Comparative Orientation of the Fluorene Residue in Native DNA Modified by *N*-Acetoxy-*N*-2-acetylaminofluorene and Two 7-Halogeno Derivatives†

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ABSTRACT: Native calf thymus DNA was reacted with *N*-acetoxy-*N*-2-acetylaminofluorene (*N*-AcO-AAF) and its 7-fluoro and 7-iodo derivatives. Different ways of purification of the fluorene modified DNA samples were checked in order to obtain a nucleic acid free from all noncovalently bound fluorene residues. The decrease in melting temperature in DNA samples modified by *N*-AcO-AAF (DNA-AAF) was carefully reinvestigated. From these experiments, we conclude that the melting temperature decrease is equal to 1.15 °C per percent of modified bases, in DNA-AAF samples. Electric dichroism measurements on sonicated DNA samples modified by the different fluorene derivatives show the fluorene ring perpendicular to the helix axis in the case of the *N*-AcO-AAF and its fluoro derivative, and lying along the phosphate-sugar backbone in the case of the iodo derivative. The results presented in this paper, along with those obtained earlier, led us to propose an "insertion-denaturation model" for the mode of binding of *N*-Aco-AAF and its fluoro derivative, and an "outside binding model" for the iodo derivative. Discrepancies with the data obtained by Chang et al. ((1974) *Biochemistry 13,* 2142-2148) concerning the melting temperature decrease and the electric dichroism results are observed and discussed.

It is well known that the carcinogenic aromatic amines bind in vivo to nucleic acids, proteins, and carbohydrates (Miller and Miller, 1967; Farber et al., 1967). The carcinogenic effect of aromatic amines has been shown (Miller, 1970) to be related to their strong electrophilic metabolites. In the case of AAF,[1] the ultimate derivative has been identified (Cramer et al., 1960; Miller and Miller, 1967, 1969) as an ester of *N*-OH-AAF. The synthetic acetic ester *N*-OH-AAF is currently used for the in vitro modification studies of nucleic acids. In this paper native DNA was reacted with three analogues of AAF: *N*-AcO-AAF and *N*-AcO-AAFF, two ester derivatives of the two carcinogens AAF and AAFF (Miller et al., 1955; Miller, 1970). The third analogue was *N*-AcO-AAIF, which is according to the now well-established mechanism of activation of arylamides (Miller, 1970), a possible metabolite of the noncarcinogen AAIF (Morris et al., 1960).

In the case of *N*-AcO-AAF, the main addition product has been shown to occur at position C-8 of guanine (Kriek et al., 1967; Miller et al., 1966). A conformational change from anti to syn was observed for AAF modified guanine in oligonucleotides (Nelson et al., 1971). The three-dimensional conformation of modified native DNA is not yet clear. In order to explain the local denaturation sites induced by the fluorene ring as seen by melting temperature decrease (Kapuler and Michelson, 1971; Fuchs and Daune, 1971, 1972; Troll et al., 1969) and by formaldehyde unwinding kinetic (Fuchs and Daune, 1973, 1974), we proposed an "insertion-denaturation model", where the fluorene ring is accommodated between the two nearest base plates (Fuchs and Daune, 1971, 1972). An analogous model was recently named by Levine et al. (1974) and Weinstein and Grunberger (1974) the "base displacement" model. More recently, however, Chang et al. (1974) measured the orientation of the fluorene in T_4 renatured DNA and found a value which is consistent with the AAF residue being constrained to lie along the helix winding angle.

In this paper we measured by electric dichroism the orientation of the fluorene ring covalently bound to native DNA at low substitution levels. The results clearly indicate the fluorene ring perpendicular to the helix axis in the case of DNA-AAF and DNA-AAFF. On the other hand, for the iodo derivative we found the angle between the transition moment of the fluorene molecule at 305 nm and the helix axis to be 60°.

Different purification techniques of the carcinogen modified DNA samples have been investigated in order to answer the Chang et al. (1974) objections concerning the possibility of noncovalently bound fluorene residues in our DNA samples. The results show that the different possible ways of purification of a given DNA sample all led to the same percentage of modified bases. Chang et al. (1974) also claimed the decrease in DNA thermal stability as being equal to 1.8 °C per percent of modified bases in DNA-AAF. We carefully measured the melting temperature of the different DNA samples, purified by two different ways. Accordingly, we maintain that the melting temperature depression value is equal to 1.15 °C per percent of modified bases for DNA-AA', in agreement with the value we published earlier (Fuchs and Daune, 1973).

Materials and Methods

Reagents. All chemicals were reagent grade (Merck). AAF has been synthesized as previously described (Lotlikar et al., 1966). The way of synthesis of the two compounds AAFF and AAIF will be described in a subsequent paper.

DNA Preparation. Native DNA was prepared from calf thymus (Kay et al., 1952) and had the following characteristics: hypochromicity at 260 nm, 42%; $s_{20,w}$ = 21 S; protein

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[1] Abbreviations used: AAF, AAFF, and AAIF, *N*-2-acetylaminofluorene, and 7-fluoro and 7-iodo derivatives; *N*-OH-AAF, *N*-hydroxy-*N*-2-acetylaminofluorene; *N*-AcO-AAF, *N*-AcO-AAFF, and *N*-AcO-AAIF, *N*-acetoxy-*N*-2-acetylaminofluorene and 7-fluoro and 7-iodo derivatives; DNA-AAF, DNA-AAFF, and DNA-AAIF, DNA modified covalently with *N*-Aco-AAF, *N*-AcO-AAFF, *N*-AcO-AAIF, respectively; NMR, nuclear magnetic resonance.

TABLE I: Different Purification Techniques of the DNA-AAF Samples.

	3 Ethyl Ether Extractions (without Dialysis)		Method 1[a]		Method 2[a]		Method 3[a]	
Sample No.	$(OD)_{305}/(OD)_{260}$	x^b	$(OD)_{305}/(OD)_{260}$	x^b	$(OD)_{305}/(OD)_{260}$	x^b	$(OD)_{305}/(OD)_{260}$	x^b
1	0.0877	4.0	0.0810	3.8	0.0815	3.8	0.084	3.9
2	0.136	6.6	0.1345	6.6	0.1335	6.5	0.134	6.6
3	0.220	13.0	0.216	12.5	0.213	12.3	0.216	12.5

[a] The different methods are described in Materials and Methods. [b] x is the percentage of modified bases determined from the $(OD)_{305}/(OD)_{260}$ ratio (Fuchs and Daune, 1972).

TABLE II: Melting Temperature Values of Different DNA-AAF Samples.

DNA-AAF Purified by Method 1[a]		DNA-AAF Purified by Method 2[a]	
x^b	T_m (°C)	x^b	T_m (°C)
0	58.5	0	58.9
3.8	53.4	3.8	54.1
6.9	49.9	6.8	50.0
12.5	44.5	12.3	44.5

[a] The different methods as described in Materials and Methods. [b] x is the percentage of modified bases determined from the A_{305}/A_{260} ratio (Fuchs and Daune, 1972). All the melting experiments have been done in sodium citrate buffer, 2×10^{-3} M/l. (pH 7).

content as determined by the method of Lowry, 0.38% in weight.

Sonicated DNA was obtained by treating native DNA in 0.1 M NaCl, 2×10^{-3} M sodium citrate buffer (pH 7), during 4 min, at 20 kHz (Litzler, 1967) to yield molecule fragments of about 500 000 daltons. To avoid DNA single-stranded ends as eventually produced during the sonication step, we treated the sonicated DNA with S_1 endonuclease from *Aspergillus oryzae* (generous gift from Professor Chambon, Strasbourg) in the conditions described by Vogt (1973). Finally the sonicated DNA was dialyzed against 2×10^{-3} M sodium citrate buffer (pH 7) before the carcinogen reaction step.

Carcinogen Reaction and Purification of the Modified DNA Samples. Reactions of DNA with AAF, AAFF, and AAIF were performed in a way similar to that described by Miller et al. (1966) by incubating the aqueous ethanol solution (2×10^{-3} M sodium citrate buffer (pH 7)-ethanol, 80:20 (v/v)) during 3 h at 37 °C under argon atmosphere. The purification step (i.e., preparation of a modified nucleic acid where all noncovalently bound fluorene residues are removed) was achieved by three different methods: (method 1) three extractions with 2 volumes of ethyl ether, followed by a dialysis against 2×10^{-3} M sodium citrate buffer (pH 7) (four buffer changes); (method 2) three extractions with 2 volumes of ethyl ether, followed by a dialysis against 2×10^{-3} M sodium citrate buffer (pH 7)-ethanol, 50:50 (v/v) (four buffer changes), and finally against 2×10^{-3} M sodium citrate buffer (pH 7) (one buffer change); (method 3) three extractions with 2 volumes of ethyl ether, followed by CsCl gradient purification.

In each method, the purification was followed by a short centrifugation at 20 000 rpm during 20 min.

CsCl gradient purification was performed in an analytical ultracentrifuge Beckman E type equipped with a scanning device (Neimark, 1975). The DNA-AAF samples (3 μg) were run in 0.5 ml of a 7.7 *m* CsCl solution during 28 h at 44 000 rpm at 18.8 °C. Each cell was scanned at 260 and 305 nm by steps of 3.61×10^{-3} and 3.00×10^{-3} cm, respectively. The area under each peak was determined and the ratio of the area at 305 nm over the area at 260 nm was calculated.

Melting Temperature. The melting curves were obtained on a XY recorder giving absorbance vs. temperature (Wilhelm et al., 1970).

Electric Dichroism. All the electric dichroism measurements have been done with sonicated DNA, with an apparatus built by Sturm (1974).

The electric pulse, applied between 1-mm distant electrodes, has an intensity equal to 500 V, a duration of 800 μs, and a short rise time (0.1 μs). The response of the molecule is measured as the variation $\Delta I_\parallel$ of transmitted light, polarized parallel to the electric field. The steady-state or plateau value depends upon the degree of orientation of the macromolecule and upon the directions of the contributing transition moment with respect to the DNA molecule axis. For a fibrous or a rod-like molecule, the general expression $\Delta I_\parallel/I$, at steady state is (Sturm, 1974):

$$\Delta I_\parallel/I = (3\cos^2\chi - 1)(2.3/15)(\mathrm{OD})AE^2 \qquad (1)$$

where I is the transmitted light intensity in absence of field, χ the angle between the transition moment observed and the DNA molecule axis, OD the optical density, and AE^2 the product of an orientation function A and the square of the electric field. The optical anisotropy of modified DNA was measured at three wavelengths. At 265 nm, the absorption is mainly due to the DNA bases, owing to the fact that we use fluorene modified DNA samples having only ~2% of their bases modified. Accordingly, the fluorene absorption never exceeded 5% of the total absorbance. Therefore, at this wavelength the optical anisotropy is due to the $\pi \rightarrow \pi^*$ transition of the DNA bases, which lies in or close to the base planes. At 302 and 313 nm, the contribution of DNA bases absorption is negligible. The optical anisotropy is therefore due to the fluorene $\pi \rightarrow \pi^*$ transition. This type of transition lies in the plane of the aromatic ring.

These wavelengths correspond to peaks of intensity in the xenon-mercury lamp spectrum, which we used for these measurements. In such a way, the error on optical density due to the bandwidth of the monochromator is minimized. As we consider that the base planes are perpendicular to the DNA axis, we can write:

$$\left(\frac{\Delta I_\parallel}{I}\right)_{265\mathrm{nm}} = -\frac{2.3}{15}(\mathrm{OD})_{265\mathrm{nm}}AE^2 \qquad (2)$$

correspondingly, at 302 or 313 nm we have:

TABLE III: Electric Dichroism Measurements on Different Fluorene Modified DNA Samples.[a]

		$(\Delta I_\parallel/I)$/OD			χ^c (degrees)	
	x^b	265 nm	302 nm	313 nm	302 nm	313 nm
DNA	0	7×10^{-2}				
DNA-AAF	0.7	4.54×10^{-2}	4.24×10^{-2}		81.5	
DNA-AAFF	2.0	2.98×10^{-2}	2.67×10^{-2}	2.71×10^{-2}	79	80
DNA-AAIF	2.0	7.77×10^{-2}	3.57×10^{-2}	3.2×10^{-2}	65	64

[a] All the DNA samples have been sonicated prior to the modification step (Materials and Methods). [b] x is the percentage of modified bases determined from the $(OD)_{305}/(OD)_{260}$ ratio (Fuchs and Daune, 1972). [c] χ is the angle between the transition moment of the fluorene ring and the DNA axis.

$$\left(\frac{\Delta I_\parallel}{I}\right)_{302nm} = -\frac{2.3}{15}(1 - 3\cos^2\chi)AE^2(OD)_{302nm} \quad (3)$$

the direct ratio yields the following expression:

$$\frac{\left(\frac{\Delta I_\parallel}{I}\right)_{302nm}}{\left(\frac{\Delta I_\parallel}{I}\right)_{265nm}} = (1 - 3\cos^2\chi)\frac{(OD)_{302nm}}{(OD)_{265nm}} \quad (4)$$

which gives us the value of the angle χ. At each wavelength, dilutions were made in order not to exceed an optical density of 0.5.

Results

Purification of the DNA-AAF Samples. We tried three different ways of purification of the DNA after the carcinogen reaction (see Material and Methods) in order to verify the affirmation made by Chang et al. (1974) concerning the preparation of a DNA-AAF sample free from all noncovalently bound fluorene residues.

Method 1 was commonly used by us in all our previous work, method 2 takes account of the suggestion made by Chang et al. (1974) (i.e., dialysis against a buffer containing 50% ethanol), and finally method 3 was designed according to the suggestion of Maher et al. (1968). The results (Table I) clearly show that all the different ways of purification led to a modified nucleic acid having the same ratio OD_{305}/OD_{260} and, therefore, the same percentage of modified bases (Fuchs and Daune, 1972). As mentioned in Table I, even after the ethylic ether extractions, the DNA-AAF was almost freed from the noncovalently bound residues.

Melting Temperature Depression. The melting temperature was measured for the DNA-AAF samples obtained by methods 1 and 2, in 2×10^{-3} M, sodium citrate buffer (pH 7), in order to test the data obtained by Chang et al. (1974). These authors found a value of 1.8 °C of destabilization by percent of modified bases in DNA-AAF samples purified by a method similar to method 2. Our results (Table II) agree with those we have published previously (Fuchs and Daune, 1973). The value which can be derived from these data is 1.15 °C of destabilization by percent of modified bases.

Electric Dichroism. We measured by electric dichroism, the orientation of the fluorene ring, expressed as the angle χ between the fluorene ring and the helix axis of the DNA. The values of this angle χ were calculated by mean of the results obtained at two wavelengths: 302 and 313 nm (see Materials and Methods). For each fluorene derivative, these values (Table III) were the mean value derived from three experiments. The greatest divergence between the results was found

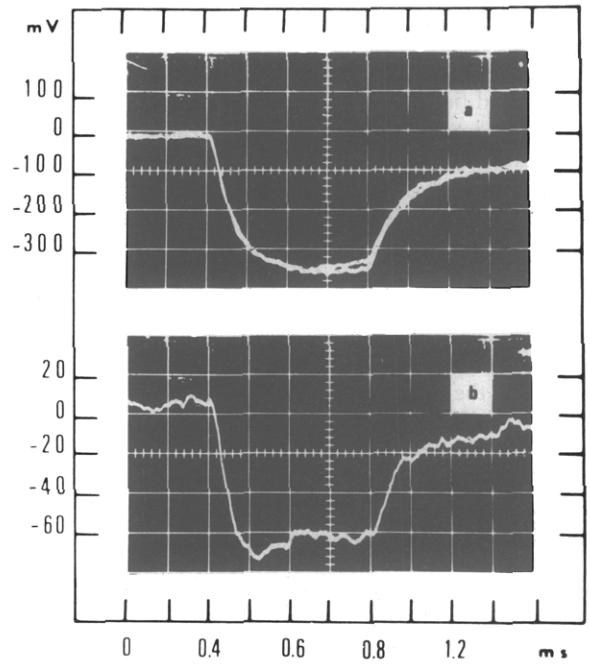

FIGURE 1: Electric dichroism experiment; example of oscilloscope traces showing the optical response to an electric pulse (for its characteristics, see Materials and Methods) of a DNA-AAIF sample having 2% of modified bases in 10^{-3} M/l. sodium citrate buffer (pH 7). (a) At 265 nm, $(OD)_{265}^{1cm} = 0.47$; (b) at 302 nm, $(OD)_{302}^{1cm} = 0.23$. The plateau value corresponds to $\Delta I_\parallel$; the intensity I of transmitted light is maintained constant and equal to 10 V in all experiments.

to be 4°. We found the angle between the fluorene ring and the helix axis to be $\simeq$80° in the case of DNA-AAF and DNA-AAFF, and equal to $\simeq$65° in the case of DNA-AAIF. An example of oscilloscope traces obtained with the DNA-AAIF sample is shown in Figure 1. In order to compare the orientation under electric field of the different modified DNA, we reported in Table III the value of $(\Delta I_\parallel/I)$/OD which depends on the angle χ, and on the orientation of the macromolecule (orientation function A), the electric field E being kept constant in all experiments.

$$\frac{\Delta I_\parallel/I}{OD} = \frac{2.3}{15}(3\cos^2\chi - 1)AE^2 \quad (5)$$

At 265 nm, as χ for the DNA base plate is equal to 90°, the expression 5 becomes proportional only to the orientation function:

$$\left(\frac{\Delta I_\parallel/I}{OD}\right)_{265nm} = -\frac{2.3}{15}AE^2 \quad (6)$$

It appears (Table III) that the orientation of the macromolecule depends on the nature of the fluorene derivative bound to the DNA. This point will be discussed in the next section.

Discussion

Purification and Melting of DNA-AAF Samples. The three different methods of preparation of a modified DNA sample free from the noncovalently bound fluorene residues (see Materials and Methods) led all to a nucleic acid having the same percentage of modified bases (Table I). Therefore, in the condition used, dialysis against an ethanol-containing buffer (Chang et al., 1974) or purification by CsCl banding (Maher et al., 1968) does not remove more fluorene derivatives than does dialysis against a nonethanol-containing buffer.

Concerning the melting temperature depression in DNA-AAF samples, we confirm (Table II) the value (−1.15 °C/% of modified bases) we published in a previous paper (Fuchs and Daune, 1973). Therefore, we do not agree with the value (−1.8 °C/% of modified bases) found by Chang et al. (1974). It may be that the greater value proposed by these authors can be explained by the fact they melted DNA samples which have been renatured after the AAF modification step. Therefore, it is likely that the renaturation is not perfect, leading to a general destabilization which is greater than that we observed by melting native DNA-AAF samples (i.e., samples which have been reacted with AAF in a native state).

Angle of the Fluorene Ring with Respect to the Helix Axis. As shown (Table III) DNA-AAF and DNA-AAFF give approximately the same orientation for the fluorene nucleus with respect to the DNA axis (80°). Here again we are in apparent contradiction with the results of Chang et al. (1974), who found in the case of DNA-AAF an angle χ equal to 60°. However, as we mentioned above, these authors measured the orientation of the fluorene ring after renaturation of the DNA sample which has been reacted with the carcinogen in a denatured state. Thus, during the renaturation step, the rearrangement between the base plates and the fluorene rings (formation of the correct hydrogen-bonded secondary structure) is possibly not as faithful as is the secondary structure of a DNA sample modified in the native state. Moreover, Chang et al. (1974) used for their electric dichroism measurements a DNA-AAF sample having 12% of modified bases. This large amount of modified bases is, in our point of view, highly prejudicial for the correct measure of the orientation of the fluorene ring. In fact, a renatured DNA sample having randomly each eight bases plates a modified base (which brings the modified base from its anti conformation to a syn conformation by rotation around the glycosyl linkage) is not comparable to a native DNA modified to a low extent (<2% in this work). Thus the experimental values found by Chang et al. (1974) are probably not in error, but the extreme conditions (renaturation of a highly modified DNA) used by these authors do not allow any speculation relative to the orientation of the fluorene bound at low extent to a native DNA sample. Our results are compatible with the fluorene insertion followed by the DNA local denaturation (Fuchs and Daune, 1972). This model has later been confirmed by Levine et al. (1974).

In the case of DNA-AAIF, the values of 65° (Table III) for the angle between the fluorene and the helix axis is not compatible with the insertion model. The iodine atom, which constitutes a bulky atom, does probably hinder the insertion of the fluorene ring (Fuchs and Daune, 1973, 1974). It is interesting to note the correlation with the van der Waals diameters of H, F, and I that are equal to 2.4, 2.7, and 4.3, respectively. These values can be compared with 3.4 Å which is

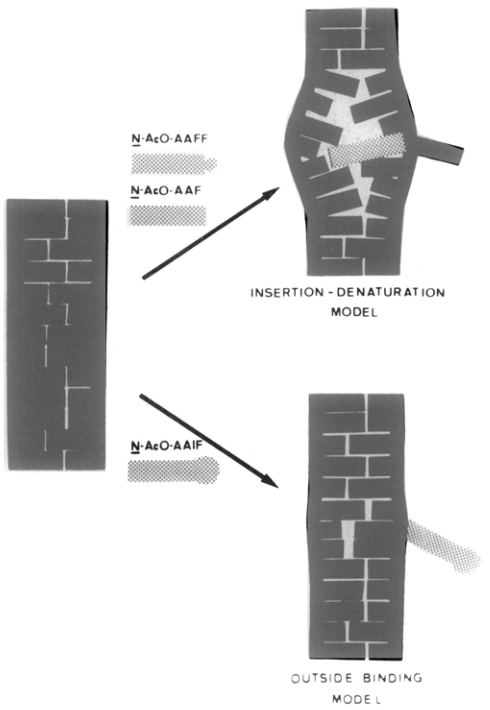

FIGURE 2: Insertion–denaturation and outside binding model. Schematic model of a native DNA sample after reaction with the different fluorene derivatives. The DNA base plates and the fluorene derivatives are represented by the black and the grey rectangles, respectively. The upper part shows the local denaturation produced by the insertion of either *N*-AcO-AAF or its 7-fluoro derivative. The lower part shows the outside binding of the 7-iodo derivative which does not induce important local melting. As shown the iodine atom confers to the molecule a "molecular thickness" which probably hinders the insertion. The disrupted base pairs depicted in this figure represent approximatively the number of open base pairs calculated from the formaldehyde unwinding experiments at 15–20 °C below the melting temperature (Fuchs and Daune, 1974).

the distance between two base plates in native DNA. It remains, however, to establish clearly the chemical nature of the addition products in this case (work in progress). Analogous results (insertion of AAF and AAFF between the nearest base plates and exclusion of the iodo fluorene ring) were obtained with two fluorene modified deoxytrinucleotides (AGA and TGA) (circular dichroism and NMR studies, work in progress). On the other hand, in the case of DNA-AAF and DNA-AAFF, we do not find the fluorene ring exactly perpendicular to the DNA axis (Table III). This can be explained in two different ways.

1. The insertion of the fluorene ring induces local denaturation. For steric reasons, in these sites, the base plates and consequently the fluorene ring may not be exactly perpendicular to the helix axis.

2. Kriek (1974) recently showed that two addition products are found, in vitro as well as in vivo, in DNA-AAF samples. Eighty percent of the AAF residues are linked to C-8, and 20% to N-2 in guanine. Kriek speculates, on the basis of molecular models, that the fluorene ring attached to N-2 in guanine, does not require a conformational change of the DNA, the fluorene ring lying in the small groove. Therefore, the signal of linear dichroism would be issued from two different types of fluorene: One exactly perpendicular to the helix axis ($\chi = 90°$, 80% of the total fluorene); and one lying along the phosphate–sugar backbone of the DNA ($\chi = 60°$, 20% of the total fluorene).

Taking in account, the linearity of $\Delta I_{\parallel}/I$ with the square of cos χ, we calculated the apparent angle corresponding to this

signal. The theoretically obtained value in such a model (77°) is close to the experimentally measured one (80°).

Orientation Properties of the Different DNA Samples. The comparison at 265 nm of the different $(\Delta I_{\parallel}/I)$OD values (Table III) inquires about the orientation properties of the different modified DNA samples in an electric field. The greater this value, the better the macromolecule orientation. In fact, at 265 nm this value becomes directly proportional to the orientation function A (eq 6). The DNA-AAIF sample is oriented as well as the DNA sample itself. On the other hand, the values obtained for DNA-AAF and DNA-AAFF are much smaller, indicating a poorer orientation of these two samples. This observation has to be related to the introduction of bending sites (as seen by viscosity and light scattering) in native DNA modified by the AAF residue (Fuchs and Daune, 1972). The local denaturation introduced by the AAF and AAFF residues gives rise to hinge points, preventing a good orientation of the macromolecule. This effect is no longer observed in the case of the DNA-AAIF sample, which is as stiff as native DNA itself.

In conclusion, we can distinguish two different addition products (Figure 2).

1. The first is denaturating addition products (mainly DNA-AAF and DNA-AAFF), in which the fluorene ring gives rise to single-stranded like regions; these regions have recently been shown recognized and excised by the S_1 endonuclease from *Aspergillus oryzae*. This type of covalent binding is described as the "insertion-denaturation model" (Fuchs, 1975).

2. The other is nondenaturating addition products (DNA-AAIF) in which the insertion of the fluorene ring is probably hindered (in this case by the bulky iodine atom). The fluorene residue is probably lying along the phosphate-sugar backbone (outside binding).

The mechanism of repair of these two kinds of addition products is currently being investigated in a cell culture system.

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