Bifunctional Photobinding of Psoralen to Single Stranded Nucleic Acids

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Psoralen, nucleic acids, cross-linkings

As psoralen and other furocoumarin derivatives, intercalated between two base pairs of native DNA, under irradiation at 365 nm form inter-strand cross-linkings as a consequence of bifunctional addition, the writers have investigated the ability of psoralen to give such bifunctional photoadditions, too, with nucleic acids with disordered or partilly disordered structure (denatured DNA and r-RNA).

On the basis of fluorimetric, light-scattering, viscosimetric measurements and of the renaturation ability of denatured bacterial DNA, certain results have been obtained. In addition to monofunctional photoadditions, psoralen can give bifunctional binding by irradiation at 365 nm both with denatured DNA and with r-RNA. However, when irradiation of denatured DNA in the presence of psoralen was performed in a concentrated solution (0.4%), the formation of bifunctional additions between two different strands was demonstrated by the increase (50%) of molecular weight of denatured DNA. However, when irradiation of denatured DNA was performed in more dilute solutions (0.1%), the bifunctional photoaddition of psoralen took place producing only bifunctional additions in the same strand, very probably with the formation of loops, as has been shown by the absence of increase of molecular weight of DNA and by the more restricted structure assumed by the macromolecule, revealed by the light-scattering and viscosimetric measurements. The formation of these bifunctional additions was confirmed by the reduced rate of renaturation shown by denatured bacterial DNA after irradiation in the presence of psoralen.

In the case of r-RNA, psoralen, when irradiated can form bifunctional additions only in the same strand.

Introduction

It is well-known that furocoumarins under irradiation at 365 nm photoreact with the 5,6-double bond of the pyrimidine bases giving C_4-cycloadducts; they have two reactive sites, namely the 3,4- and 4′,5′-double bonds and therefore can give two types of photoadducts [1–3] (see Fig. 1).

psoralen **1** thymine **2**

3,4-photoadducts

4′,5′-photoadducts

Furocoumarins, photoreact in an analogous way with the pyrimidine bases of native DNA [4–7].

Moreover it has recently been shown [8–13] that furocoumarins when photoreacting with native DNA can act as bifunctional reagents, forming cross-linkings between the two strands of the macromolecule. This is possible in native DNA because of a preliminary complex in the dark through an intercalation of the planar furocoumarin molecules between the planes formed by two base pairs; when furocoumarins have a linear structure, such as that of psoralen, it is possible for their reactive double bonds to be aligned with the 5,6-double bonds of two pyrimidine bases appertaining to two opposite

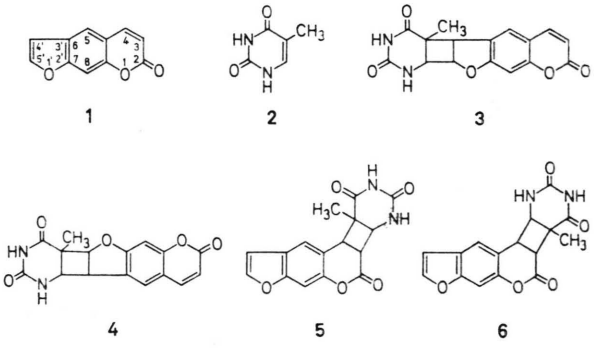

Fig. 1. Molecular structure of psoralen (**1**), thymine (**2**), and respectively of 4′,5′ (**3, 4**) and 3,4-photoadducts (**5, 6**) between the two substances.

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strands and therefore, by irradiation, both can photoreact and give cross-linkings.

In previous research it was been evidenced that furocoumarins can also photoreact with pyrimidine bases of denatured DNA and of r-RNA [4–7, 14]. In these cases, too, the formation of molecular complexes has been shown [6, 15, 16], but very probably, with a different spatial situation because of the disordered or partially disordered structure of the macromolecules.

In the present paper we are reporting on experiments carried out so as to determine whether or not bifunctional photoadditions of psoralen to pyrimidine bases may occur in these conditions, as well. The results have shown that bifunctional additions can be formed by psoralen in both denatured DNA and r-RNA. In r-RNA and in dilute (0.1%) solutions of denatured DNA, these take place between two pyrimidines of the same strand, while in more concentrated solutions (0.4%) of denatured DNA, these can also occur between two pyrimidines appertaining to different strands.

Materials and Methods

Psoralen

A sample extracted from fig-leaves [17], and tritiated following a procedure described elsewhere [18] was used; specific activity $8.7 \cdot 10^9$ dpm/mmole.

Nucleic acids

Yeast r-RNA, sodium salt, highly polymerized was purchased from Calbiochem (Los Angeles). Calf thymus native DNA, sodium salt, highly polymerized, purchased from Mann Research Laboratories, New York, was used for the fluorimetric, light scattering and viscosimetric studies. Its hypochromicity was over 40%, determined according to Marmur and Doty [19]. Denaturation was carried out by heating a solution for 10 min in a boiling water bath immediately cooling, and then keeping it in ice where it remained for 20 min.

Highly purified (A grade) DNA extracted from *E. coli* purchased from Calbiochem (Los Angeles) was employed for the renaturation experiments; its hypochromicity was over 42%, determined according to the method suggested by Marmur and Doty [19]; denaturation was carried out as indicated above.

Irradiation procedure

Aqueous solutions 0.1% or 0.4% of denatured DNA, and of r-RNA containing 2 mM NaCl, were added to tritiated psoralen (small volumes of the concentrated alcoholic solution). Portions (4 ml) of these solutions were irradiated in calibrated glass tubes, 12 mm in diameter, immersed in a small cell with glass walls, around which thermostatically controlled water circulated. The specimens were then irradiated with two HPW 125 Philips lamps, which emit light at almost exclusively 365 nm (the intensity of incident radiation was $1.4 \cdot 10^{16}$ $h\nu/cm^2/sec$ determined by means of an actinometric system [20]).

After irradiation NaCl was added to the specimens up to 1 M final concentration and nucleic acids were precipitated by adding two volumes of ethyl alcohol, collected and washed with 80% ethyl alcohol and redissolved again in the initial volume of water; radioactivity and fluorescence were then determined.

Fluorimetric and radiochemical measurements

To determine the intensity of fluorescence acquired by the single stranded nucleic acids, 0.5 ml of the irradiated specimens was added to 2 ml of 0.1 M phosphate buffer pH 6.98; the specimens were then examined with an Aminco Bowman spectrophotofluorimeter; activating wavelength: 330 nm; maximum fluorescent wavelength: 400 nm.

Radiochemical determinations were carried out using a liquid scintillation spectrometer Packard Model 3375 – 0.2 ml of irradiated specimens was diluted with 1 ml of water and added to 10 ml of dioxane base scintillator (P.P.O. 5 g, P.O.P.O.P. 0.075 g, naphthalene 120 g, dioxane up to 1000 ml).

Apparatus efficiency was within the range of 34 – 42 per cent for counting tritium.

Light scattering measurements

Light scattering determinations were performed using a photometer Sofica 42,000, with cylindrical cells, immersed in highly purified toluene. The determinations were carried out at a wavelength of 546 nm, using the value $dn/dc = 0.17$ [21, 22].

The instrument was standardized with highly purified benzene employing the value $16.3 \cdot 10^{-6} = R$ (546 nm) according to Carr and Zimm [23] and the experimental determinations were performed within a range between 30 and 120° [11, 24, 25].

The specimens to be examined were diluted to obtain various different samples with concentrations between $1.5 \cdot 10^{-3} - 4.5 \cdot 10^{-3}$ in the case of r-RNA and $1 \cdot 10^{-4} - 4 \cdot 10^{-4}$ in the case of denatured DNA; measurements were carried out using a phosphate buffer pH 6.8, 0.195 M in sodium ion, containing 0.006 M Na_2HPO_4, 0.002 M NaH_2PO_4, 0.001 M sodium EDTA, and 0.179 M NaCl [26].

The solutions before the light scattering determinations were clarified by prolonged shaking with a chloroform-isoamyl alcohol mixture, followed by centrifugation (2 hours) at $25{,}000 \times g$ [27].

Viscosimetric determinations

The viscosity of the denatured DNA was measured using a multigradient Ubbelhode type viscosimeter at 25 °C (± 0.01) in a phosphate buffer pH = 6.8, 0.013 M in sodium ion. The extrapolation to zero shear was carried out according to the method of Holtzer *et al.* [28].

Viscosity in the case of r-RNA was determined by means of a capillary Ubbelhode type viscosimeter, at the solvent shear time of about 200 sec at 25 °C (± 0.01) in NaCl $2 \cdot 10^{-3}$ M. The extrapolation to zero concentration was performed following the method of Fuoss *et al.* [29].

Spectrophotometric measurements

The spectrophotometric measurements for the renaturation experiments were performed using a single beam Optica CF4 spectrophotometer, provided with a thermostatically controlled cell holder.

The same spectrophotometer was used to control the concentrations of the nucleic acid solutions, by reading their optical density at 260 nm.

Results and Discussion

Fluorimetric determinations

As we have previously stated (see Introduction) when psoralen or other furocoumarin derivatives photoreact under irradiation with long wavelength ultraviolet radiation with the pyrimidine bases of nucleic acids, they can form either 3,4-photoadducts or 4′,5′-photoadducts (see Fig. 1). While the former have no absorption at wavelengths longer than 320 nm and therefore cannot be excited by 365 nm radiation (in other words they are not fluorescent when observed at this wavelength), the 4′,5′-photoadducts have a well-defined absorption at 365 nm and therefore, by irradiation at this wavelength, they are fluorescent and can photoreact further, engaging the 3,4-double bonds of their furocoumarin moieties as well, and forming double photoadducts, in which one psoralen molecule is linked to two pyrimidine bases. We can assume that these double photoadducts, in common with the 3,4-photoadducts, have no absorption in the long UV region and therefore are not fluorescent at these wavelengths.

The possibility of the formation of these double photoadducts in native DNA was suggested at an early stage by the particular behaviour of the intensity of fluorescence acquired by DNA during irradiation at 365 nm in the presence of labelled psoralen. We observed that in a first period of time, there was a somewhat parallel increase both of the amount of psoralen linked to native DNA and of the intensity of fluorescence of the macromolecule. A specimen of the macromolecule was irradiated in the presence of the furocoumarin for a time corresponding to maximum fluorescence intensity acquired; successively by reirradiation of the specimen separated from the unbound psoralen, we have observed that the amount of psoralen linked to the macromolecule remained constant, the intensity of fluorescence decreased dramatically by increasing the period of reirradiation. This behaviour was interpreted as indicating the possibility of the formation of double photoadducts. The double photoadditions (inter-strand cross linkings in the case of native DNA) first suggested by the fluorescence was successively confirmed employing various other methods [8–13].

We have now investigated whether psoralen, which photoreacts with denatured DNA and with r-RNA [6–30] could also give double photoadditions when it is irradiated in the presence of these macromolecules.

Therefore we have worked out the following experiments with denatured DNA and with r-RNA: An aqueous 0.1% solution of calf thymus heat-denatured DNA, containing 10 μg/ml of ^{3}H-psoralen, was irradiated at 365 nm for 20 min at 2 °C. Previous studies [6] had shown that, in the photoreactions between furocoumarins and denatured DNA or r-RNA, the temperature at which irradiation is performed influences to a marked degree the types of photoadducts formed; at 2 °C denatured DNA acquires the highest intensity of fluorescence, indicating a maximum formation of 4′,5′-photoadducts. After this period of irradiation, denatured DNA was precipitated from the solution by means of ethyl alcohol, washed and redissolved in water (0.1%).

After having determined both its radioactivity and its intensity of fluorescence, the solution was subdivided into various samples which were reirradiated at 365 nm at 22 °C for increasing periods of time. From every reirradiated sample DNA was

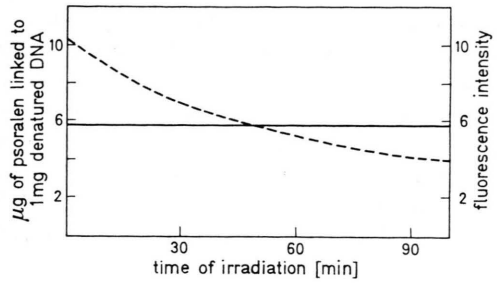

Fig. 2. Results obtained by reirradiating at 365 nm a denatured DNA-^{3}H psoralen combination: — — — Fluorescence intensity (activating wavelength: 330 nm; maximum fluorescent wavelength: 400 nm); ——— amount of ^{3}H-psoralen linked to denatured DNA (μg/mg DNA).

precipitated, washed, and redissolved again in water. The solutions thus obtained were used to determine both the radioactivity and the intensity of fluorescence. The results are reported in Fig. 2.

It may be observed that while the amount of psoralen linked remains constant, the intensity of fluorescence is noticeably decreased.

The results now obtained are analogous to that already obtained with native DNA and in this case, too, this behaviour may be explained by assuming that some molecules of psoralen, initially linked to pyrimidine bases with their 4′,5′-double bond have further photoreacted with a second pyrimidine molecule through their 3,4 double bond, in such a way forming a double photoadduct.

An analogous behaviour was shown by a solution of r-RNA irradiated in the presence of psoralen.

These results therefore indicate that in the presence of disordered molecules of nucleic acids, too, psoralen can act as a bifunctional reagent and give double photoadducts in which one molecule is linked to two pyrimidine bases. However, while in the case of native DNA, because of the intercalation of the molecule of psoralen between the planes of two base pairs, the double photoaddition leads necessarily to the cross-linking formation between the two strands, in the cases now examined there are different possibilities; in fact, the two pyrimidine bases involved in the formation of this double photoadduct may appertain either to the same single strand or to two different separated strands.

Light scattering and viscosimetric measurements

If the two photoreacting pyrimidine bases appertain to two different strands an increase in the molecular weigth of the macromolecule will be observed after irradiation; if, on the other hand, the two bases appertain to the same chain, no modification of molecular weight will occur; in this case, however, the double photoaddition should occur mainly with the formation of loops, determining a decrease in the average size of the irradiated chains, which should attain a more restricted structure.

In order to confirm that psoralen acts as a bifunctional agent and moreover to arrive at these results, the following experiments were performed:

1. Aqueous solutions 0.1% or 0.4% of heat-denatured DNA containing labelled psoralen (30 μg/ml and 50 μg/ml respectively) were irradiated at 365 nm at 2 °C for 20 min; denatured DNA was then precipitated with ethyl alcohol, washed and redissolved in the original volume of water. The solutions obtained were irradiated again at 22 °C for 120 min, repeating then the precipitation of denatured DNA and its redissolution in water. The final solutions were used for radiochemical determinations (from which the amount of psoralen linked has been calculated) and for light-scattering and viscosimetric measurements.

For verification and comparison, aqueous solutions of denatured DNA not irradiated, but twice precipitated and redissolved in water in the same experimental conditions, were used.

The results obtained as regards molecular weight, gyration radius and viscosity are reported in Table I.

Table I. Data obtained from light scattering and viscosimetric determinations on denatured DNA solutions.

Solutions examined	Number of molecules of psoralen linked: Nucleotides present in denatured DNA	Molecular weight $\cdot 10^6$	Gyration radius [Å]	$[\eta]$ dl/g
0.1% solution not irradiated	—	1.37	1180	15.20
0.1% solution irradiated in the presence of psoralen	1 : 51	1.45	990	11.25
0.4% solution not irradiated	—	2.6	—	—
0.4% solution irradiated in the presence of psoralen	1 : 67	3.8	—	—

It may be observed that the molecular weight of denatured DNA irradiated in 0.4% solution in the presence of psoralen shows noticeable increase (about 50%); this fact demonstrates that some bifunctional additions between two different strands have been formed.

The same irradiated solutions 0.4% of denatured DNA have also been examined by means of light-scattering measurements in an acidic medium at pH 2.4, following the procedure of A. I. Krasna *et al.* [25]. In these conditions, too, the increase of molecular weight was practically identical to that observed in neutral solutions and reported in Table I. This excludes the fact that the observed increase of the molecular weight should depend on a simple aggregation or partial renaturation of certain molecules of denatured DNA.

On the other hand, when DNA was irradiated in the presence of psoralen in 0.1% solution, it showed only a very slight increase of molecular weight, indicating that in this condition practically no bifunctional photo-additions occurred between two different strands.

However, in this case, the gyration radius value of the irradiated sample was definitely smaller than that of the unirradiated one. Moreover, the viscosity of the irradiated denatured DNA was clearly lower than that of the unirradiated sample (see Table I). These data are in agreement with a more restricted conformation assumed by the macromolecule after photoreaction and strongly suggest the formation of loops in the strands of the macromolecule, provoked by the double photoreaction of psoralen with two pyrimidines of the same strand.

2. Analogous experiments have been worked out on aqueous solutions of r-RNA, containing labelled (tritiated) psoralen. The results are reported in Table II. As may be seen, by operating with 0.1% solutions, the results are analogous to those obtained with 0.1% solutions of denatured DNA; in fact there is no increase of molecular weight after irradiation and a small, but significant, decrease in viscosity (in this case, the gyration radius cannot be determined with accuracy, due to the low molecular weight of RNA).

Table II. Data obtained from light scattering and viscosimetric measurements on RNA solutions.

Solutions examined	Number of molecules of psoralen linked : Nucleotides present in RNA	Molecular weight $\cdot 10^4$	$[\eta]$ dl/g
0.1% solution not irradiated	—	7.5	0.943
0.1% solution irradiated in the presence of psoralen	1 : 68	7.0	0.770

Adopting 0.4% solutions of r-RNA, the results (not reported in Table II) were more or less the same, that is to say, no increase of molecular weight was observed.

It may be concluded that in r-RNA, irradiated in aqueous solution in the presence of psoralen, bifunctional photobinding of psoralen can occur only with two pyrimidines appertaining to the same strand.

Experiments on the renaturation capacity of denatured DNA after the photoreaction with psoralen

To obtain further data supporting the suggestion of the formation of loops (in the same strand) by irradiation of denatured DNA in dilute aqueous solution and in the presence of psoralen, we have tested its ability to renature after this photoreaction.

An aqueous 0.04% solution of heat-denatured *E. coli* DNA, containing 2 mM NaCl and tritiated psoralen (30 μg/ml) was irradiated and treated in the same way as described for light-scattering measurements. The final solutions was prepared in such a way as to obtain a concentration of 20 μg/ml of nucleic acid, in the presence of 0.3 M NaCl and 0.03 M Na citrate. On the basis of radioactivity measurements performed on this solution, the de-

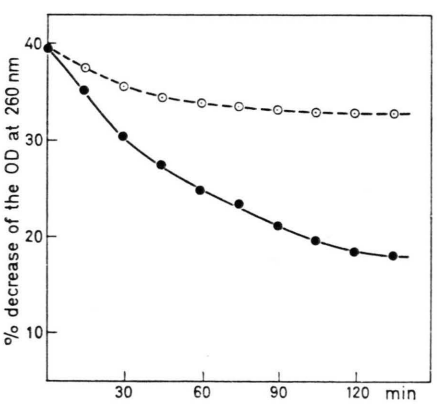

Fig. 3. Variations of the OD of solutions of denatured *E. coli* DNA samples by keeping them at 71 °C. — — — Irradiated in the presence of psoralen; ——— not irradiated.

natured DNA contained 1 psoralen molecule linked to every 51 nucleotides.

An equal solution of denatured *E. coli* DNA, treated in the same way but not irradiated, was used for reference.

The renaturation experiment was performed according to the method used by Marmur and Doty [31], that is by keeping the solutions of denatured DNA at a temperature 20 °C lower than that corresponding to the Tm value of the original native DNA, observing the variations of the optical density at 260 nm. Both irradiated and reference solution were kept at 71 °C for 130 min, and their optical density was measured every 10 min. The results are reported in Fig. 3. It may be observed that the renaturation rate of the irradiated sample is clearly lower than that of the reference sample. This fact further supports the theory that psoralen forms bifunctional additions in the same strand; undoubtedly the presence of loops in the single stranded macromolecules is a delaying factor, which reduces possibility of re-annealing.

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