Comparative Studies on the 7-Iodo and 7-Fluoro Derivatives of *N*-Acetoxy-*N*-2-acetylaminofluorene: Binding Sites on DNA and Conformational Change of Modified Deoxytrinucleotides†

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ABSTRACT: GMP and native DNA were reacted with 7-iodo and 7-fluoro derivatives of *N*-acetoxy-*N*-2-acetylaminofluorene. It was shown that the 7-halogeno derivatives react on C-8 of guanine. Furthermore the respective amount of arylamidation (covalent linkage on the C-8 of guanine) and arylation (covalent linkage on 2-NH_2 groups of C_3 of guanine) addition products was determined in both native and denatured DNA-[^{14}C]AAIF. Two G containing deoxytrinucleotides modified by either AAFF or AAIF were studied comparatively by means of circular dichroism, and as a function of several parameters known to affect the conformation of the deoxytrinucleotides. The induced optical activity in fluorofluorene ring seemed to be very sensible to the conformational changes of the deoxytrinucleotides. On the other hand, the AAIF residue exhibit a lower induced optical activity which remained unchanged when the deoxytrinucleotides conformation was affected. The results presented in this paper led us to conclude that the AAFF and AAIF modified deoxytrinucleotides adopt a conformation which nicely fits with the insertion-denaturation and outside-binding model, respectively.

It is now widely recognized that the reaction of chemical carcinogens with nucleic acid reconstituents is an important and probably crucial exent in the process of neoplastic transformation. The relative ease of synthesizing various analogues of the carcinogen AAF[1] offers a good opportunity to study the relationship between the chemical structure and the carcinogenic properties of these molecules.

The 7-fluoro derivative of *N*-AcO-AAF is a model for the ultimate metabolite of the strong carcinogen AAFF (Miller et al., 1955). On the other hand, according to the general mechanism of activation of *N*-arylacetamides (Miller, 1970) the 7-iodo derivative of *N*-AcO-AAF may represent a metabolite of AAIF. AAIF has been found to be essentially noncarcinogenic in rats (Morris et al., 1960). However, recent studies have shown that the myristic acid ester of *N*-OH-AAIF is at least as potent a local carcinogen as the corresponding ester of *N*-OH-AAF (Miller & Miller, personal communication). Lack of metabolic activation of AAIF accounts probably for these observations. Precedent studies from this laboratory (Fuchs & Daune, 1973; Fuchs, 1975; Fuchs et al., 1976) have shown a different conformational change induced locally in native DNA on the one hand by the binding of *N*-AcO-AAF or its 7-fluoro derivative (insertion-denaturation model) and on the other hand by the binding of *N*-AcO-AAIF (outside binding model). The insertion-denaturation model states that the fluorene ring is accommodated between the adjacent base pairs (Fuchs & Daune, 1971, 1972) leading to a local denaturation of the double helix. Similar conclusions have been reached for the structure of oligonucleotides modified with *N*-AcO-AAF (Nelson et al., 1971). In the outside binding model, the iodofluorene residue is believed to lie along the phosphate sugar backbone of the DNA double helix (Fuchs et al., 1976). In this latter case the bulky iodine atom probably prevents the insertion of the fluorene ring leading therefore to an almost nondenaturing addition product (Fuchs & Daune, 1973).

In this paper we show that similarly to *N*-AcO-AAF (Miller et al., 1966; Kriek et al., 1967), the 7-halogeno derivatives mainly react on C-8 of guanine. By means of the assay described by Fuchs (1977, submitted) the respective amount of arylamidation and arylation addition products was determined in both native and heat denatured DNA-[^{14}C]AAIF.

The comparative conformations of two G containing deoxytrinucleotides modified by either *N*-AcO-AAFF or *N*-AcO-AAIF were explored by means of circular dichroism. The induced optical activity in each fluorene derivative was studied as a function of several parameters known to affect the conformation of the deoxytrinucleotide. The AAFF and AAIF modified deoxytrinucleotides adopt a conformation which nicely fits the insertion-denaturation and the outside binding model, respectively.

Material and Methods

All common chemicals were reagent grade (Merck).

Synthesis of N-Acetoxy-N-2-acetylamino-7-fluorofluorene (N-AcO-AAFF) and N-Acetoxy-N-2-acetylamino-7-iodofluorene (N-AcO-AAIF). The two products were obtained from 2-nitro-7-fluorofluorene (Miller et al., 1955) and 2-nitro-7-iodofluorene (Weisburger, 1950) by a reductive acetylation similar to that described by Cramer et al. (1960).

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[1] Abbreviations used: AAF, AAFF, and AAIF, *N*-2-acetylaminofluorene, and 7-fluoro and 7-iodo derivatives; *N*-OH-AAF and *N*-OH-AAIF, *N*-hydroxy-*N*-2-acetylaminofluorene and 7-iodo derivatives; *N*-AcO-AAF, *N*-AcO-AAFF, and *N*-AcO-AAIF, *N*-acetoxy-*N*-2-acetylaminofluorene and 7-fluoro and 7-iodo derivatives; *N*-AcO-[^{14}C]AAIF, *N*-acetoxy-*N*-2-[^{14}C]acetylamino-7-iodofluorene; [^{14}C]AAIF, *N*-2-[^{14}C]acetylamino-7-iodofluorene; DNA-[^{14}C]AAIf, DNA which is reacted with *N*-AcO-[^{14}C]AAIF; G-AF, G-AFF, and G-AIF, 8-(*N*-2-fluorenylamino)guanine and 7-fluoro and 7-iodo derivatives; GMP, guanosine 5′-monophosphate; GMP-AAF, GMP-AAFF, and GMP-AAIF, GMP substituted on C-8 with AAF, AAFF, and AAIF, respectively; $TG_{AAFF}A$ and $AG_{AAFF}A$, $TG_{AAIF}A$, and $AG_{AAIF}A$, deoxytrinucleotides modified covalently with *N*-AcO-AAFF and *N*-AcO-AAIF, respectively; DNA-AAF, DNA-AAFF, and DNA-AAIF, DNA modified covalently with *N*-AcO-AAF, *N*-AcO-AAFF, and *N*-AcO-AAIF, respectively; ^{1}H NMR, proton magnetic resonance.

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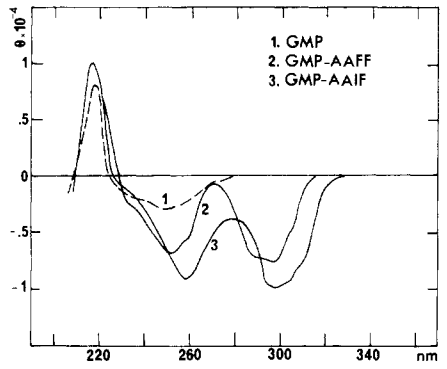

FIGURE 1: Circular dichroism spectra of (1) GMP; (2) GMP-AAFF; and (3) GMP-AAIF.

A modification of this catalytic hydrogenation procedure proved to be more practical.

2-Nitro-7-fluorofluorene (0.4 mM) was dissolved in a mixture of 4 mL of ethyl acetate and 4 mL of pyridine. 0.4 mL of acetic anhydride and 14 mg of charcoal containing 5% of palladium were added. This mixture was hydrogenated at room temperature at atmospheric pressure until 0.8 mM of hydrogen had been absorbed. After removal of the catalyst by filtration, the *N*-acetoxy compound was directly purified from this mixture by column chromatography in the dark on silica gel (Kieselgel, Merck) eluted by pure $HCCl_3$.

N-AcO-AAFF. This procedure gave yields of crude product (chromatographically pure) of 70 to 80% of theory. After crystallization this product melted at 113–114 °C. Anal. Calcd for $C_{17}H_{14}O_3NF$: C, 68.25; H, 4.68; N, 4.68. Found: C, 68.24; H, 4.73; N, 4.77. The UV spectrum in ethanol had the following characteristics: λ_{max}(nm) 304.5 (ϵ 14 100); 273 (ϵ 24 600); λ_{min}(nm) 301 (ϵ 11 700).

N-AcO-AAIF. The reduction was performed in pure pyridine owing to the poor solubility of the nitro compound in ethyl acetate. The crude yield was 35% of theory. This product was further purified by thick layer chromatography on silica gel (solvent: $HCCl_3$). A slightly coloured oil crystallized upon storage at −20 °C in the dark, under argon atmosphere. Anal. Calcd for $C_{17}H_{14}O_3NI$: C, 50.15; H, 3.44; N, 3.44. Found: C, 49.95; H, 3.56; N, 3.53. The UV spectrum in ethanol had the following characteristics: λ_{max}(nm) 310 (ϵ 23 000), 284 (ϵ 27 800); λ_{min}(nm) 305 (ϵ 20 500).

Synthesis of N-Acetoxy-N-2-[^{14}C]-acetylamino-7-iodofluorene (N-AcO-[^{14}C]AAIF). *N*-Hydroxy-*N*-2-[^{14}C]-acetylamino-7-iodofluorene has been obtained from the above described reduction mixture starting from 2-nitro-7-iodofluorene and [^{14}C]acetic anhydride (CEA) by the purification procedure described by Poirier et al. (1963). The ^{14}C-labeled hydroxamic acid is then acetylated with acetyl chloride in chloroform, in presence of triethylamine. Using Unisolve liquid scintillator (Koch Light Laboratories, Ltd.), the specific activity of *N*-AcO-[^{14}C]AAIF was equal to 1230 cpm/nM.

DNA. Native DNA was prepared from chicken erythrocytes (Kay et al., 1952) and had the following characteristics: hyperchromicity at 260 nm, 41%; $s_{20,w}$, 21 S; ϵp^{260} = 6400. Protein content was lower than 0.8% by weight. Denatured DNA was obtained by heating buffered solution (2.10 −3 M/L sodium citrate buffer, pH 7) of native DNA, in a sealed vial up to 100 °C for 10 min. To avoid DNA renaturation the sample was rapidly chilled in ice. The hyperchromicity at 260 nm of the denatured DNA was equal to 28%, but the melting profile showed no cooperativity.

Deoxytrinucleotide Preparation. Deoxytrinucleotides of known sequence (AGA and TGA) were prepared in a way similar to that described by Rushizky et al. (1972), starting from a DNA micrococcal nuclease hydrolysate. The sequence identification was performed on the basis of the respective position of each deoxytrinucleotide on the chromatograms and on the basis of the R_f relative to 3′-adenylic acid values obtained on cellulose paper chromatography in solvent (40 g of $(NH_4)_2SO_4$ added to 100 mL of 0.1 M Tris-HCl, pH 7.5). We obtained the R_f values relative to 3′-adenylic acid indicated by Rushizky et al. (1972) (AGA, 0.31; TGA, 0.64).

TABLE I: R_f Values of the Fluorene-GMP Adducts.[a]

	Solvent A[b]	Solvent B[c]
GMP	0.10–0.11	0.20–0.21
GMP-AAF	0.50	0.55–0.57
GMP-AAFF	0.54	0.56
GMP-AAIF	0.54	0.57

[a] The R_f values were obtained from TLC on cellulose plates. [b] Solvent A: 1-butanol:glacial acetic acid:water (50:11:25). [c] Solvent B: isopropyl alcohol:concentrated ammonia:water (6:3:1).

CD, UV, and NMR Spectra. Circular dichroism measurements have been done with a Dichrograph Jouan II. Samples for circular dichroism spectra were prepared at a concentration of $\sim 5 \times 10^{-5}$ M in water, or in 5×10^{-3} M Tris-HCl buffer, pH 7. Circular dichroism melting curves were obtained on a XY recorder giving the signal intensity versus temperature (Wilhelm et al,. 1974). The curves presented are normalized according to the following expression:

$$\theta(t) = \frac{\theta_t - \theta_0}{\theta_0}$$

were θ_0 is the ellipticity at low temperature, θ_t the ellipticity at temperature t. Ellipticities are expressed in deg cm^2 dmol^{-1} and corrected with a solvent blank.

Spectrophotometers Cary 15 or Cary 118 C were used to perform all absorption spectra.

NMR spectra were taken on a pulsed Fourier transform spectrometer (Varian XL 100).

Reaction of the N-Acetoxy-N-arylacetamides with Nucleic Acids Constituents. Reaction with Guanosine 5′-Phosphate (GMP). One volume of GMP (1 mg/mL) in 2×10^{-3} M sodium citrate (pH 7) is mixed with 1 volume of either *N*-AcO-AAFF or its iodo analogue (1 mg/mL) in ethanol and incubated at 37 °C, during 3 h, under argon atmosphere in the dark. Most of the fluorene solvolysis products are removed by ethylic ether extractions and the fluorene modified GMP (GMP-AAFF or GMP-AAIF) are purified on LH 20 Sephadex column chromatography under the conditions described by Nelson et al. (1971). For sake of comparison we prepared 8-(*N*-2-fluorenylacetamido)guanosine 5′-phosphate (GMP-AAF) according to the procedure described by Kriek et al. (1967). Thin-layer chromatography was carried out on cellulose layer in 1-butanol-glacial acetic acid-water (50:11:25) (solvent A) and isopropyl alcohol-concentrated ammonia-water (6:3:1) (solvent B). The R_f values of GMP and of the different GMP adducts are given in Table I. There is a striking similarity between the ultraviolet absorption spectra of GMP-AAFF and GMP-AAF. Small shifts are observed in the position of the 300-nm shoulder of GMP-AAF (Kriek et al., 1967). This shoulder is located at 305 nm in the case of GMP-AAFF and at 310 nm in the case of GMP-AAIF.

Circular Dichroism. The induction of a relatively intense negative band (Figure 1) in the 300-nm range in all the GMP adducts is attributed to optical activity induced in fluorene as

a result of its covalent attachment to GMP (Nelson et al., 1971).

Proton Magnetic Resonance. The proton magnetic resonance (^{1}H NMR) spectra in D_2O of GMP-AAFF and GMP-AAIF suggested that the reaction occurred on the nitrogen of *N*-AcO-AAFF and of *N*-AcO-AAIF, respectively, since the integration in the region of the aromatic protons showed the correct number of protons for an unsubstituted fluorene nucleus. The ^{1}H NMR spectra of GMP exhibit the absorption signal of the proton on carbon C-8 at −3.52 ppm (the chemical shifts are refered to the signal of water). The spectra of GMP-AAFF and GMP-AAIF showed no absorption between −3.3 ppm and −6.0 ppm, suggesting that substitution had occurred at carbon C-8 of guanine. N-7 substitution can theoretically not be ruled out since it would also give rise to a highly exchangeable C-8 proton (Tomasz, 1970). This seems, however, unlikely since C-8 substitution has been proved unequivocally in the case of the analogous compounds GMP-AAF (Kriek et al., 1967) and guanosinyl-*N*-methyl-4-aminoazobenzene (Lin et al., 1975).

Reaction with the Deoxytrinucleotides (TGA and AGA). The incubation conditions are those described for the reaction with GMP, except for the percentage of ethanol which was equal to 33%. After three ethylic ether extractions of the noncovalently bound fluorene residues, the modified deoxytrinucleotides are purified by BD-cellulose column chromatography under the conditions described by Grunberger et al. (1974). The nonreacted deoxytrinucleotides are eluted with 1.3 M ammonium bicarbonate and the fluorene modified trinucleotides came out in 1.3 M ammonium bicarbonate:ethanol (2:1). The ammonium bicarbonate salt was subsequently removed by multiple lyophilization–redissolution cycles. The ultraviolet absorption spectra of $TG_{AAFF}A$ (Figure 2) and $AG_{AAFF}A$ showed the same shoulder around 305 nm as GMP-AAFF. This absorption band is shifted up to 310 nm in the spectra of the corresponding iodo modified trinucleotides ($TG_{AAIF}A$ (Figure 2) and $AG_{AAIF}A$). The absorption in the 300–310-nm range is only due to the fluorene nucleus, and the intensity of this band showed no variation upon heating (from 20 up to 80 °C). We considered therefore the same molar extinction coefficient in this region of wavelengths for both the modified deoxytrinucleotide and the modified nucleotide ($\epsilon^{305nm}_{GMP-AAFF}$ = 18 000; $\epsilon^{310}_{GMP-AAIF}$ = 23 200).

Reaction with DNA. The incubation conditions are those described for the reaction with GMP except for the percentage of ethanol which was equal to 20%. After three ethylic ether extractions the remaining noncovalently bound fluorene residues were removed by extensive dialysis against 2×10^{-3} M sodium citrate buffer. The ultraviolet absorption spectra of DNA-AAFF and DNA-AAIF showed a shoulder at 307 and 313 nm, respectively. The determination of the percentage of modified bases was made either spectrophotometrically as described by Fuchs & Daune (1972) or by radioactivity counting when using the ^{14}C-labeled product.

Acidic Depurination of the Fluorene Modified DNA Samples. Native DNA samples (40 optical units at 260 nm) having around 5% of modified bases were depurinated in 1.2 M HCl at 100 °C for 60 min in sealed vials. The hydrolysates were lyophilized and dissolved in 400 μL of 0.5 N HCl–ethanol (1:1). These solutions were spotted on silica gel thin-layer chromatography plates (with a fluorescent indicator F 254) and developed in formic acid–1-butanol–water (7:77:16) (solvent C).

In this system each hydrolysis mixture was resolved into four spots having almost the same R_f values regardless whether DNA was modified with AAF or its 7-fluoro and 7-iodo derivatives. Product I (R_f 0.14) and product II (R_f 0.18) were easily identified as guanine and adenine, respectively. Product IV (R_f 0.55–0.57) had the same R_f values and UV spectra as the products obtained by depurination (1.2 M HCl, 100 °C, 60 min) of GMP modified with the corresponding fluorene derivative. The GMP-AAF depurination product had UV spectra identical with that published by Miller et al. (1966) for 8-(*N*-2-fluorenylamino)guanine (G-AF). The 7-fluoro and 7-iodo derivatives 8-(*N*-2-fluorenylamino)guanine (G-AFF and G-AIF, respectively) showed similar UV spectra as G-AF with bathochromic shifts of 5 and 10 nm, respectively. Product III (R_f 0.47–0.48) which appeared as a minor spot (probably another fluorene–purine adduct) has so far not been identified (see Discussion).

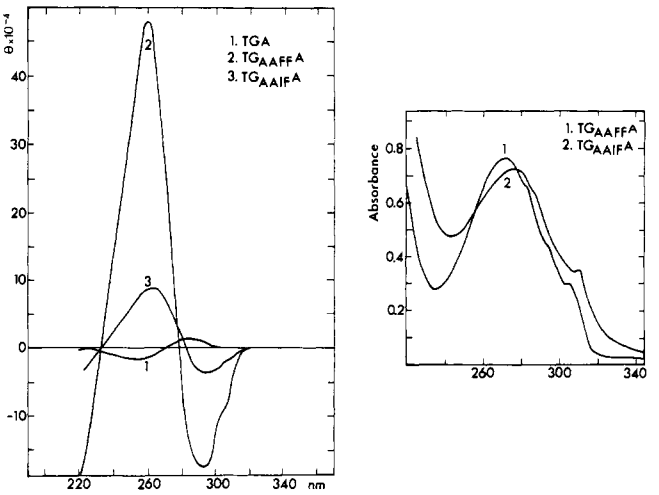

FIGURE 2: Comparison of the circular dichroism spectra of TGA, $TG_{AAFF}A$ and $TG_{AAIF}A$ in water and UV absorption spectra of TGA modified with AAFF and AAIF, in water.

Determination of the Percentage of Arylamidation and Arylation of DNA-AAIF. According to the procedure published previously (Fuchs, submitted), we determined the relative amount of arylamidation and arylation products in both native and heat denatured DNA modified with *N*-AcO-[^{14}C]AAIF.

Results and Discussion

Identification of the Addition Products. The 7-fluoro and 7-iodo derivatives of *N*-AcO-AAF showed almost the same chemical reactivity toward GMP and native and heat-denatured DNA as the parent compound itself. Moreover, as shown in this paper, the reaction products of *N*-AcO-AAFF and *N*-AcO-AAIF with GMP exhibit similar UV, ^{1}H NMR spectra, and chromatography data as 8-(*N*-2-fluorenylacetamido)guanosine 5′-phosphate (GMP-AAF) which is the well-defined addition product of *N*-AcO-AAF with GMP (Kriek et al., 1967). It is therefore tempting to identify the addition products between GMP and the 7-halogeno derivatives of *N*-AcO-AAF to the 7-fluoro and 7-iodo derivatives of GMP-AAF, respectively. Nevertheless, a direct synthesis, similar to that described for G-AF (Kriek et al., 1967), would be needed to prove unequivocally these structures. The conversion of GMP-AAFF and GMP-AAIF to G-AFF and G-AIF, respectively, by acidic hydrolysis led us to identify (by UV spectra and R_f values on silica gel TLC) the products with the major addition product (product IV) obtained from the depurination mixture of native DNA-AAFF and DNA-AAIF. The TLC plates show, however, a minor addition product (product III). Since this latter derivative is also present in the depurination mixture of native DNA-AAF, it is

TABLE II: Respective Amount of Arylamidation and Arylation in Native and Heat-Denatured DNA-[^{14}C]AAIF Samples.

	Native DNA-[^{14}C]-AAIF	Denatured DNA-[^{14}C]-AAIF
% of modified bases[a]	0.11–2.45	6.0
% of bases modified via arylamidation[b]	77	97
% of bases modified via arylation[c]	23	3

[a] The percentage of modified bases is obtained by the ratio [C]/[P] where [C] is the concentration of [^{14}C]AAIF and [P] the concentration of nucleotides. [b] The relative amounts of counts in the supernatant fraction of the ethanol precipitation are given. [c] The relative amounts of counts in the pellet fraction of the ethanol precipitation are given.

tempting to identify product III with the acidic hydrolysis product of 3-(deoxyguanosine-N^2-yl)-AAF, the minor addition product identified by Westra et al. (1976). If so, product III obtained by chromatography of the depurination mixture of native DNA–AAFF and DNA–AAIF would be the arylation addition products obtained by covalent linkage to the 2-NH_2 group of guanine of carbon C-3 of AAFF and AAIF, respectively. This interpretation seems to be correct since by means of the radiochemical determination we found that 24% of the [^{14}C]AAIF residues are linked via an arylation reaction in native DNA-[^{14}C]AAIF (see the results in the next section).

Radiochemical Determination of the Amount of Arylamidation and Arylation in Native and Heat-Denatured DNA-[^{14}C]AAIF. The overall reactivity of denatured DNA is three to four times greater than that of native DNA; this result is similar to that observed with *N*-AcO-AAF (Miller et al., 1966; Lang et al., 1978).

We determined the amount of arylamidation and arylation according to the assay described previously (Fuchs, submitted). Native and heat denatured chicken erythrocyte DNA have been modified with *N*-AcO-[^{14}C]AAIF. Native DNA-[^{14}C]AAIF samples with different percentages of modified bases (ranging from 0.11% to 2.45%) showed almost all the same modification pattern. In fact, the arylation reaction represents 23–24% of the total amount of bound [^{14}C]AAIF residues (Table II). On the other hand, a heat-denatured DNA sample exhibits almost only AAIF addition via arylamidation (97% of arylamidation) (Table II). As a consequence of this result we can be sure that the AAIF modified deoxytrinucleotides only contain arylamidation adducts (i.e., adducts to C-8 of guanine). Since the same situation occurs with AAF (denatured DNA reacts only on C-8 with N-AcO-AAF) (Fuchs, submitted), we will assume that it is also true in the case of AAFF modified deoxytrinucleotides.

A second hydrolysis of the pellet fraction of a given native DNA-[^{14}C]AAIF shows that almost all (93%) of the nonalkali labile [^{14}C]acetyl groups present in this fraction are recovered in the second pellet fraction. This is in good agreement with the results obtained previously with other *N*-acylacetamides (Fuchs, submitted; Fuchs et al., submitted). The distribution of the covalently linked AAIF residues to both native and heat denatured DNA is very similar to that obtained previously with *N*-AcO-AAF (Fuchs, submitted).

Circular Dichroism Spectroscopy on AAFF and AAIF Modified Deoxytrinucleotides. Deoxytrinucleotide (AGA and TGA) had only poor optical activity in the 220 to 300 nm range (Figures 2 and 3). Specific attachment of AAFF or AAIF to

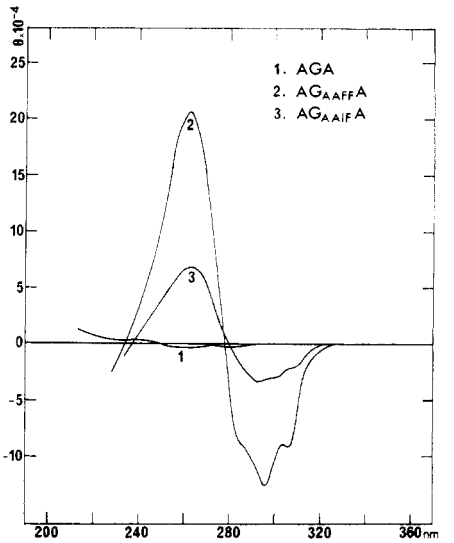

FIGURE 3: Comparison of the circular dichroism spectra of AGA, AG$_{AAFF}$A, and AG$_{AAIF}$A in water.

the central guanosine residue of AGA or TGA caused spectacular changes in their circular dichroism spectra (Figures 2 and 3). This effect had already been observed in AAF modified trinucleotides (Nelson et al., 1971).

From a qualitative point of view the shapes of the spectra of TG$_{AAFF}$A, TG$_{AAIF}$A, AG$_{AAFF}$A, and AG$_{AAIF}$A were similar. We noticed a red shift when the fluoro derivative was replaced by its iodo analogue.

Let us consider two regions in each spectrum: (1) below 300 nm which is the common absorption region of both nucleic bases and fluorene residue (in this range, the CD spectrum is a combination of the dichroic bands induced by the coupling of all the transition dipole moments); (2) above 300 nm, where only the fluorene residue exhibits optical transitions. At 305 nm, which corresponds to a vibronic band of the absorption spectrum of *N*-AcO-AAFF, there is a shoulder in the CD spectra of TG$_{AAFF}$A (Figure 2) and AG$_{AAFF}$A (Figure 3) with a negative ellipticity value. In the case of the corresponding AAIF modified deoxytrinucleotides this shoulder appears at 310 nm (Figures 2 and 3). In this spectral range, the CD spectrum could be interpreted on the basis of a one-electron theory (Condon et al., 1937) as induced by the asymmetric environment of the fluorene chromophore.

The intensity of the CD signal depends firstly on the nature of the fluorene derivative attached to the guanosine residue in a given deoxytrinucleotide (see the spectra of TG$_{AAFF}$A and TG$_{AAIF}$A for instance, Figure 2), and secondly on the sequence of the triplet in which guanosine is modified by the same fluorene derivative (sequence effect: see the spectra of TG$_{AAFF}$A, Figure 2, and AG$_{AAFF}$A, Figure 3).

In order to investigate the first effect, we normalized the ellipticity value at 305 or 310 nm of TG$_{AAFF}$A or TG$_{AAIF}$A with the corresponding ellipticity value of GMP-AAFF or GMP-AAIF, respectively. We obtained:

$$\frac{\theta^{305}_{TG_{AAFF}A}}{\theta^{305}_{GMP\text{-}AAFF}} = 18 \text{ and } \frac{\theta^{310}_{TG_{AAIF}A}}{\theta^{310}_{GMP\text{-}AAIF}} = 2$$

Similar results are obtained with AGA triplet:

$$\frac{\theta^{305}_{AG_{AAFF}A}}{\theta^{305}_{GMP\text{-}AAFF}} = 22 \text{ and } \frac{\theta^{310}_{AG_{AAIF}A}}{\theta^{310}_{GMP\text{-}AAIF}} = 2.5$$

It is clear, from these results, that the induced CD in fluorene chromophore is greater for AAFF than for AAIF. This

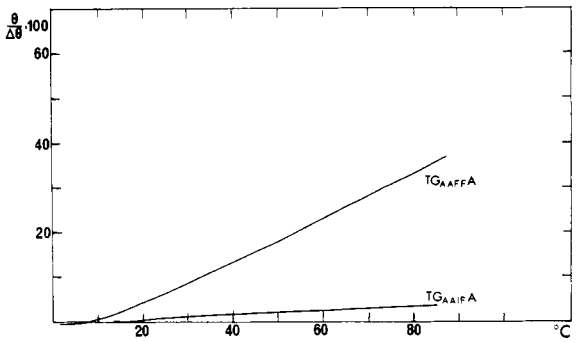

FIGURE 4: Temperature dependence of the normalized ellipticity ($\theta_t - \theta_0/\theta_0$) ($\theta_0$ was taken at 5 °C), at 305 nm for $TG_{AAFF}A$ and at 310 nm for $TG_{AAIF}A$. The modified deoxytrinucleotides were dissolved in 0.005 M Tris-HCl buffer, pH 7.

suggests that AAFF interacts more closely with the nucleic bases than AAIF.

The second effect, referred to as sequence effect, can be observed when comparing the induced optical activity in deoxytrinucleotides with different base sequence modified with a given fluorene derivative. This is illustrated by the magnitude of the ellipticity at 260 nm of $TG_{AAFF}A$ which is about two times as great as that of $AG_{AAFF}A$. On the other hand, the corresponding AAIF modified deoxytrinucleotides exhibit almost the same ellipticity values over all the spectra. The AAIF chromophore seems thus to be insensitive to the replacement of a thymidine residue by an adenosine residue, in a position adjacent to the modified guanosine.

We suggest, from these observations, that the fluorofluorene ring interacts very closely with the other constituents of the deoxytrinucleotides, so that the CD spectrum depends strongly on the nature of the bases and their respective position. The results obtained with the iodo derivative (no sequence effect and lower induced CD signal) suggest that the iodofluorene ring does not so closely interact with the neighboring bases as compared with the fluoro derivative. It looks as if the AAIF chromophore only experiences the average field of the oligonucleotide.

A tentative model would be to assume the AAFF residue stacked with the adjacent bases while the guanosine is shifted outside (unstacked). Analogue conclusions have previously been reached on AAF modified oligonucleotides (Nelson et al., 1971). On the other hand, the AAIF residue is expected to be unstacked, leaving the bases well stacked. This scheme would therefore fit to the general insertion–denaturation or outside binding model described for the conformation of the DNA–AAF (Fuchs & Daune, 1971, 1972) and DNA–AAFF (Fuchs & Daune, 1973) or DNA–AAIF (Fuchs & Daune, 1973; Fuchs et al., 1976), respectively. We followed the circular dichroism of modified deoxytrinucleotides under conditions known to alter the conformation of oligonucleotides (temperature and solvent). The purpose was to investigate the induced optical activity in the fluorene residue in relation with the conformational changes experienced by the trinucleotide.

Temperature Effect. The conformational change induced by increasing the temperature in trinucleotides has been interpreted as an unstacking of the bases (Glaudiber et al., 1968; Ts'o et al., 1969; Warshaw & Cantor, 1970; Johnson et al., 1972).

We followed the CD signal, as a function of temperature, at 305 nm or 310 nm of AAFF or AAIF modified TGA, respectively (Figure 4). Between 5 and 80 °C, the relative variation of the 305 nm ellipticity of $TG_{AAFF}A$ showed a linear and

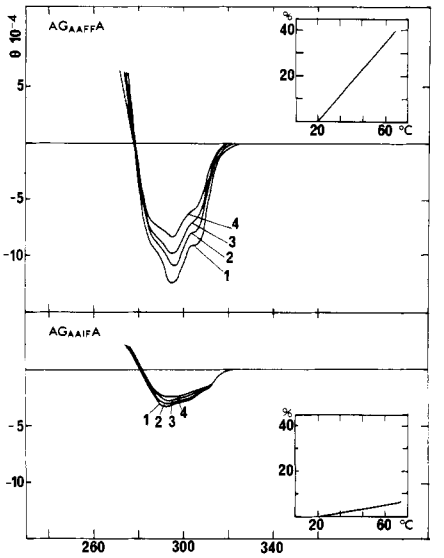

FIGURE 5: CD spectra in the 260–320-nm range, of $AG_{AAFF}A$ and $AG_{AAIF}A$, in 0.005 M Tris-HCl buffer, pH 7, at (1) 20 °C; (2) 40 °C; (3) 60 °C; (4) 80 °C. The temperature dependence of the normalized ellipticity ($\theta_t - \theta_0/\theta_t$) is shown in the inserts, at 305 nm for $AG_{AAFF}A$ and at 310 nm for $AG_{AAIF}A$ (θ_0 was taken at 20 °C).

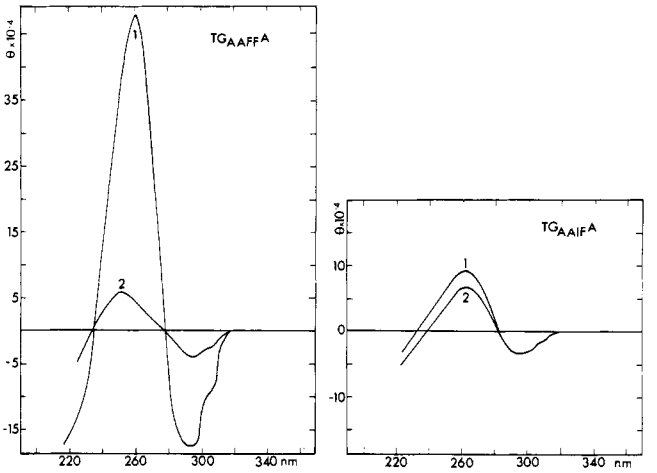

FIGURE 6: Comparison of CD spectra of $TG_{AAFF}A$, at 20 °C (1) in water and (2) in 1 M NaCl. Comparison of CD spectra of $TG_{AAIF}A$, at 20 °C (1) in water and (2) in 1 M NaCl.

reversible variation with temperature reaching a final value of 40%. The corresponding relative variation of $TG_{AAIF}A$ at 310 nm was almost negligible (Figure 4). Very similar results were obtained with the correspondingly modified AGA triplet (Figure 5). The relatively intense variation of the CD signal in the region of specific absorption of the fluorofluorene ring showed that this residue was directly involved in the unstacking process of the whole oligonucleotides. On the other hand, the iodofluorene residue appeared to be insensitive to the progressive base unstacking.

Effect of Ionic Strength. It is well known that, by increasing the ionic strength, one changes the conformation of the oligonucleotides, by decreasing the electrostatic repulsive forces between the phosphate groups. This conformational changes can be followed by circular dichroism (Johnson & Schleich, 1974; Brahms et al., 1969).

The ellipticity at 305 nm of $TG_{AAFF}A$ in H_2O decreased about four times when this modified deoxytrinucleotide was dissolved in 1 M NaCl (Figure 6). In the 260-nm range, the magnitude of the CD signal was also drastically lowered

(eightfold). However, the general shape of the spectrum was maintained. Similar results were obtained with $AG_{AAFF}A$. These data showed again that the conformational properties of the AAFF modified deoxytrinucleotides involve close interactions between fluorene ring and bases. In the case of $TG_{AAIF}A$ (Figure 6) and $AG_{AAIF}A$, only a small solvent effect was detected in the 260-nm range, while the ellipticity value remained unchanged at 310 nm. Although the structure of the trinucleotide was modified by increasing salt concentration (changes at 260 nm), the optical activity induced in fluorene by nucleic bases (ellipticity at 310 nm) did not change. Therefore, no close interaction occurs between AAIF and the deoxytrinucleotide constituents.

Conclusion

From a general point of view, the two 7-halogeno derivatives of *N*-AcO-AAF appeared to have a similar global reactivity toward native or heat denatured DNA as the parent compound itself. Moreover, the major binding site was also the C-8 of guanine as previously demonstrated for *N*-AcO-AAF (Miller et al., 1966; Kriek et al., 1967). By means of the radiochemical assay described in a previous paper (Fuchs, submitted), heat-denatured DNA was shown to react with *N*-AcO-[^{14}C]AAIF to give almost only addition via arylamidation. An arylation addition reaction (24% of the total amount of binding) occurred in native DNA-[^{14}C]AAIF. Similar results have been obtained with DNA reacted with *N*-AcO-[^{14}C]AAF (Fuchs, submitted). The binding site of *N*-AcO-AAIF or *N*-AcO-AAFF on the single-stranded deoxytrinucleotides was therefore assumed to be almost exclusively on C-8 of guanine. The AAFF and AAIF modified deoxytrinucleotides showed a large circular dichroism signal mutually induced in both the fluorene ring and the nucleic acid bases. Above 300 nm the induced circular dichroism signal is specific of the fluorene ring. We studied the variation of this signal as a function of several parameters (base sequence, temperature, solvent, . . .) known to affect the conformation of the oligonucleotides. All the results showed the circular dichroism signal induced in AAFF to be very sensible to the oligonucleotide conformational changes, whereas the optical activity induced in AAIF remained almost unchanged. These results are in good agreement with both the insertion-denaturation model and the outside binding model.

Let us summarize the general features of these two models: the insertion-denaturation model states that the fluorene ring is stacked between the two nearest base plates leading to a local melting of the double helix. It requires the passage of the guanine from its anti conformation to its syn conformation by rotation around the glycosyl linkage (Nelson et al., 1971). This model is valid for the major binding site (C-8 of guanine) in both DNA-AAF and DNA-AAFF (Fuchs & Daune, 1972, 1973; Fuchs et al., 1976).

In the outside binding model the aromatic nucleus is believed to lie in one of the DNA grooves. This model, which does not require such a large local denaturation of the double helix, has been coined in order to fit all the experimental data obtained for DNA-AAIF (Fuchs & Daune, 1973, Fuchs, 1975; Fuchs et al., 1976). It is at first look surprising to find that these models which have been established on the basis of studies with double helical DNA are valid in the cases of single-stranded deoxytrinucleotides. In fact, one could have been expecting that the differences in the conformational changes induced in DNA by the binding of the different fluorene derivatives were related to sterical restrictions imposed by the double helix. We have therefore not expected such differences to occur in the case of the modification of the more flexible structure of the deoxytrinucleotides. The results, however, tend to prove that the AAIF residues bound to the deoxytrinucleotides were unstacked. This is in contrast with both the AAFF modified deoxytrinucleotides and the AAF modified oligonucleotides (Nelson et al., 1971) in which cases the fluorene ring comes in stacking interaction with the adjacent bases.

On the basis of the double helical model of DNA (CPK), it was possible to fit an AAIF residue in the large groove along the phosphate-sugar backbone, without rotating guanine from an anti to a syn conformation. In this model the geometry of the double helix is only slightly affected in agreement with the findings that: (i) the thermal destabilization is weak (Fuchs & Daune, 1973); (ii) DNA-AAIF is almost insensible to the hydrolysis by the single-stranded endonuclease S_1 (Fuchs, 1975). To confirm this hypothesis, NMR studies on the AAIF modified deoxytrinucleotides are in progress. From the molecular model it appears that the angle between the fluorene ring and the helix axis is approximatively equal to 60°, a value consistent with our previous data (Fuchs et al., 1976). On the other hand, when the guanine comes in its syn conformation (for example, if modified by AAF or AAFF) all the three atoms implied in the hydrogen bonds of the Watson-Crick base pair become accessible from the large groove. This has been experimentally confirmed by the finding that kethoxal, a reagent specific for the non-H-bonded guanine residues (Shapiro & Hachmann, 1966) reacts preferentially with DNA-AAF in contrast with the low reactivity of both DNA-AAIF and native DNA (Fuchs, unpublished results).

We want to stress the fact that though AAIF reacts mainly on the same position as AAF in guanine residues (the C-8) the conformational change induced by both derivatives is quite different. As already mentioned (Fuchs & Daune, 1973; Fuchs et al., 1976), the bulky iodine atom accounts probably for this difference by preventing the insertion of the fluorene ring.

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Replication of Antibiotic Resistance Plasmid R6K DNA in Vitro†

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ABSTRACT: A soluble extract prepared from cells of an *Escherichia coli* strain carrying the antibiotic resistance plasmid R6K is capable of carrying out the complete process of R6K DNA replication. DNA synthesis in vitro is dependent on the four deoxyribo- and ribonucleotide triphosphates and is sensitive to rifampin and streptolydigin, inhibitors of DNA-dependent RNA polymerase. The incorporation of deoxyribonucleotides into R6K DNA also is sensitive to actinomycin D, novobiocin, arabinofuranosyl-CTP, and *N*-ethylmaleimide. Kinetics of synthesis are linear for 60 to 120 min. Replication proceeds semiconservatively and supercoiled closed-circular DNA molecules are synthesized. Analysis by alkaline sucrose gradient centrifugation indicated that the early R6K DNA products contain DNA fragments of approximately 18 S in size, corresponding to the length between the R6K α origin of replication and the terminus of replication observed in vivo. Addition of exogenous supercoiled R6K DNA is inhibitory to the in vitro system, whereas the addition of R6K DNA in the form of relaxation complex stimulates R6K DNA synthesis to a small extent.

Bacterial plasmids, including the antibiotic resistance (R) plasmids, are circular duplex DNA molecules that are present stably in the extrachromosomal state in host cells. These elements have the fundamental character of autonomous replication as covalently closed circular DNA molecules and segregation to the daughter cells of the host bacterium (Clowes, 1972; Helinski, 1973, 1976). Plasmid DNA replication is a complex process that proceeds through several successive steps: initiation of DNA replication, semiconservative DNA synthesis, termination and segregation of daughter strands. An important approach to the study of the complex process of DNA replication is the systematic analysis of a replication system in vitro (Kornberg, 1974; Geider, 1976). With regards to plasmid replication, soluble systems have been derived from *Escherichia coli* cells that are capable of carrying out the replication of the plasmid ColE1 DNA (Sakakibara & Tomizawa, 1974a; Tomizawa et al., 1975; Staudenbauer, 1976). In these systems, cell extracts are made from chloramphenicol-treated *E. coli* cells and no plasmid-coded protein is required for ColE1 replication.

Plasmid R6K is a naturally occurring conjugative plasmid that determines resistance to the antibiotics ampicillin and streptomycin (Kontomichalou et al., 1970). The molecular weight of R6K is 25×10^6, and it exhibits a relaxed mode of replication in *E. coli* cells in that it is present as 11 to 13 copies per chromosome in log-phase cells (Kontomichalou et al., 1970). The mode of replication is unique in that it is bidirectional and proceeds sequentially to an asymmetric terminus from either of two possible origins (α and β) (Lovett et al., 1975; Crosa et al., 1975, 1976). It has also been reported that catenated molecules of this DNA are replicative intermediates (Kupersztoch & Helinski, 1973) and replicating R6K DNA molecules are found preferentially associated with the folded chromosome of *E. coli* (Wlodarczyk & Kline, 1976). Of the known host replication functions, at least the *dnaB, dnaC, dnaD* and *polC* (*dnaE*) genes are required for R6K DNA replication (Arai & Clowes, 1975). Finally, a large proportion of R6K DNA molecules can be isolated from cells in the form of a relaxation complex (Kupersztoch-Portnoy et al., 1974). The unique bidirectional mode of replication in vivo and other replication properties make R6K especially attractive for studying the molecular mechanism and regulation of replication of plasmid DNA in vitro. In this paper the properties of a cell-free extract system derived from *E. coli* cells and capable of replicating supercoiled R6K DNA are described.

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