 0.10, 1.00 M in NaCl. They were incubated at 25°. Aliquots (0.2 ml) were withdrawn at times indicated in the figure and each diluted to 1.0 ml with 0.001 M sodium citrate buffer (pH 7.4), 0.01 M NaCl. The aliquots were then extracted thrice with ether and their ultraviolet spectra recorded on a Cary 15 spectrophotometer. The increase in absorption at 305 nm relative to 270 nm is used as a criterion of reaction. b. T_m as a function of substitution. The thermally induced optical transitions of the DNA-AAF conjugates formed in the absence of added NaCl and in 0.01 M NaCl prepared as described above were measured at 265 nm. The T_m's are plotted as a function of the extent of reaction by using the ratio of absorption at 305 to 270 nm as a criterion for the latter. Thermal dissociation in 0.01 M NaCl (×–×) and in 0.008 M NaCl (●–●). c. T_m as a function of percentage of dGMP-AAF. The samples used in the previous experiment (b) were depurinated (1.2 M HCl at 100° for 30 min) and their ultraviolet spectra measured. As described in the text, the amount of dGMP-AAF in the original DNA-AAF was estimated from the spectra. Thermal dissociation in 0.01 M NaCl (×–×) and in 0.008 M NaCl (●–●).

extensively under the conditions used. Reaction of GMP residues in poly rG · poly rC is inhibited compared with poly rG and this structural inhibition is even more apparent with poly rA · poly rU compared with free poly rA. It may be noted that this is not due to substitution at a hydrogen-bonding site since poly rA-AAF (with at least 95 % of the residues substituted) readily complexes with poly rU and poly rI (A. M. KAPULER, F. POCHON AND A. M. MICHELSON, unpublished). As with DNA, an increase in salt concentration decreases the extent of the reaction of AAAF with both poly rG and the complex poly rG · poly rC (Table IV). This suggests that the stability of the double helix and hence the respiration of hydrogen bonds, *i.e.* the life-time of local fluctuating distortion, plays a significant role in the mechanism of substitution. That DNA is also less reactive with increasing salt, but is more reactive than these ribopolymers suggests a property unique to DNA. If helical structure (imposing the form *anti* on the nucleotides) is destroyed as in the copolymer (G, BrG) containing 89 % 8-bromoguanylic acid, then all 11 % guanine residues are rapidly substituted (Table V). This also demonstrates the specificity of site of reaction since only GMP residues were substituted. Alkaline hydrolysis of poly G-AAF (15 %) gave a mixture of the 2'- and 3'-phosphates of guanosine and guanosine-AF, the latter showing a characteristic absorption band at 310–350 nm (Fig. 9).

The corresponding deoxyribopolynucleotides, poly dG and poly dA also react with AAAF but at a lower rate than the ribonucleotides. Thus to obtain the same pro-

TABLE III

REACTION OF AAAF WITH RIBOPOLYNUCLEOTIDES

All reactions were performed at room temperature in 1.0 ml of 20 % (v/v) propylene glycol; 0.001 M citrate (pH 7.4), 0.01 M NaCl with 1.1 μmoles of AAAF. Aliquots of 100 μl were removed, diluted to 1.0 ml in buffer, extracted with ether and analysed spectrophotometrically. Poly rA · poly rU, poly rI · poly rC and poly rG · poly rC were mixed 1 : 1.05 (purine nucleotide : pyrimidine nucleotide) in 0.05 M NaCl, 0.002 M cacodylate (pH 7.0) at a final nucleotide concentration of 5 μmoles/ml. They were kept at room temperature for 24 h before use. In poly rG (b) and poly rG · poly rC (b), the product isolated from the first reaction after 24 h was treated with AAAF a second time. The initial reaction was extracted with ether, dialyzed against water and aliquots of 300 nmoles of either poly rG-AAF or poly rG · poly rC-AAF in 1 ml 0.001 M cacodylate (pH 7.0) added to 1.1 μmoles of AAAF in 300 μl propylene glycol. After 2 h at room temperature, they were extracted 3 times with ether and analyzed spectrophotometrically for rGMP-AAF after alkaline hydrolysis.

Polymer	*Amount (nmoles)*	*% Purine residues reacted with AAF*		
		First reaction		*Second reaction*
		2 h	*24 h*	*2 h*
Poly rA** (a)	600	8	9	—
(b)	500	—	—	16
Poly rI	600	< 1	< 1	—
Poly rG (a)	600	15	16	—
(b)	300	—	—	27
Poly rA · poly rU	500	< 1	< 1	—
Poly rI · poly rC	500	< 1	< 1	—
Poly rG · poly rC (a)	500	6	6	—
(b)	300	—	—	12*

* In a third reaction, this value was increased to 14 %.

** An estimate of the amount of AAF covalently bonded to poly rA is obtained by assuming an ε at 302 nm = 20 000 for the rAMP-AAF product and an $A_{300\text{ nm}}/A_{256\text{ nm}}$ ratio of 1.20. The product of poly rA with AAF has a shoulder at 302 nm similar to that in dGMP-AAF, suggesting that the ring systems and hence extinction coefficients are comparable.

TABLE IV

EFFECT OF SALT ON REACTION OF POLY rG · POLY C AND POLY rG WITH AAAF

Reactions were in a final volume of 1.0 ml 40 % (v/v) in propylene glycol. Each reaction contained 0.5 μmole of polymer, 2.2 μmoles of AAAF, 0.001 M sodium cacodylate (pH 7.0) and NaCl as indicated in the table. After incubation at room temperature for 2 h, they were extracted with ether, dialyzed against water and analyzed for rGMP-AF spectrophotometrically.

NaCl in reaction (M)	*% rGMP-AAF in polymer*	
	Poly rG · poly C	*Poly rG*
0.002	6.9	22
0.200	2.5	6.9

portion of modification 5 times as much carcinogen must be used, again suggestive of the influence of conformation on the reactivity of the purine residues. Similarly, the reactivity of poly d(A–T) to attack by AAAF is markedly less than that of DNA (Fig. 10) and reaction only occurs in the absence of salt with 65 % organic solvent (*i.e.* single-stranded form of poly d(A–T). In addition a 40-fold excess (rather than

TABLE V

REACTION OF AAAF WITH COPOLYMER (rG, BrG)

Copolymer (rG, BrG) (11 : 89) was prepared as described previously[5]. The reactions were performed in series. 220 nmoles each of poly rG or copolymer (rG, BrG) (11 : 89) in 0.7 ml water were added to 0.3 ml of propylene glycol containing 3.0 μmoles of AAAF and incubated at room temperature for 2 h. After ether extraction and dialysis, composition of the product was estimated spectrophotometrically after alkaline hydrolysis. Aliquots of each product were also treated with AAAF a second time under the same conditions.

	Ratio AAF/rGMP in reaction	*Composition of product (% residues rGMP-AAF)*
Copolymer (G, BrG) (11 : 89) (1)	135	13
(2)	330	13
Poly rG (1)	15	24
(2)	25	27

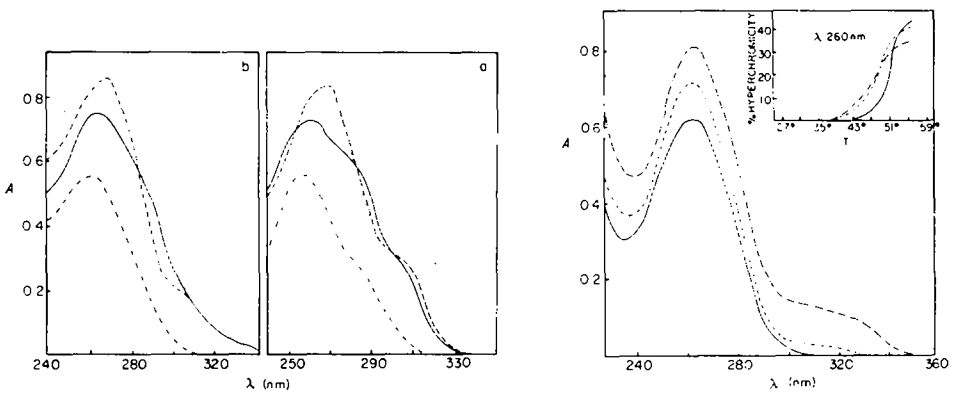


Fig. 9. a. Ultraviolet absorption spectra of copolymer (rG, G-AAF) (76 : 24) at pH 7.0 (— · —), at pH 13.0 (- - -) and heated at 80° at pH 13 for 45 min to convert rGMP-AAF residues to rGMP-AF (. . .). The spectrum of poly rG at pH 7.0 (- · -) is included for reference. The ultraviolet spectrum of the copolymer (rG, rG-AAF) (76 : 24) is unchanged following addition of ethanol to 20 %. b. Ultraviolet absorption spectra of copolymer (rG, G-AAF) (88 : 12)· poly rC at pH 7.0 (— — —), at pH 13 (- - -) and after heating at 80° (pH 13) for 45 min (. . .). The spectrum of poly rG · poly rC at pH 7.0 (- · -) is included for reference.

Fig. 10. Ultraviolet absorption spectra of poly d(A-T) containing increasing amounts of dAMP AAF. The products corresponding to the ratios of AAAF to dAMP are 0 (— — —), 4 (- - -), 16 (- - -) and 40 (. . .). Insert: T_m's of the different poly dAT-AAF preparations in 0.01 M NaCl, 0.001 M sodium cacodylate (pH 7.0) at 265 nm. The respective T_m's for the polymers are 50° (— —) 47.5° (- - -), 45.5° (- - -) and 43.5° (. . .) and their corresponding hyperchromicities were 44, 39, 33 and 30 %. All thermal dissociations were completely reversible. A reversible hyperchomicity at 305 nm of 20 % was shown by the most highly substituted polymer.

2-fold) of the carcinogen must be used to obtain approximately the same amount of substitution as in DNA. As with DNA, modified poly d(A–T) shows a lower T_m and an increased breadth of transition, though optical rotation dispersion spectra remain relatively unchanged (Fig. 11).

It thus appears from these studies that the most reactive polynucleotide with respect to AAAF is DNA by a factor of about 10. With sufficient carcinogen, up to 50 % of the purines can be substituted without complete loss of secondary structure,

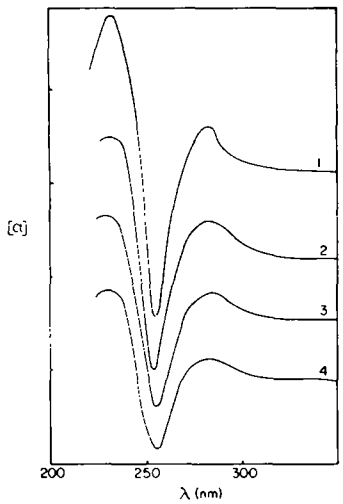


Fig. 11. ORD spectra of different poly dAT-AAF conjugates in 0.01 M NaCl, 0.005 M cacodylate (pH 7.0). Ratio of AAAF to dAMP are 0 (Curve 1), 4 (curve 2), 16 (Curve 3) and 40 (Curve 4). Spectra are recorded in a 1-cm path length cuvette with an absorbance of 0.6 at λ_{max}. One division corresponds to a rotation of 0.01°.

the T_m is lowered and is less co-operative and the optical rotation dispersion spectrum remains conservative[21].

Since reaction of AAAF with DNA occurs at both AMP and GMP residues, yet AMP residues in homopolymer double-helical complexes are resistant, it is likely that reaction in DNA takes place first at GMP residues followed by AMP residues in the vicinity (*cf.* the mutagenicity of AAAF[22]). It is thus possible that local regions of the DNA will be denatured by the carcinogen in the absence of a co-operative denaturation affecting the entire molecule. That GMP residues, conjugated to AAAF are preferentially in the *syn* rather than the *anti* conformation is likely, but still unproven[8,16].

Studies of static models of DNA predict that C-8 of purine residues will be protected from chemical reaction by virtue of its relative inaccessibility due to the *anti* conformation[8]. The reactivity of DNA with AAAF implicates a dynamic aspect of the DNA double helix. In this sense, local liberation of bases from the rigid helix, even though of a very short duration, and with a distortion certainly limited by a number of factors, is presumably necessary for successful attack by the carcinogen. This dynamic liberty is favoured by low salt concentrations (or by addition of organic solvents) and thus increases reactivity. Rotational distortion about the glycosyl linkage in the absence of H-bond breaking, a type of freedom not easily appreciated in static models of DNA, would account for the reactivity. Then too, an exceptional nucleophilicity of C-8 in dGMP residues in a helix might also facilitate the reaction. In this case, C-8 would have some *ylide* character reminiscent of that shown by the thiazole nucleus in thiamine pyrophosphate[23] (compare exchange with that of C-8 in purine nucleosides)[25]. In a high salt concentration, there is still reaction with AAAF, perhaps at easily denaturable regions or at helix ends. In either case, the reagent cannot be used to distinguish double-stranded nucleic acids from single-stranded

ones; rather to demonstrate differences in geometry and respiration. In this respect, the lower reactivity of poly rG · poly rC relative to DNA can be interpreted in terms both of the difference in geometry of their structures and of the smaller deformational amplitude possible in the synthetic polymer (higher T_m and higher alkaline pK dissociation[24]).

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