PHYSICAL BASIS OF CHEMICAL CARCINOGENESIS BY *N*-2-FLUORENYLACETAMIDE DERIVATIVES AND ANALOGS

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1. Introduction

Analogs and derivatives of the chemical carcinogen *N*-2-fluorenylacetamide (AAF), after metabolic activation, are known to induce tumors in many different tissues [1–8].

By using synthetic analogs of these metabolites, we have a unique opportunity to study *in vitro* the relationship between carcinogenic activity and physical modification of DNA.

Calf thymus DNA was reacted *in vitro* with *N*-acetoxy-*N*-2-acetylamino-7-fluorofluorene (AAAFF), an ultimate metabolite of the strong carcinogen *N*-2-acetylamino-7-fluorofluorene (AAFF) [2] and with *N*-acetoxy-*N*-2-acetylamino-7-iodofluorene (AAAIF) a possible metabolite of the non-carcinogen *N*-2-acetylamino-7-iodofluorene (AAIF) [7].

In the case of *N*-acetoxy-*N*-2-acetylaminofluorene (AAAF) and AAAFF, the covalently linked fluorene induces local denaturation of the double helix. In fact, the modified base is shifted outside the double helix, while the fixed carcinogen is inserted. By means of kinetic experiments, we have shown that "breathing" of the DNA duplex takes place preferentially in the neighborhood of each modified base plate. Such an effect was no longer observed with AAAIF, which affects only slightly the thermal stability of the DNA molecule and its dynamic properties.

2. Materials and methods

The way of synthesis of the two new compounds AAAFF and AAAIF will be described elsewhere [9]. DNA was the same as described in a preceding paper [10].

The reaction of DNA with AAAF, AAAFF and AAAIF, the extraction and the purification of the modified DNA were performed in a way similar to that described by Miller et al. [11]. In the ultraviolet spectrum of the modified DNA, a shoulder appears at 305 nm, 310 nm and 315 nm with AAAF, AAAFF and AAAIF, respectively.

The determination of the iodine in the DNA–AAIF samples by the ultramicromethod described by Spitzy et al. [12] enables us to calculate the specific extinction coefficient of the addition product at 310 nm. The percentage of modified bases in AAAIF-reacted DNA was calculated according to our method [13]. In the case of DNA–AAFF, work is now in progress to titrate, after mineralisation, the fluorine ion by means of a specific electrode.

3. Results and discussion

3.1. *Circular dichroism*

Upon binding of the fluorene derivatives, a negative band appears at 300 nm (fig. 1). It has been shown [13] that for a given carcinogen, the intensity of the signal is only dependent of the total amount of carcinogen bound and not of the ratio carcinogen/DNA.

3.2. *Melting curves*

As in the case of DNA–AAF [13–15], we observed with DNA–AAFF a linear decrease of T_m of about 1.1C° per 1% of modified bases.

With DNA–AAIF, the destabilizing effect is three times smaller (fig. 2). At 305 nm, a cooperative melt-

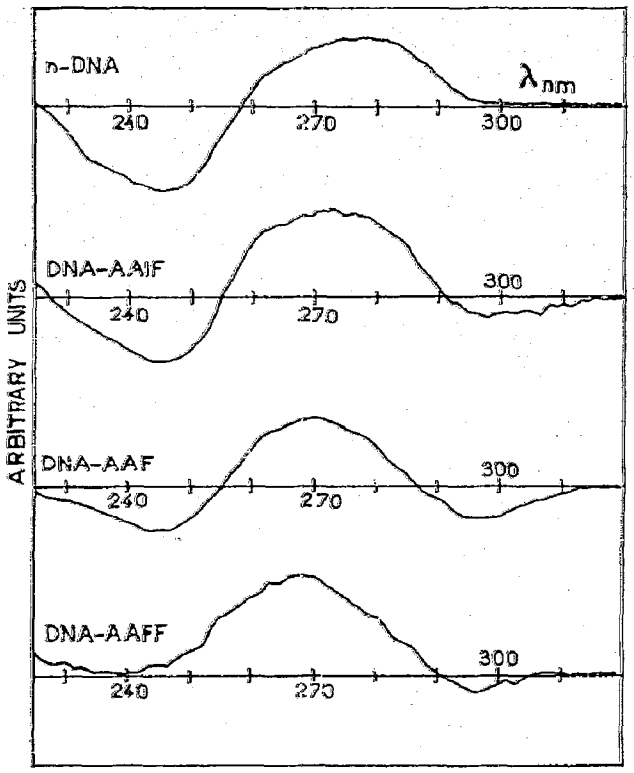

Fig. 1. Circular dichroism spectra of native and modified calf thymus DNA in 2×10^{-3} M sodium citrate buffer (pH 7). The spectra were recorded with a Jouan II dichrograph. n-DNA: native DNA; DNA–AAIF: 8.85% of modified bases. DNA–AAF: 13.0% of modified bases. DNA–AAFF: $\simeq$ 13% of modified bases. The concentration of DNA was in each case of $\simeq 1.5 \times 10^{-4}$ mol of phosphorus/ℓ.

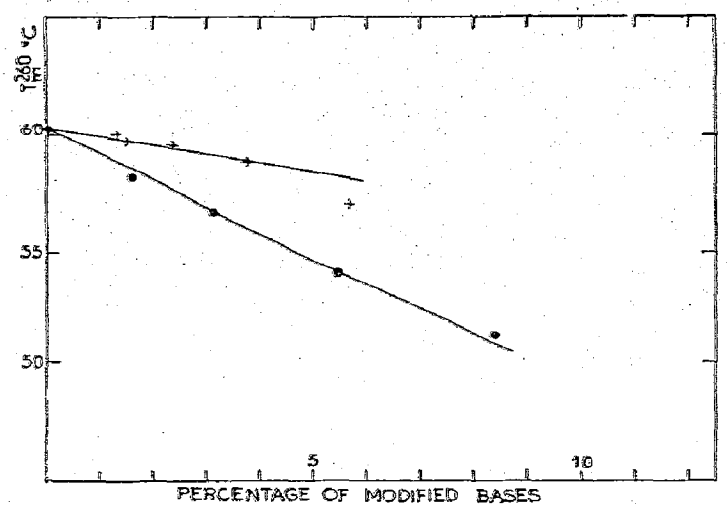

Fig. 2. Melting temperature at 260 nm vs. percentage of modified bases in 2×10^{-3} M/ℓ sodium citrate buffer (pH 7). +: DNA–AAIF. •: DNA–AAF. The decrease of T_m^{260} with AAAFF modified DNA is similar to that observed with DNA–AAF.

ing curve is still observed in every case. The melting temperature T_m is similar at the two wavelengths 260 and 305 nm.

When a large amount of AAAFF is bound to DNA ($\simeq$ 13% of modified bases) the decrease of T_m is as high as 20C° but only 5C° in the case of AAAIF. In the same conditions, the negative band at 240 nm of the CD spectrum disappears with DNA–AAFF (fig. 1). Thus, the hypochromicity, the cooperative melting and the CD measured at 305 nm are present in each case. Therefore these physical parameters do not appear as directly related to the carcinogenic properties. The apparition of optical activity and hypochromism in the absorption band of the fluorene derivatives which was considered as an evidence of intercalation, indicated only that the aromatic chromophore experiences the internal field of the double helix.

More indicative is the change of T_m with the relative amount of modified bases, since in this case a strong linear decrease is found only with DNA modified by a carcinogen like AAAF and AAAFF.

3.3. *Kinetic experiments*

Finally the dynamic state of the DNA, as determined from kinetics of unwinding is directly related to the carcinogenic activity.

Utiyama and Doty [16] and Von Hippel and Wong [17] have shown how it is possible to get very useful indications about the dynamic structure of DNA, by following the kinetics of unwinding induced by HCHO in given experimental conditions. At the same time Lazurkin et al. [18] were using the same experimental procedures to detect the concentration of "weak" points in a DNA sample.

We have compared the initial rate constant of unwinding k_o for native DNA, modified DNA (fig. 3) and denatured DNA. Table 1 gives the initial rate of unwinding in the case of DNA–AAF, DNA–AAFF and DNA–AAIF with different amounts of modified bases, and the same rate obtained with native (n-DNA)

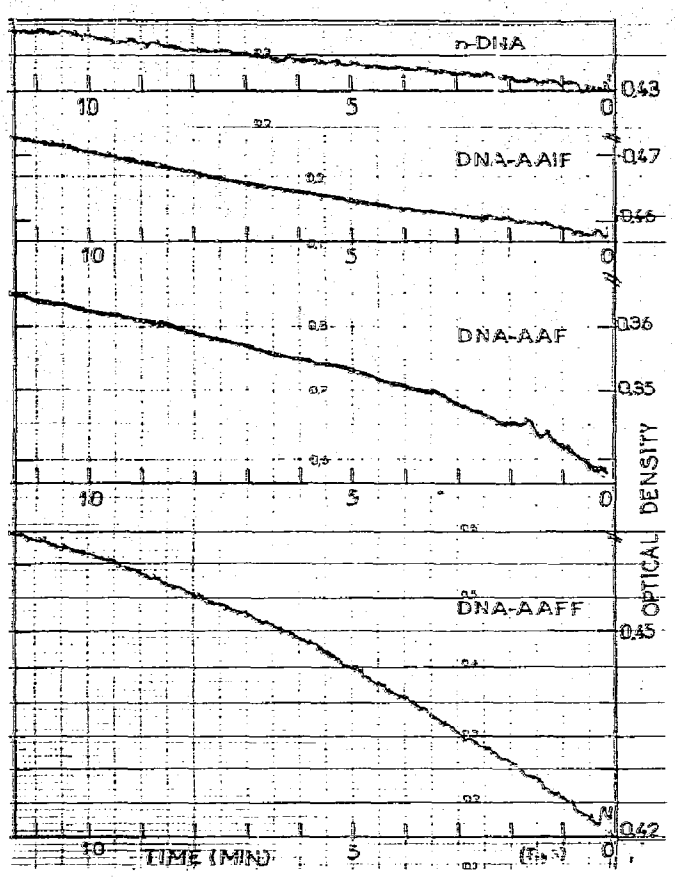

Fig. 3. Increase in optical density of native and modified calf thymus DNA followed at 251 nm. The reaction mixture contains 0.042 mol/ℓ sodium borate buffer pH 8.9 and 1.05 mol/ℓ formaldehyde. All experiments were performed at 49.0 ± 0.2°C. n-DNA: native DNA; DNA–AAIF: 1.3% of modified bases; DNA–AAF: 1.7% of modified bases; DNA–AAFF: ≃ 1.4% of modified bases. The initial rate constant k_0 is determined in each case by: k_0 = (initial slope)/($A_0^{251} \times H^{251}$), where A_0^{251}: absorbance at 251 nm at time zero (corrected for formaldehyde absorbance) and H^{251}: total hypochromicity due either to complete denaturation or to complete reaction at 251 nm. This parameter was determined in each case.

and denatured (d-DNA) in the same conditions of temperature and ionic strength. According to Von Hippel and Wong [17], the ratio $k_{n\text{-DNA}}/k_{d\text{-DNA}}$ represents the relative amount $\theta(T)_{n\text{-DNA}}$ of "open" base plates in native DNA at a given temperature.

If $\theta(T)_{c\text{-DNA}}$ represents the corresponding relative amount in the case of modified DNA, one finds always:

$$\theta(T)_{c\text{-DNA}} > \theta_{n\text{-DNA}}$$

Table 1
Calculation of the number of open base plates $x(T)$ introduced by a given single fluorene derivative bound to native DNA.

T = 49°C	Percentage of modified bases	$k_0 \times 10^4$ min^{-1}	$\theta_n(T)$	$\theta_c(T)$	$x(T)$
d-DNA	0	1700			
n-DNA	0	80	0.047		
DNA–AAF	1.7	450		0.264	12–13
DNA–AAFF	1.4	460		0.270	15–16
DNA–AAIF	1.3	110		0.065	1–2
DNA–AAF	3.7	880		0.517	12–13
DNA–AAIF	2.5	180		0.106	2

All unwinding measurements were performed in sodium borate buffer 0.042 mol/ℓ, pH 9. The melting temperature of native calf thymus DNA in this buffer is 70°C.

The difference $\theta_c - \theta_n$ can thus be attributed to the additional "breathing" induced by the presence of open G–C pairs. The ratio $x(T) = (\theta_c - \theta_n)/\%$ modified bases will be therefore a measure of the local desorganization in the vicinity of the covalently bound molecule of carcinogen expressed as an average number of open base plates. Results are given in table 1.

At a given temperature, i.e. 49°C (20C° lower than T_m) we can notice that the number x of open base plates in the vicinity of a covalently bound fluorene residue, is independent of the percentage of modified bases and appears therefore as a characteristic parameter of a given molecule. As shown in table 1, the iodo-derivative introduces only a weak destabilization (1–2 base plate per residue) whereas the carcinogenic derivatives AAAF and AAAFF induce much larger loops (12–13 base plates per bound residue).

The number x of desorganized nucleotide pairs in the neighborhood of a binding site, can thus be considered as a physical measurement of the carcinogenic activity.

It remains however to explain why carcinogens like AAAF and AAAFF introduce such open loops, whereas non carcinogens like AAAIF do not. Several steps may be introduced in this process:

i) the covalent binding of AAAF to DNA was shown [12] to be only possible if the base pair G–C is open in order to make C_8 sterically accessible. It is reasonable to assume the same initial step for the chemical reaction between each fluorene derivative and DNA. Work is in progress to study the relative

reactivity of each carcinogen toward DNA bases, and to determine the chemical nature of the addition products.

ii) The covalent binding is followed by a rotation of the group "G-fluorene" around the glycosyl bond $N_9-C'_1$, in order to put the fluorene ring in the space available between the two nearest neighbours of the G–C pair, and to push out the guanine residue. This model is in agreement with recent works on tRNA [19] and trinucleotides [20]. This second step is only possible if the insertion of the aromatic ring is not hindered either for sterical reasons (non-planarity, "bulky" substituent) or for energetical reasons. In fact, the stacking energy of aromatic rings decreases quickly if their distance is kept greater than 3.2 Å [21]. Both effects are indeed present together and for the iodo-derivative the total energetical balance is unfavourable to insert the fluorene nucleus in the double helix (the Van der Waals diameter of iodine is 4.3 Å). This second step is the most important to make the distinction between carcinogenic and non-carcinogenic molecules.

iii) The fluorene ring is unable to be paired with the C-residue and such a local distortion induces conformational and energetical changes in the neighbouring pairs of nucleotides. Around each covalently bound carcinogen, able to be intercalated in place of guanine, the secondary structure of the DNA is therefore permanently opened. The existence of these local desorganized groups of nucleotides would be the key of the carcinogenic properties. Such effect was no longer observed with molecules like AAAIF unable to be inserted because of their molecular thickness.

The model which is presented and discussed here, relative to the mechanism of action of a series of chemical carcinogens, could be extended to any process giving rise to a local and permanent destabilization of a sequence of bases inside the DNA.

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