Light Scattering and Flow Dichroism Studies on DNA After the Photoreaction with Psoralen

S. MARCIANI *, M. TERBOJEVIC **, and F. DALL'ACQUA *

Institute of Pharmaceutical Chemistry, Cattedra di Chimica farmaceutica applicata *
and Institute of Organic Chemistry ** of the Padua University — Padova (Italy)

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Light scattering measurements performed on DNA after irradiation in the presence of psoralen clearly show that inter strand cross linkings are present in the macromolecule. In fact after heat denaturation and successive cooling irradiated macromolecule shows a molecular weight practically unchanged while a DNA sample after the same treatment shows a molecular weight half of the intact native DNA. Also the general conformation of irradiated DNA undergoes practically to no modifications after the same heat treatment while native DNA shows itself to have been strongly modified. Moreover, on the basis of flow dichroism determinations, DNA cross-linked by psoralen after heat denaturation showed to be able to restore its ordered double helix structure, during the successive cooling.

It is well known that furocoumarins photoreact both in vitro 1–5 and in vivo 6,7 with nucleic acids by irradiation at 365 nm, and that this photoreaction can explain the biological photosensitizing properties of furocoumarins. A C1-cyclo-addition of the substances to the 5,6-double bond of the pyrimidine basis of DNA takes place; furocoumarins (e.g. psoralen) can react either with their 3,4- or with their 4',5'-double bond, forming therefore two types of photoadducts 8–10.

Fig. 1. Molecular structure of psoralen.

Experimental evidences have been recently obtained 11–13 demonstrating that when psoralen or other furocoumarins are intercalated between two base pairs in double-stranded DNA and are irradiated at 365 nm, they can react with both their two double bonds linking to two pyrimidine bases appertaining to opposite strands and forming therefore inter-strand cross-linkages.

In the present paper we have further investigated this new very interesting aspect of the photoreactions between furocoumarins and DNA. By light-scattering and flow-dichroism measurements we have obtained new information about the properties of the double stranded DNA after irradiation in the presence of psoralen, concerning its molecular weight, its general conformation and its ability to renature spontaneously after heat-denaturation. The results obtained fully confirm the previous observations on the ability of furocoumarins to form inter-strand cross-linkages in native DNA.

Material and Methods

Psoralen

A sample extracted from fig-leaves 14 was used, m.p. 164°. This substance had been tritiated by the Wilzbach method and purified following a procedure described elsewhere 15: specific activity 4.5 × 10^6 dpm/ mMol.

DNA

Two samples have been used:

a) Calf thymus DNA, highly polymerized, purchased from Mann Research Laboratories, New York; Tm =87°; hypochromicity higher than 40 percent. This sample was used for the light scattering measurements.

b) Salmon sperm DNA, highly polymerized, furnished by Calbiochem, Los Angeles, California; Tm 86°; hypochromicity higher than 38 percent. This sample was used for linear flow-dichroism determinations.

Preparation of the DNA-psoralen combination

To DNA 0.1% aqueous solutions containing 2 mM NaCl were added small volumes of a concentrated ethanolic solution of ^3H-psoralen to obtain a final concentration of 10 μg per ml (the ethanolic concentration was always lower than 0.3%).

Portions of 60 ml of these solutions were placed in a beaker (10 cm in diameter) and irradiated in a cold room (+1°) for 2 hours, using a Philips HPW 125 lamp, which emits almost exclusively at 365 nm, placed at a distance of 16 cm; the intensity of incident radiation was 1.25 × 10^15 hv/cm²/sec, deter-
mined by means of a potassium ferrioxalate actinometric system. After irradiation, solid NaCl to 1 M final concentration was added to the solutions and then 2 volumes of cold anhydrous ethyl alcohol were slowly added stirring with a glass rod; precipitated DNA was collected, washed with cold 70% ethyl alcohol and dissolved again into 60 ml of aqueous solution of 2 mM NaCl. 0.2 ml of this solution, diluted with 1 ml of water and added to 10 ml of dioxane base scintillator (P.P.O. g 5, P.O.P.O.P. g 0.075, naphthalene g 120, dioxane up to 1000 ml) was counted for its radioactivity with a Beckman LS 150 Liquid scintillation spectrometer; on the basis of these measurements, the amount of H-psoralen linked to DNA was calculated.

The DNA-psoralen combination so prepared contained 1 molecule of psoralen linked every 75 nucleotides.

Heat treatment

Measured portions of all the prepared samples were introduced into a flask connected with a condenser and heated for 10 min in a boiling water bath; then the flask was immersed immediately in ice and so held for 20 minutes.

Light scattering determinations

Light scattering determinations were performed using a Photometer Sofica Model 42,000, with cylindrical cells immersed in highly purified toluene.

The instrument was standardized with benzene (clarified with accuracy) using $R_{99}$ (546 nm) = 16.3 x 10^-6 18. The measurements were made at $\lambda$ = 546 nm, using the value $dn/dc$ = 0.17 19. The experimental determinations were performed in the range comprehended between 30° and 120°, as DNA MW was lower than 3 x 10^6 and consequently determinations at low angles (<30°) were not necessary 20-22.

Each sample to be examined was diluted with a phosphate buffer pH 6.8, 0.195 M in sodium ion containing 0.006 M NaHPO4, 0.002 M Na2HPO4, 0.001 M sodium EDTA, and 0.179 M NaCl, to obtain various different patterns with decreasing DNA concentration in the range 5 x 10^-5 - 15 x 10^-5 g/ml.

The solutions examined were previously clarified by prolonged shaking with chloroform-isooamyl alcohol mixture 23, followed by centrifugation (2 hours) at 25,000 g.

Flow-dichroism measurements

Differential flow-dichroism of DNA solutions was determined by a Shimadzu QV-50 Spectrophotometer equipped with the flow attachment. This last, which replaces the usual cell chamber, consists of a quartz cylindrical cell containing a rotating quartz cylinder and a calcite prism which polarizes monochromatic light parallel (||) or perpendicular (⊥) to the flow line.

The sample solution was injected into the gap (0.5 mm) between the cell wall and the cylinder and this was rotated at 1,000 r.p.m. This rotation provoked an orientation of the helix axis of native DNA parallel to the flow line 24-26.

Base pairs of the double stranded DNA which are perpendicular to its helix axis show themselves so disposed perpendicular to flow line.

Differential dichroism was determined according to WADA and KOZAWA 24, that is by the difference between the molar extinction coefficients of light polarized parallel to the flow line ($\varepsilon_{||}$) and that polarized perpendicular to it ($\varepsilon_{⊥}$); $\Delta\varepsilon = \varepsilon_{||} - \varepsilon_{⊥}$.

As the transition moment around 260 nm of both purine and pyrimidine bases of DNA 25-27 (which involves a $\pi \rightarrow \pi^*$ transition) is parallel to the ring plane of the same bases and therefore perpendicular to helix axis, $\varepsilon_{⊥}$ comes out larger than $\varepsilon_{||}$, consequently $\Delta\varepsilon$ assumes a negative value when DNA retains its ordered double helix structure 25,26.

Also the ratio $R = \Delta\varepsilon/\varepsilon$ at $\lambda_{max}$ has been calculated according to WADA 25.

Velocity gradient of the laminar flow in our experimental conditions was 2900 sec^-1.

Results and Discussion

Light-scattering measurements

In a previous research, performed using light scattering measurements 28, we have observed that native DNA, irradiated at 365 nm in the presence of psoralen, maintains practically unmodified both its tertiary structure and its molecular weight, although some furocoumarin molecules have been covalently linked to pyrimidine bases.

Now, with the aim of studying cross-linking formation by psoralen molecules linked to DNA, we have performed the following two series of experiments:

1. A DNA-psoralen combination prepared as described in the section Materials and Methods, dissolved (0.1%) in aqueous solution containing 2 mM NaCl, was heated at 100° for 10 min and then immersed in ice and kept there for 20 minutes.

After this, a little portion of the solution was utilized to determine its O.D. at 260 nm and the remaining part was employed for light scattering measurements, as described in the section Materials and Methods.

For a control and a comparison, the O.D. and light scattering measurements were performed also on the following solutions:

a) The same solution of the DNA-psoralen combination not treated by heat.
b) Aqueous solution (0.1%) of native DNA containing 2 mM NaCl.
c) The same solution b heated at 100° for 10 min and then immersed in ice for 20 minutes.
The results obtained are reported in Table I. They show that a great difference exists between the behavior of native DNA and that of the DNA-psoralen combination when they are heated at 100°.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% increase of O.D. at 260 nm after heating 10' and quenching in ice</th>
<th>MW x 10^6</th>
<th>R_G [Å]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native DNA untreated by heat</td>
<td>—</td>
<td>2.90</td>
<td>1810</td>
</tr>
<tr>
<td>Native DNA after treatment by heat</td>
<td>33</td>
<td>1.32</td>
<td>460</td>
</tr>
<tr>
<td>DNA-psoralen combination untreated by heat</td>
<td>—</td>
<td>2.99</td>
<td>1970</td>
</tr>
<tr>
<td>DNA-psoralen combination treated by heat</td>
<td>4</td>
<td>2.74</td>
<td>1750</td>
</tr>
</tbody>
</table>

Table I. Results obtained from spectrophotometric and light scattering measurements.

and then quenched in ice. Not only with reference to the optical density, as previously observed, but also with reference to the molecular weight, which in the DNA-psoralen combination remains unmodified after the heat treatment, in contrast with that of native DNA, which in the same conditions becomes less than half.

Moreover, as the gyration radius value of the DNA-psoralen combination after the heat treatment was very similar to the initial value and to that of intact native DNA, we have obtained further evidence that the cross-linked macromolecule is practically renatured.

2. For obtaining more direct evidence that in DNA-psoralen combination the two strands cannot be completely separated, we wanted to determine the molecular weight of this macromolecule also when it is swollen.

For this purpose, the solutions of the DNA-psoralen combination and of native DNA have been brought to pH 2.4*; in this condition usually DNA is almost completely denatured. The results obtained by O.D. and light-scattering measurements on these solutions are reported in Table II. It appears that in this condition the DNA-psoralen combination shows a strong increase of its optical density (about the same as shown by the solution of native DNA) and an evident change of the gyration radius value; these data demonstrate that in this medium the two strands are strongly swollen both in native DNA and in DNA-psoralen combination, and consequently the conformation of the macromolecule is greatly modified. However the molecular weight of the DNA-psoralen combination, although a little smaller than that found in a non swelling medium (see Table I) is clearly much higher than that of native DNA when its solution is brought to pH 2.4. This fact confirms that also in strongly swelling conditions the two strands of the DNA-psoralen combination cannot completely separate.

* DNA acidic solutions have been prepared according to A. I. Krasna and Coworkers 22.
indicating that they are maintained together by cross-linkings formed by psoralen molecules.

Native DNA, after the heat treatment and successive cooling, presents an evident increase of O.D. (33%) at 260 nm, a molecular weight which is about half of the initial value and a gyration radius strongly modified: these data are in connection with the denaturation of the macromolecule produced by heat treatment; the two strands underwent an almost complete separation and during the cooling period they were not able to reform the double helix structure.

By contrast, when DNA contains some psoralen molecules covalently linked (DNA-psoralen combination), after heat treatment and successive cooling, it shows only a small increase of O.D. (about 4%) and moreover a molecular weight and a gyration radius very similar to the initial values and to those of intact native DNA.

In previous studies \(^6,9\) we have observed that the O.D. at 260 nm of the DNA-furocoumarin combinations increases by increasing temperature of the solutions indicating that the two strands of DNA can swell and therefore a partial denaturation occurs; however by successive decreasing of the temperature, O.D. gradually decreases to reach almost the initial value.

This fact was interpreted as evidence that psoralen linked to DNA forms cross-linkings between the two strands of the macromolecule; the presence of this type of bonds in DNA forbids the complete separation of the two strands during the heat treatment and successively, in the cold, favours renaturation of the macromolecule.

**Flow dichroism measurements**

As pointed out by many authors \(^24-26,29\) native DNA when oriented in flow or in film presents a linear dichroism due to the regularity of purine and pyrimidine bases in its double stranded helix structure. In particular the rings of purine and pyrimidine bases are disposed perpendicularly to helix axis. When it is denatured and its ordered structure passes to a disordered state, the possibility of orientation of the macromolecule by flow decreases strongly and its linear dichroism practically disappears \(^25,26\). Flow dichroic measurements represent therefore a very sensitive way to control the ordered structure of DNA.

We have examined by flow dichroism measurements the DNA-psoralen combination, obtained as above described, in comparison with native DNA; both samples, in 0.05% solutions containing 2 mM NaCl, were examined before and after the heat treatment (see Materials and Methods).

The results are reported in Fig. 2. It is evident, at first, that DNA-psoralen combination presents at 260 nm a dichroism value practically equal to that of intact native DNA: in fact also the ratio \(\Delta e/e\) \(^24,25\) has in both the cases the same value, that is 0.33; this fact is a further confirmation that the internal ordered secondary structure of DNA is not modified by the binding of some psoralen molecules to pyrimidine bases.

One can observe moreover that after heat treatment, while native DNA loses almost completely its dichroism (Fig. 2, part b), the DNA-psoralen combination after the same treatment maintains its dichroism value practically unchanged.

Considering the high sensitivity of this method, this finding represents a valid confirmation of the capacity of the two strands of DNA cross-linked by psoralen, to reform an identical secondary structure after their partial separation caused by heat treatment.

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On the Binding of Benz[a]pyrene to DNA “in vivo”

F. CARLASSARE, C. ANTONELLO, F. BACCICHETTI, and P. MALFER

Istituto di Chimica Farmaceutica, Università di Padova, Centro di Chimica del Farmaco
del Consiglio Nazionale delle Ricerche, Padova (Italy)

The Possibility that “in vivo” a binding takes place between DNA and benz[a]pyrene was examined. After oral administration of 3H-benz[a]pyrene, radioactive DNA was isolated from the skin, spleen and liver of NCLmice, and especially from the liver. This suggest that covalent binding between DNA and benz[a]pyrene takes place.

Many studies described in the literature suggest a relationship between the chemical structure of several hydrocarbons and their carcinogenic power. It has been known since 1930 \(^1\) that it is possible to induce tumors by local treatment with 1:2,5:6-dibenzanthracene. The possibility that light may influence carcinogenesis due to benz[a]pyrene has also been studied \(^2\).

Many authors have reported that the carcinogenic activity depends upon a binding between the hydrocarbon, or one of its metabolites, and the cellular constituents. Some time ago it was suggested that binding to proteins was the beginning of the carcinogenesis; it has, moreover, also been suggested that an important part of the tumoral process may be attributed to a binding between hydrocarbons and nucleic acids.

There are different opinions on the possibility that such a binding occurs, both “in vitro” and “in vivo”. With regard to experiments “in vitro”, Heidelberger \(^3\) up till 1964 excludes that the responsibility for the beginning of the carcinogenic process could be attributed to the binding between hydrocarbons and DNA. The results of Brookes and Lowley \(^4\) are noteworthy; when mouse skin was treated with a set of tritiated hydrocarbons, radioactive nucleic acids were isolated.

Brookes \(^5\) studied in 1966 the problem quantitatively, and Goshman and Heidelberger considered it again in 1967; finding that the radioactivity supplied by tritiated hydrocarbons of high specific activity was bound to DNA, RNA, and to proteins isolated from mouse skin after topical treatment. In this case benz[a]pyrene was not considered. Brookes and Heidelberger \(^7\) found in 1969 that reaction between hydrocarbons and nucleic acids was obtained using 7,12-dimethylbenz[a]anthracene as a hydrocarbon, and rodent embryo cells in culture as a source of DNA.

Prodi et al. \(^8\) examined the radioactivity of DNA, RNA, nuclear and cytoplasmic proteins of various organs of rat after i.p. injection of tritiated 7,12-dimethylbenz[a]anthracene and benz[a]pyrene. All the cellular constituents examined showed the presence of radioactivity, indicating binding, although in limited measure.

With regard to the experiments “in vitro”, we recollect that the studies of Boyland and Green \(^9\),