The action spectrum of xanthotoxin and bergapten for the photoreaction with native DNA

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The action spectrum of two skin-photosensitizing furocoumarins, xanthotoxin and bergapten, for the photoreaction with native DNA, determined in the range 254—436 nm, showed that the maximum amount of furocoumarin was linked to DNA after irradiation at 312 nm for xanthotoxin and 334 nm for bergapten, while the quantum yields of the photoreactions in both cases had their maximum value at 365—405 nm and decreased very rapidly by decreasing the wavelength of the radiation. The action spectrum of the photoreaction in the range 254—265 nm had a very low efficiency for photoreaction, although furocoumarins exhibit a strong absorption band in this region and DNA has its highest absorption. It was demonstrated that this fact is mostly due to a filter effect exerted by DNA for these radiations.

The photoreaction between skin-photosensitizing furocoumarins and native DNA has been revealed and studied by Musajo and the Authors1—4. Following irradiation at 365 nm of an aqueous solution of the substances, a stable chemical bond of the furocoumarins to DNA takes place5. It has been ascertained that the reactive sites of the macromolecule are the pyrimidine bases2,3—5,8. A stabilization of the helix structure of DNA is produced5,9, and no breakages of the polynucleotide chains occur during

the photoreaction; however the template efficiency of DNA in RNA-polymerase reaction is decreased.

This photoreaction seems to be able to clarify the mechanism of the photosensitizing action that some furocoumarins exert on various biological substrates. Upon irradiation at 365 nm, they provoke erythema on human and guinea-pig skin, lethal and mutagenic effects on bacteria cultures, and on mammalian cells adapted to in vitro growth, inactivation of DNA-viruses, and destruction of the tumor-producing capacity of Ehrlich ascites tumor cells in mice.

Until now the photoreactions between DNA and furocoumarins were studied using radiations of 365 nm, which were found to be the most active in producing the photosensitization of the human and guinea-pig skin by xanthotoxin (1) and bergapten (2), by irradiating with various ultraviolet radiations having different wavelength, in the range 254–405 nm. We have determined the amounts of furocoumarins linked to DNA after irradiation with different increasing numbers of quanta. From the results we have obtained the action spectra of the two furocoumarins for the photoreaction with native DNA and moreover the quantum yields of the same photoreactions at the various wavelengths used.

We have now studied the photoreaction between native DNA and two skin-photosensitizing furocoumarins, that is xanthotoxin (1) and bergapten (2), by irradiating with various ultraviolet radiations having different wavelength, in the range 254–405 nm. We have determined the amounts of furocoumarins linked to DNA after irradiation with different increasing numbers of quanta. From the results we have obtained the action spectra of the two furocoumarins for the photoreaction with native DNA and moreover the quantum yields of the same photoreactions at the various wavelengths used.

**Materials and methods**

Deoxyribonucleic acid (DNA). Highly polymerized calf thymus DNA from Mann Research Laboratories, New York was used. Tm = 87°C [determined by the method of MARMUR and DOTY]. Hypochromicity was higher than 37 per cent.

Xanthotoxin (8-methoxy-psoralen). It was a commercial product, purified by sublimation and crystallization, m.p. 148°C; it was titrated with the WILZBACH method and was obtained in a radiochemical pure state with a specific activity 1.37 × 10⁸ dpm/mM.

Bergapten (5-methoxy-psoralen). The -CH₃ bergapten used was prepared in our laboratory; it had a specific activity of 8.5 × 10⁸ dpm/mM.

Irradiation procedure. The monochromatic radiations used were obtained with a Bausch and Lomb “High Intensity Grating Monochromator”, provided with a medium high pressure mercury arc lamp, and a super pressure mercury short arc lamp.

Wavelengths were chosen corresponding to the emission lines of the lamps: 254, 265, 302, 312, 334, 365, 405 and 436 nm. The monochromaticity of the radiations was checked by use of a CGA, CP 2300 spectrophotometer; half-band width was not more than 4 nm.

For irradiation, 2 ml of the solution (see below) were pipetted into a spectrophotometric quartz cuvette (optical path 1 cm) which was placed at the exit slit of the monochromator. The percent transmission of the solution was determined at the same wavelength with a CGA, CP 2300 spectrophotometer.

The actinometric measurements at various wavelengths were performed by the HATCHARD and PARKER potassium ferrioxalate method.

Preparation of the solutions. Aqueous 0.1% DNA solutions containing 0.002 M sodium chloride, were used. To these solutions labelled xanthotoxin or bergapten in concentrated ethanolic solution were added to give final concentrations of about 15 µg/ml; the final concentration of ethyl alcohol was about 1.5 per cent. The solutions were shaken at room temperature for 90 minutes and then slowly filtered through a Millipore SM 5 µ filter.

The concentration of DNA in the prepared solutions was checked by spectrophotometric determination of 100 µg DNA/ml, corresponding to an absorbance at 260 nm of 0.025.

**References**

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the O.D. at 260 nm. No significant changes in DNA concentration of the solutions were found after filtration through the Millipore SM 5 µ filter 28.

The concentration of the furocoumarins in the filtered solutions was checked both by spectrophotometric reading 29 and by determining the radioactivity. Since small quantities of furocoumarins were absorbed by the filter membrane, the concentrations of the filtered solutions were adjusted to 10 µg/ml by diluting with a 0.1% DNA solution filtered through a Millipore SM 5 µ filter.

In preceding papers 3 it was shown that by adding ethyl alcohol to an aqueous solution containing DNA and a photosensitizing furocoumarin after irradiation at 365 nm, precipitated DNA contains a small amount of linked furocoumarin, which increases by increasing the period of irradiation. On the contrary, without irradiation no trace of furocoumarin is present in precipitated DNA.

Therefore, the solution (2 ml) after irradiation was diluted with distilled water to 5 ml; 2.5 ml of this solution, with sodium chloride added to give a concentration of 1 M and with 5 ml of absolute ethyl alcohol, were shaken and then centrifuged (3000 r.p.m. for 5 minutes). The precipitated DNA, washed with 80% aqueous ethyl alcohol, was redissolved in 1 ml of distilled water.

Then 0.2 ml of this solution were added with 1 ml of distilled water and 10 ml of dioxane base scintillator (120 g naphthalene, 25 g P.O.P.O.P. [2,2'-phenylenebis(5-phenyl-oxazole)] and 4 g P.O.P [2,5-diphenyl-oxazole] in dioxane up to 1000 ml of solution). The sample so obtained was used for the determination of the radioactivity, using a liquid scintillation counting system Beckman LS-150.

From the results obtained, the quantum of furocoumarins linked to DNA, as a consequence of the photoreaction, was calculated.

*Quantum yield.* This was calculated from the ratio between the number of molecules of furocoumarins linked to DNA after an established period of irradiation and the number of quanta absorbed by the solution during the same period.

*Radiation experiments.* The experiments consisted of irradiating 0.1% DNA solutions containing 0.002 M sodium chloride and 10 µg/ml labelled xanthotoxin or bergapten at the following wavelengths: 254, 265, 302, 312, 334, 365, 405 and 436 nm. The intensity of each radiation’s beam was previously determined by chemical actinometry and then the irradiation times were normalized to give a determined number of incident quanta. After each irradiation the amount of furocoumarin linked to DNA was determined by the radioactivity measurements on DNA precipitated from the solution. The quantum yield was calculated as indicated above, considering the percent of light absorbed by the solution at the wavelengths used.


Results and discussion

The amounts of xanthotoxin linked to DNA after various period of irradiation at the different wavelengths are reported in Fig. 1. An analogous pattern was obtained in the photoreaction between bergapten and DNA. The action spectra of the two furocoumarins and the quantum yields of their photoreactions at the various wavelengths are reported in Fig. 2.
As it appears, the results obtained with the two furocoumarins are almost parallel. The more active radiations in determining the photoreactions are those lying in the region \( \lambda > 300 \text{ nm} \) with a maximum at 312 nm in the case of xanthotoxin and 334 nm in that of bergapten. In both cases the radiations at 254 – 265 nm appear to have a very low efficiency, although furocoumarins exhibit a strong absorption band in this region and DNA has its maximum of absorption (see Fig. 3).

Fig. 3. Absorption spectra (aqueous 10 mM NaCl solutions): DNA 0.1% ———, xanthotoxin (10 \( \mu \text{g/ml} \)) ———, bergapten (10 \( \mu \text{g/ml} \)) ———, DNA 0.1% + xanthotoxin 10 \( \mu \text{g/ml} \) [complex formation 25] ———, DNA 0.1% + bergapten 10 \( \mu \text{g/ml} \) [complex formation 25] ———.

The quantum yields of the photoreactions generally are low, as we had previously observed 3. However they have their maximum value at 365 – 405 nm and then decrease very rapidly by decreasing the wavelength of the radiations.

These results indicate that, even if from the point of view of the action spectrum the most effective radiations are those with \( \lambda = 312 \text{ nm} \) (for xanthotoxin) and with \( \lambda = 334 \text{ nm} \) (for bergapten), the radiations which are utilized more than others for the photoreaction are those with \( \lambda = 365 \text{ nm} \), even if they are lesser absorbed.

Moreover we may find some analogies between these action spectra and the action spectrum of xanthotoxin for the erythema on human 22 and guinea-pig 23 skin. In fact also in vivo the more active radiations are those lying in the long ultraviolet region, whereas those with \( \lambda < 300 \text{ nm} \) are ineffective.

This analogy appears to give support to the belief that this photoreaction is connected with the photosensitizing effects exerted by the furocoumarins on the skin.

In order to clarify the behaviour of the quantum yields and the fact that the short wavelength ultraviolet radiations are almost ineffective for the photoreaction, we have formulated two working hypotheses.

1) It is known that the photoadducts between furocoumarins and pyrimidine bases, which can be obtained by irradiation at 365 nm, split easily reforming the parent compounds by irradiation at 254 nm 5–7.

\[
\text{Furocoumarin} + \text{pyrimidine} \xrightarrow{365 \text{ nm}} \text{photoadduct.}
\]

As previously found 6, in the photoreactions between furocoumarins and DNA the reactive sites are the pyrimidine bases, which form with the furocoumarins photoadducts of the same kind as those obtained by irradiation of the simple compounds. Therefore we have suggested that these photoadducts between furocoumarins and DNA have the same behaviour as those between furocoumarins and pyrimidine bases toward the 254 – 265 nm radiations.

In order to verify this hypothesis, 20 ml of a 0.1% DNA solution containing 0.002 M NaCl and 10 \( \mu \text{g/ml} \) of \(^3\text{H-xanthotoxin} \) were irradiated in a cold room (2 – 4°C) at 365 nm (0.98 mW/cm²) for 3 h and 30 minutes. DNA was then precipitated, washed and redissolved in water containing 0.002 M NaCl. The amount of xanthotoxin linked to it, calculated on the base of radioactivity measurements, was 4.71 \( \mu \text{g/mg DNA} \).

Portions of this solution (2 ml) were irradiated at 365, 334, 312, 302 and 254 nm and then treated as above described for the determinations of the radioactivity. The results obtained are reported in Table I.

<table>
<thead>
<tr>
<th>Wavelength used [nm]</th>
<th>No. of quanta incident</th>
<th>( \mu \text{g/mg DNA} ) found after irradiation</th>
<th>% of splitting</th>
</tr>
</thead>
<tbody>
<tr>
<td>302  ( \times 10^{18} )</td>
<td>6 ( \times 10^{18} )</td>
<td>4.63</td>
<td>1.70</td>
</tr>
<tr>
<td>9 ( \times 10^{18} )</td>
<td>4.59</td>
<td>2.55</td>
<td></td>
</tr>
<tr>
<td>12 ( \times 10^{18} )</td>
<td>4.55</td>
<td>3.40</td>
<td></td>
</tr>
<tr>
<td>18 ( \times 10^{18} )</td>
<td>4.48</td>
<td>4.88</td>
<td></td>
</tr>
<tr>
<td>254  ( \times 10^{18} )</td>
<td>4.43</td>
<td>5.94</td>
<td></td>
</tr>
<tr>
<td>9 ( \times 10^{18} )</td>
<td>4.32</td>
<td>8.28</td>
<td></td>
</tr>
<tr>
<td>12 ( \times 10^{18} )</td>
<td>4.22</td>
<td>10.40</td>
<td></td>
</tr>
<tr>
<td>18 ( \times 10^{18} )</td>
<td>4.00</td>
<td>15.07</td>
<td></td>
</tr>
</tbody>
</table>

Table I. Splitting of the photoadducts between xanthotoxin and DNA by irradiation at 302 and 254 nm; 4.71 \( \mu \text{g of furocoumarin} \) were initially linked to 1 mg of DNA. No splitting was observed by irradiation at 365, 334 and 312 nm.
They show that a little splitting of the photoreaction, however, is observed at 254 nm and at an even more reduced extent at 302 nm. At the other wavelengths no breakages occur.

We think that the little splitting of the photoreaction, which occurs at 254 nm (about 8% in the irradiation conditions used for determining the action spectrum) may contribute but it is not sufficient to explain completely the very low photoreactivity at this wavelength.

Considering that the quantum yield of the photoreaction is relatively high when the absorption of the radiation is due only to the furocoumarins (406–365 nm) and it decreases until very low values when also DNA absorbs (λ < 300 nm), we have suggested that at least the main part of the radiation absorbed by DNA is ineffective for the photoreaction and therefore that DNA, which is present in the solutions in a much larger concentration than furocoumarins, exerts a filter effect.

In order to verify this hypothesis we have irradiated at the various wavelengths a solution containing the same concentration of xanthotoxin (10 μg/ml) as previously used but a smaller one of DNA (0.015%), with a ratio furocoumarin: DNA = 1:15, instead 1:100 as in previous solutions. The quantum yield of the photoreaction also in these conditions had the maximum value at 365 nm, but its decrease by decreasing the wavelength of the radiation was considerably smaller than in the previous conditions, as Table II shows.

Moreover, irradiating a 0.1% DNA solution at 265 nm in the presence of different increasing amounts of bergapten (6, 12, and 18 μg/ml) the quantum yield of the photoreaction augments with the increasing bergapten’s concentration (see Table III).

Table II. Behaviour of quantum yields of the photoreactions between DNA and xanthotoxin obtained by irradiation at various wavelengths with different ratios between the two substances. * Calculated always after the absorption of 3 × 1018 quanta.

<table>
<thead>
<tr>
<th>Wavelength [nm]</th>
<th>Ratio DNA-xanthotoxin</th>
<th>Quantum yield</th>
<th>% decrease of quantum yield</th>
<th>Ratio DNA-xanthotoxin</th>
<th>Quantum yield</th>
<th>% decrease of quantum yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>365</td>
<td>15:1</td>
<td>1.28 × 10⁻³</td>
<td>—</td>
<td>100:1</td>
<td>3.20 × 10⁻³</td>
<td>—</td>
</tr>
<tr>
<td>334</td>
<td>40:1</td>
<td>1.23 × 10⁻³</td>
<td>4.0</td>
<td>100:1</td>
<td>2.86 × 10⁻³</td>
<td>10.6</td>
</tr>
<tr>
<td>312</td>
<td>80:1</td>
<td>1.17 × 10⁻³</td>
<td>8.6</td>
<td>100:1</td>
<td>2.55 × 10⁻³</td>
<td>20.3</td>
</tr>
<tr>
<td>302</td>
<td>160:1</td>
<td>0.98 × 10⁻³</td>
<td>23.5</td>
<td>100:1</td>
<td>1.43 × 10⁻³</td>
<td>55.3</td>
</tr>
</tbody>
</table>

Table III. Quantum yields of the photoreaction between DNA and bergapten obtained by irradiation at 265 nm of a 0.1% DNA solution containing different increasing amounts of bergapten, after the absorption of 3 × 1018 quanta.

We point out that at this wavelength the radiation is completely absorbed by the solution and this fact is due overall to DNA. Augmenting the concentration of bergapten increases the amount of radiation absorbed by its molecules. The parallel increase of the quantum yield confirms our previous hypothesis that the radiation absorbed by furocoumarins is more effective for the photoreaction than that absorbed by DNA.

After these results we may conclude that the very low ability of the short wavelengths in provoking the photoreaction between furocoumarins and DNA is due to a minor part to the little stability of the photoadducts to these radiations and to a main part to the filter effect exerted by DNA itself for the same radiations.

We may suggest that also on the skin the ineffectiveness of the short wavelengths in producing the photosensitization by furocoumarins is due to these facts; concerning the filter action, on the skin it may be exerted, beside DNA, also by proteins and by other absorbing substances which are present.

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