Formation of Inter-Strand Cross-Linkings in the Photoreactions between Furocoumarins and DNA

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A furocoumarin intercalated between two base pairs of native DNA can photoreact with two pyrimidine bases engaging both its 4',5' and its 3,4-double bond. This fact was evidenced studying the behaviour of the fluorescence acquired by DNA during irradiation at 365 nm. When this double reaction occurs, a cross-linking is formed between the two strands of DNA, as denaturation and renaturation experiments have demonstrated. The various furocoumarin derivatives have a very diverse ability to form cross-linkages, which is independent of their photobinding capacity. This different ability is due mainly to the structure of furocoumarins and to the steric relationships that the intercalated furocoumarin molecules have with the stacked pyrimidine bases.

Under irradiation at long wavelength ultraviolet light furocoumarins photoreact with nucleic acids (native or denatured DNA, r-RNA) giving a covalent combination with them. Pyrimidine bases (thymine, cytosine, uracil) are the reactive sites of the nucleic acids. A C₄-cyclo-addition reaction takes place involving the 5,6-double bond of pyrimidine bases and the 3,4- or the 4',5'-double bond of furocoumarins. Until now two types of photoadducts have been isolated and identified from the irradiation products of mixtures of pyrimidine bases and furocoumarins as well as from the products of hydrolysis of DNA irradiated in the presence of furocoumarins: they are the 3,4-formulas and (references) and the 4',5'-photoadducts (6, 7; see Fig. 1).

Both these two types of photoadducts derive from the addition of one pyrimidine molecule to one furocoumarin molecule. In recent times we have published a preliminary report on some evidences indicating that psoralen in photoreaction with DNA can react both with the 3,4- and 4',5'-double bond, giving therefore a third type of photoadduct in which one psoralen molecule is linked to two pyrimidine molecules. Behaving as a bifunctional reagent, psoralen can give interstrand cross-linkages in native DNA. On this possibility more recently Cole has also published other experimental results obtained using 4,5',8-trimethyl-psoralen.

Cross-linking formation in native DNA is a new aspect of the photoreactions between furocoumarins and nucleic acids. In the present paper we report on experiments we have performed using not only psoralen but a wider group of 12 furocoumarins. The various substances showed very different capacities to form cross-linkages; these depend mainly on the molecular structure of furocoumarins and the steric relationships with the pyrimidine bases when they are intercalated in DNA.

Materials and methods

DNA. Calf-thymus DNA highly polymerized (Mann Research Laboratories, New York), having an hypochromicity higher than 37% was used.

Furocoumarins. Bergapten (5-methoxy-psoralen) was 14C labelled (—O14CH3), prepared by methylation of...
5-hydroxy-psoralen with J$^{14}$CH$_3$. All other furocoumarins were tritiated, prepared by the Willzbach method, as described elsewhere$^{12}$. Specific radioactivity (disint/min per mmole): 5-methoxy-psoralen (bergapten) 8.5 x 10$^8$; psoralen 4.26 x 10$^8$; 8-methoxy-psoralen (xanthotoxin) 1.29 x 10$^8$; 5-methyl-psoralen 2.01 x 10$^8$; 8-methyl-psoralen 2.65 x 10$^8$; 4,8-dimethyl-psoralen 3.91 x 10$^8$; 5,8-dimethyl-psoralen 4.88 x 10$^8$; 4,5,8-trimethyl-psoralen 4.23 x 10$^8$; 4,4',8-trimethyl-psoralen 1.7 x 10$^8$; angelicin 3.1 x 10$^9$; 7-methyl-allocoumarin 1.7 x 10$^9$; 5',8-dimethyl-psoralen 4.88 x 10$^9$; 3',4'-dimethyl-psoralen 4.23 x 10$^9$; 3,4',6-trimethyl-psoralen 3.71 x 10$^9$.

Irradiation. All irradiations were performed placing 2 ml of the samples to be irradiated in glass calibrated tubes, 1.2 cm in diameter, immersed in a cell with glass walls, in which thermostatically controlled (22°C) water circulated. Irradiation was made by means of two HPW125 Philips lamps, which emit almost exclusively at 365 nm, placed one on each side of the cell at a distance of 3.5 cm. The total incident radiation on the 2 ml of the solutions was equivalent to 2.9 x 10$^{16}$ quanta/sec.$^{14}$

Fluorimetric measurements. For determining the fluorescence, 0.5 ml of the samples were diluted with 2 ml of 0.1 mol phosphate buffer pH 7.0 and examined in an Amino-Bowman spectrophotofluorimeter.

Spectrophotometric measurements. An Optica CF$_4$ spectrophotometer was used. For the determination of the denaturation and renaturation curves it was provided with the attachment for the determination of the optical density at various controlled temperatures. Quartz cuvettes with an optical path of 1 mm were used.

Radioactivity measurements. These were performed using a LS-150 Beckman liquid scintillation spectrophotometer: 0.2 ml of the samples were diluted with 1 ml of water and added to 10 ml of dioxane base scintillator (PPO g 4, POPOP g 0.075, naphthalene g 120, dioxane up to 1000 ml).

Non-spontaneously renaturable fraction of DNA. Samples of 2 ml of aqueous 0.05% DNA solutions, containing 2 mM NaCl and 10 μg/ml of a labelled furocoumarin, were kept in the dark or irradiated at 365 nm. From 0.5 ml of the samples DNA was precipitated and washed as described in previous papers$^{11,13,14,15}$, to remove the excess of nonreacted compounds; DNA was then redissolved in 0.5 ml of water and its radioactivity was measured, thus determining the amount of linked furocoumarin.

Results and discussion

Fluorescence of DNA after the photoreaction with psoralen

As mentioned previously, when some furocoumarins are irradiated at 365 nm in the presence of pyrimidine bases, they can give two types of photoadducts, engaging either their 3,4- or their 4',5'-double bond.

The 3,4-photoadducts (Fig. 1, formulas 4 and 5) are not fluorescent when observed at W o o d’s light. They have ultraviolet absorption spectra which show no absorption at wavelengths longer than 330 nm$^3,4$; therefore by irradiation at 365 nm they
are not excited and cannot give fluorescence as well as a further photoreaction.

By contrast the 4',5'-photoadducts (Fig. 1, form. 6 and 7), which have a brilliant violet fluorescence when observed at Wood's light, have ultraviolet spectra which show definite absorptions at 365 nm. Therefore these compounds by irradiation at 365 nm can be excited and can further photoreact.

This further photoreaction could be a splitting of the photoadduct, giving again the two parent compounds; however this possibility was not observed by irradiation at 365 nm, while it occurs by irradiation at shorter wavelengths (253 nm)\(^5\)\(^14\).

Another possibility is that these photoadducts photoreact with the 3,4-double bond of their furocoumarin moieties. Until the present no photoreaction products of furocoumarins have been described in which both 4',5'- and 3,4-double bonds are involved. However the suggested possibility represents a way (perhaps the only way) of explaining the particular behaviour of the fluorescence of DNA during the irradiation at 365 nm in the presence of furocoumarins.

It has been reported in previous papers\(^1\),\(^11\),\(^13\) that DNA after irradiation at 365 nm in the presence of some furocoumarins acquires a violet fluorescence (for instance, in the case of psoralen, \(\lambda_{\text{max}} = 400\) nm; exciting wavelength 330 nm), which was attributed to the formation of 4',5'-photoadducts. It was observed that in the first period of irradiation fluorescence increased by increasing the number of furocoumarin molecules linked to DNA; however, by further irradiation, while a further increasing of the amount of linked furocoumarin occurred, fluorescence remained constant or decreased. This behaviour has not hitherto been explained.

We have now performed the following experiment*. Samples of a 0.05% aqueous solution of DNA containing 2 mM NaCl and 10 \(\mu\)g/ml of \(^3\)H-psoralen (solution A) were irradiated for increasing periods up to a maximum of 10 minutes. From the irradiated samples DNA was precipitated to remove the excess of unbound psoralen, washed and redissolved in water, operating as described in previous papers\(^11\),\(^13\),\(^15\) and measuring both the fluorescence and the radioactivity. The results obtained are reported in part a of Fig. 2. They show that in the initial period of the photoreaction (10 minutes of irradiation) there is a rather parallel increase of the amount of psoralen molecules linked to DNA and of the fluorescence that these molecules produce in DNA.

Now, from a pool of several samples of the solution A irradiated for 10 minutes DNA was precipitated, washed and redissolved in the original volume of water (solution B). On the basis of radio-

![Fig. 1. Molecular structures of psoralen (1), isopsoralen or angelicin (2), allopsoralen (3), 3,4-photoadduct psoralen-thymine (4, 5), 4',5'-photoadduct psoralen-thymine (6, 7).](attachment:image)

![Fig. 2. Amount of psoralen (\(\mu\)g/mg DNA) linked to DNA —x—x—x—x— and fluorescence intensity of DNA (arbitrary units; activating wavelength: 330 nm; maximum fluorescent wavelength: 400 nm) —o—o—o— by irradiation at 365 nm: a — 0.05% DNA solution containing 10 \(\mu\)g/ml of psoralen; b — 0.05% solution of a DNA-psoralen combination containing 3.86 \(\mu\)g of psoralen linked to 1 mg of DNA; c — reirradiation of the DNA-psoralen combination irradiated for 80 minutes, precipitated and redissolved in water.](attachment:image)
activity measurements DNA contained covalently bound 3.86 µg of psoralen per mg of DNA, corresponding to 7.04 molecules of psoralen for every 1000 nucleotides (P atoms). Samples (2 ml) of the solution of this DNA-psoralen combination (solution B) were irradiated at 365 nm for different increasing times. After irradiation DNA of each sample was precipitated, washed, again redissolved in water and both its radioactivity and its fluorescence were measured. The results obtained are reported in part b of Fig. 2. They show that by re-irradiation of the DNA-psoralen combination (isolated from excess of unbound psoralen) its radioactivity remained constant: there was therefore no splitting effect and the number of psoralen moieties linked to DNA remained constant. By contrast fluorescence decreased very rapidly especially in the first period of reirradiation, becoming then rather constant.

These results can be explained assuming that some furocoumarin moieties, already linked to pyrimidine bases of DNA by means of their 4',5'-positions, by reirradiation at 365 nm photoreact again with other pyrimidine bases engaging also their 3,4-double bond. In such a way possibly photoproducts are formed containing one psoralen molecule linked to two pyrimidine bases. Although this new type of photoproducts has not yet been isolated, we can assume that it is not fluorescent on the basis of the properties of the 3,4-photoproduct 3. Therefore its formation appears to be able to explain the marked decrease of the fluorescence.

To ascertain that fluorescence really remained rather constant after the strong decrease of the initial period of reirradiation, a pool of samples (2 ml) of the solution B were irradiated for 80 minutes: DNA was then precipitated, washed and redissolved in the initial volume of water (solution C). Samples of this solution were again irradiated and, after precipitation and redissolution of DNA in water, both radioactivity and fluorescence were measured. The results are reported in part C of Fig. 2. No great variations are evident either in the radioactivity or in the fluorescence of the sample. Therefore, as DNA-psoralen combination retains a small part of its fluorescence even after a very long re-irradiation, we may conclude that not all fluorescent furocoumarin moieties have the possibility of giving a further photoreaction with a second pyrimidine base (see later for a comment).

Denaturation and renaturation curves of DNA after irradiation in the presence of psoralen

It is known that when a furocoumarin is added to an aqueous solution of DNA a molecular complex is formed (without any irradiation) in which very weak bonds are involved 20, 21. Various properties of these complexes suggest that an intercalation of the planar furocoumarin molecules takes place between the planes formed by the bases in double helix DNA structure. Therefore when a psoralen molecule so intercalated photoreacts firstly with its 4',5'-double bond and then with its 3,4-double bond, the two reacting pyrimidine bases must belong to two different strands of DNA: in such a way a cross-linking is formed between the two strands (see Fig. 6).

In respect to the possibility of cross-linking formation, we have examined the behaviour of DNA, after irradiation in the presence of psoralen, towards denaturation and renaturation. We operated in the experimental conditions normally used for the determination of the Tm value, as described by MARMUR and DOTY 23, measuring the optical density of aqueous 2 mM NaCl solutions of DNA at various increasing temperatures; after having reached the temperature at which the optical density remained constant, we gradually decreased the temperature of the solutions, determining again the optical density.
The results obtained with non-irradiated DNA and with DNA irradiated in the presence of psoralen for 1, 10 and 60 minutes are shown in Fig. 3. We can observe a progressive modification of the denaturation and renaturation curves from the non-irradiated to the irradiated DNA: 1. denaturation takes place at temperatures progressively higher; this is in agreement with the higher Tm values of DNA irradiated in the presence of various furocoumarins found in a preceding research; 2. the total increase per cent of the optical density reached at high temperature becomes progressively smaller, indicating that after irradiation in the presence of psoralen a fraction of DNA cannot be denatured. This fraction, calculated from the results reported in Fig. 3 (it is indicated as C', C", C‴) is 1% after 1 minute of irradiation; 14.5% after 10 minutes and 34.1% after 60 minutes. 3. by decreasing the temperature, renaturation takes place only to a small degree in the non irradiated DNA, while after irradiation it becomes more and more easy: after 10 minutes of irradiation it is fairly complete.

This behaviour is characteristic of DNA containing inter-strand cross-linkages. We can assume that while in non-irradiated DNA after denaturation the two strands can easily separate and therefore renaturation does not easily occur, after irradiation in the presence of psoralen, when some cross-linkings are present, the two strands can swell on heating and therefore a partial denaturation can also occur, but the two strands cannot completely separate and therefore by decreasing the temperature renaturation can occur easily.

Non-spontaneously renaturable fraction present in DNA after irradiation in the presence of psoralen and 4',5'-dihydro-psoralen

The spontaneous renaturation capacity acquired by DNA after the photoreaction with psoralen can be evaluated in a more simple but strictly reproducible way by heating the DNA solution at 100°C for 10 minutes, quenching the solution in ice for 10 minutes and then determining its optical density. The procedure is described in detail in the section “Materials and Methods”.

In this way we can obviously detect only the initial and final steps of a process which is analyzed in greater detail by the denaturation and renaturation curves reported in Fig. 3. Measuring the increase of the optical density of the solution after the treatment, we practically measure the value of b (see Fig. 3), that is the non-spontaneously renaturable fraction present in DNA before and after irradiation in the presence of psoralen. Generally we have expressed this value as a percentage in respect of the value presented by the solution of native untreated DNA (non-renaturable fraction per cent).

Using labelled (tritiated) psoralen it was possible to determine the amount of psoralen molecules linked to DNA after each period of irradiation and to correlate these amounts with the effects observed in DNA.

The results are shown in Fig. 4, in which the number of psoralen molecules (and of other furocoumarins: see later) per 1000 nucleotides present in DNA is plotted against the log of the “non-renaturable fraction per cent” of DNA. We can see that a linear relationship exists.

We have also examined 4',5'-dihydro-psoralen which differs from psoralen only by having the

Fig. 4. Cross-linking formation in native DNA by various furocoumarins under irradiation at 365 nm. The number of the furocoumarin molecules linked to DNA per 1000 nucleotides are plotted against the log of the percent non renaturable fraction of DNA.

Non-spontaneously renaturable fraction present in DNA after irradiation in the presence of psoralen and 4',5'-dihydro-psoralen

The spontaneous renaturation capacity acquired by DNA after the photoreaction with psoralen can be evaluated in a more simple but strictly reproducible way by heating the DNA solution at 100°C for 10 minutes, quenching the solution in ice for 10 minutes and then determining its optical density. The procedure is described in detail in the section “Materials and Methods”.

23 J. MARMUR and P. DOTY, J. molecular Biol. 5, 109 [1962]. * The simple addition of psoralen to the DNA solution without irradiation did not practically change the behaviour of DNA.

4',5'-positions hydrogenated. Evidently it can give a photo-cyclo-addition reaction only with the 3,4-double bond and therefore it cannot give cross-linkages. This furocoumarin photoreacts with DNA very slowly, but after a long period (5 hours) of irradiation at 365 nm an incorporation of 2.5 molecules per 1000 nucleotides of DNA was obtained. The determination of this number was possible using the tritiated compound.

After the photoreaction with this furocoumarin DNA did not change its behaviour in the previously described denaturation and renaturation experiments, as shown in Fig. 5.

This result is an evident confirmation that the modifications observed in the behaviour of DNA after the photoreaction with psoralen are really due to cross-linking formation and not to the simple monofunctional linkage of the furocoumarin molecules. We can therefore assume that the determination of the non-renaturable fraction per cent of DNA gives data which are correlated with the cross-linking formation.

**Cross-linking formation capacity of some other furocoumarins**

It is known that many other furocoumarin derivatives, other than psoralen, can photoreact with DNA; some methyl-derivatives of psoralen were found to have a very high photoactivity, higher than that of psoralen. The fluorescence of DNA after the photoreaction with some furocoumarins (5-methoxy-psoralen or bergapten, 8-methoxy-psoralen or xanthotoxin, 8-methyl-psoralen) was studied in a previous research; it showed a behaviour rather similar to that observed after the photoreaction with psoralen, that is its intensity increased in the first period of irradiation and then decreased.

Therefore to investigate the capacity of some other furocoumarins to form cross-linkings in the photoreaction with DNA, we have performed with these substances a series of experiments analogous to those described for psoralen. The furocoumarins used are reported in Table I; all compounds were labelled (tritiated, with the single exception of 5-methoxy-psoralen, which was 14C).

To obtain comparable data, the experimental conditions were strictly the same for the various substances. The solutions of DNA (0.05%) and furocoumarins (10 μg/ml; only 5',4,8-trimethyl-psoralen had a concentration of 5 μg/ml, because it is not soluble in a higher concentration) were irradiated for different increasing times and then worked out in the same way as described for the determination of non-spontaneously renaturable fraction present in DNA in the case of psoralen.

The results obtained are reported in Fig. 4. The intensities of the effects observed in this way for the various furocoumarins are rather different, indi-

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PHOTOREACTIONS BETWEEN FUROCOUMARINS AND DNA

Table I. Non-renaturable fractions per cent of DNA observed after 2.5 molecules of furocoumarins per 1000 nucleotides were linked by irradiation at 365 nm and times of irradiation necessary to obtain their linkage.

<table>
<thead>
<tr>
<th>Furocoumarins</th>
<th>% non-renaturable</th>
<th>Times of irradiation necessary to obtain the linkage of 2.5 molecules of furocoumarins per 1000 nucleotides [seconds]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bergapten</td>
<td>3.9</td>
<td>583</td>
</tr>
<tr>
<td>Psoralen</td>
<td>5.7</td>
<td>135</td>
</tr>
<tr>
<td>8-methyl-psoralen</td>
<td>8.1</td>
<td>36</td>
</tr>
<tr>
<td>Xanthotoxin</td>
<td>9.5</td>
<td>276</td>
</tr>
<tr>
<td>5-methyl-psoralen</td>
<td>10.9</td>
<td>70</td>
</tr>
<tr>
<td>5',8-dimethyl-psoralen</td>
<td>16.2</td>
<td>54</td>
</tr>
<tr>
<td>4,8-dimethyl-psoralen</td>
<td>18.7</td>
<td>30</td>
</tr>
<tr>
<td>4',4,8-trimethyl-psoralen</td>
<td>21.6</td>
<td>24</td>
</tr>
<tr>
<td>5',4,8-trimethyl-psoralen</td>
<td>22.3</td>
<td>20</td>
</tr>
<tr>
<td>Angelicin</td>
<td>30.0</td>
<td>1125</td>
</tr>
<tr>
<td>7-methyl-allopsooralen</td>
<td>30.0</td>
<td>2250</td>
</tr>
<tr>
<td>Control (non-irradiated DNA)</td>
<td>30.0</td>
<td>—</td>
</tr>
</tbody>
</table>

Table I. Non-renaturable fractions per cent of DNA observed after 2.5 molecules of furocoumarins per 1000 nucleotides were linked by irradiation at 365 nm and times of irradiation necessary to obtain this incorporation.

cating a different capacity of the various substances to form cross-linkings. On the other hand, we know that furocoumarins have a very different capacity to photoreact with DNA and therefore the binding of an equal number of molecules to DNA is obtained after very different periods of irradiation. To evidence whether an eventual correlation exists between these two properties, in Table I are reported the values of the non renaturable fraction per cent present in DNA after 2.5 molecules of the various furocoumarins were linked and the times of irradiation necessary to obtain their linkage.

As is apparent, the most active furocoumarin in forming cross-linkings is bergapten, even if it photoreacts rather slowly. By contrast, the dimethyl- and trimethyl-psoralen which have the highest photoreactivity have a small capacity to give cross-linkings. However, apart the mentioned cases, no very close, direct or indirect, correlation appears between the capacity of the substances to form cross-linkings and their photoreactivity. We point out the fact that all the linear furocoumarins form cross-linkings in a more or less great amount; by contrast the two angular furocoumarins (angelicin and 7-methyl-allopsooralen) did not form any crossing-linking.

The different capacities of furocoumarins to form cross-linkings, which appear not to be in relation with the simple capacities of the substances to photobind to DNA, from a speculative point of view could depend on various factors, such as: a) the ability of the furocoumarin molecules to intercalate more or less freely between two base pairs of DNA (complex formation); b) the steric relationships between the intercalated furocoumarins and the pyrimidine bases of DNA, which can determine the possibility of reaction of the two double bonds of furocoumarins, bearing in mind that the molecules have an almost rigid position when they are trapped in the complex; c) the preferential photoreactivity of the 4',5'- or the 3,4-double bond of the intercalated furocoumarin; as we have previously said, this fact may lead to photoadducts which are able to photoreact further or, by contrast, remain inactive by subsequent irradiation at 365 nm.

**Formation of molecular complexes between furocoumarins and DNA**

The formation of molecular complexes between some furocoumarins (psoralen, xanthotoxin, bergapten, angelicin and others) and native DNA has been already studied employing various experimental methods, some of which have led to the determination of the amount of furocoumarin molecules, which were bound to DNA in the complex. As, however, for several compounds now used (namely for the methyl-derivatives of psoralen) no data were available, we have now extended to these substances also our earlier studies on the complex formation ability.

We have determined, at controlled temperature (25 °C), the solubility of the substances in water and in DNA solution: the increase of the solubility in the presence of DNA is generally assumed as due to a complex formation and the amount of substance solubilized more than in water is con-
sidered bound to DNA. Using these data, for each furocoumarin the ratio
\[
\frac{[\text{furocoumarin bound to DNA}]}{[\text{free furocoumarin}]}
\]
was calculated; as it is correlated with the stability constant of the complex *, it gives a good indication of the ability of the substance to form the complex.

The results now obtained are reported in Table II. We can see that the complex formation ability of psoralen and its methyl-derivatives increases by increasing the number of the methyl-groups. From the data of Table I we see that the cross-linking formation capacity, by contrast, decreases in the same order; therefore the loss of cross-linking formation ability by introduction of methyl-groups in the psoralen molecule cannot be attributed to a loss of the ability to intercalate in DNA.

The data now obtained by contrast appear well able to contribute to the explanation of the high photoreactivity of the various methyl-derivatives of psoralen with native DNA; in fact going from psoralen to their mono-, di- and tri-methyl-derivatives we can observe (Table II) that both capacity to photoreact and ability to form complexes increase in an almost parallel way. This is in agreement with the fact pointed out in other papers¹¹,¹³,¹⁵,²¹, that the furocoumarin molecules which are bound in the complex with DNA are in a more suitable condition to photoreact than the free molecules.

### Furocoumarins

<table>
<thead>
<tr>
<th>Furocoumarins</th>
<th>Complex formation with DNA</th>
<th>Relative photoreactivities with DNA¹⁵ (psoralen = 100)</th>
<th>Solubilities in water at 25°C [µg/ml]</th>
<th>Solubilities in a 0.1% DNA solution at 25°C [µg/ml]</th>
<th>Bound furocoumarin</th>
<th>Free furocoumarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psoralen</td>
<td></td>
<td></td>
<td>36.5</td>
<td>54.5</td>
<td>0.49</td>
<td>100</td>
</tr>
<tr>
<td>5-methyl-psoralen</td>
<td></td>
<td></td>
<td>35.8</td>
<td>66.6</td>
<td>0.86</td>
<td>233</td>
</tr>
<tr>
<td>8-methyl-psoralen</td>
<td></td>
<td></td>
<td>19.7</td>
<td>33.4</td>
<td>1.71</td>
<td>373</td>
</tr>
<tr>
<td>5',8-dimethyl-psoralen</td>
<td></td>
<td></td>
<td>4.6</td>
<td>16.3</td>
<td>2.54</td>
<td>466</td>
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<tr>
<td>4,8-dimethyl-psoralen</td>
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<td></td>
<td>3.4</td>
<td>21.2</td>
<td>5.23</td>
<td>589</td>
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<tr>
<td>5',4,8-trimethyl-psoralen</td>
<td></td>
<td></td>
<td>0.8</td>
<td>5.0</td>
<td>5.25</td>
<td>710*</td>
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<tr>
<td>4',4,8-trimethyl-psoralen</td>
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<td></td>
<td>2.0</td>
<td>21.5</td>
<td>9.75</td>
<td>517*</td>
</tr>
</tbody>
</table>

Table II. Complex formation with DNA by psoralen and various its methyl-derivatives. * These are new data, not previously reported. About the photoreactivity of 5',4,8-trimethyl-psoralen with DNA see also l. c. ²⁶, ²⁷.

* In fact it can be considered a simplification of the equation:
\[
K = \frac{[\text{Furocoumarin} - \text{DNA}]}{[\text{Furocoumarin}][\text{DNA}]}
\]

Steric relationships of the intercalated furocoumarins with the pyrimidine bases of DNA

Using a molecular model of the double stranded native DNA (Crystal Structure Limited, Bottisham-Cambridge, England) and molecular models of furocoumarins on the same scale, we have studied the various positions that an intercalated furocoumarin can assume and the possibilities of a photoreaction between the two reactive double bonds of the furocoumarin and the 5,6-double bonds of the pyrimidine bases. In this way we have observed various interesting situations.

a) As COLE¹⁰ pointed out, when psoralen is intercalated between two stacked base pairs, it can assume two different positions in which it has both the 4',5'- and 3,4-double bonds aligned with the 5,6-double bonds of the two pyrimidine bases of the opposite strands. Therefore in these two positions psoralen can form cross-linkings; no steric difference is apparent between the thymine and cytosine. One of these two positions was well represented by COLE¹⁰, the second, strictly analogous, is shown by the Fig. 6.

b) The presence of a methyl-group in the 5 or 8 positions does not seem to provoke a well defined steric hindrance.

c) By contrast the presence of a methyl-group in the position 4 provokes an interference with the methyl-group of thymine; as Fig. 7 shows, in this case very probably the furocoumarin derivative is shifted into a position which makes difficult the photoreaction with DNA in which the concentration of DNA was omitted. The omission is possible because the concentration of DNA solution was the same for all furocoumarins. The values thus obtained for the various substances are comparable.
Fig. 6. Projection of a psoralen molecule intercalated between two base pairs in DNA. Only two thymines belonging to the opposite strands are shown, omitting the two complementary purine bases, that is the adenines. The thymines are rotated $24^\circ$ because of the intercalation. Both the 4',5'- and the 3,4-double bond of psoralen can photoreact with the 5,6-double bond of the two thymines, requiring only a little distortion of the DNA structure.

Fig. 7. Projection of a 4,8-dimethyl-psoralen molecule intercalated between two base pairs in DNA (see Fig. 6). The interference of the methyl-group in the 4-position with the methyl-group of thymine makes probable a position shifted in respect to that of psoralen, which hinders the photoreaction of the 3,4-double bond.

photo reaction between the 3,4-double bond and the 5,6-double bond of thymine. On the contrary, no steric hindrances occur when cytosine is present, instead of thymine.

Very analogous is the situation when a methyl-group is present in the 4'-position; in this case the photoreaction of the 4',5'-double bond with thymine is hindered.

This steric hindrance, which decreases the possibility of furocoumarin derivatives to photoreact freely, may explain the low ability to form cross-linkings when one or two methyl-groups in the 4- and 4'-positions are present.

d) Very interesting appear the two angular furocoumarins, namely angelicin and 7-methyl-allo-psoralen. As is reported in Table I, these two substances did not form cross-linkings; this fact was confirmed working out an experiment analogous to that reported for 4',5'-dihydro-psoralen (see Fig. 5); the results obtained in these cases were strictly analogous.

Angelicin differs from many other furocoumarins also by another particular behaviour in the photoreactions with DNA: although it can photobind to DNA under irradiation at 365 nm, it does not produce any characteristic fluorescence in DNA. Recalling the properties of the photoadducts 3,4- (non fluorescent) and 4',5'- (very fluorescent), we are led to conclude that angelicin photoreacts only with its 3,4-double bond. Since 3,4-photoadducts cannot further photoreact by irradiation at 365 nm, as we have previously seen, we could explain in this way the inefficiency of angelicin in forming cross-linkages.

However this inefficiency derives very clearly also from the examination of the molecular models. When angelicin is intercalated in DNA, it can have at least two positions suitable for photoreacting by means of the 3,4-double bond and two other positions for reacting by means of the 4',5'-double bond. However in any of these positions the second reactive double bond is so far from the 5,6-double bond of the opposite pyrimidine base as to make the photoreaction impossible. There are no possible positions in which angelicin can photoreact with the two double bonds (see Fig. 8) and therefore angelicin cannot form cross-linkages.

Fig. 8. Projection of an angelicin molecule intercalated between two base pairs of DNA (see Fig. 6). In this position (which is one of the most probable) angelicin can photoreact only with its 3,4-double bond.

The situation of 7-methyl-allo-psoralen is analogous to that of angelicin.

e) We have previously seen that by irradiation of a DNA-psoralen combination its fluorescence decreases, but does not completely disappear after a very long irradiation, indicating that some fluorescent psoralen-moieties cannot further photoreact. We can say now that this fact occurs when in the opposite strand of DNA, above and below the intercalated psoralen moiety, there exists no pyrimidine base, but both are purine bases.