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Changes in the melting curve of DNA after the photoreaction with skin-photosensitizing furocoumarins

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Chimica. — *Changes in the melting curve of* DNA *after the photoreaction with skin-photosensitizing furocoumarins* (*). Nota di FRANCESCO DALL'ACQUA e GIOVANNI RODIGHIERO, presentata (**) dal Corrisp. L. MUSAJO.

RIASSUNTO. — Nell'ambito delle ricerche sulle interazioni tra DNA e furocumarine, gli Autori trovano che l'irradiazione a 3.655 Å di DNA estratto da timo di vitello in presenza di una furocumarina fotosensibilizzatrice (psoralene, xantotossina, bergaptene) provoca un netto aumento del valore del Tm del DNA, mentre l'irradiazione in presenza di una furocumarina priva di attività fotosensibilizzatrice (bergaptolo, isopimpinellina) non altera il valore del Tm. Questo fatto è un'altra prova della fotoreazione tra DNA e furocumarine fotosensibilizzatrici, che avviene per irradiazione a 3.655 Å e forma uno stabile legame chimico tra le furocumarine ed il DNA.

INTRODUCTION.

In recent papers [1–5] have been published the results of the studies on the interaction between nucleic acids and furocoumarins, worked out in continuing the researches on the mechanism of the photosensitizing effects that some of these substances (the so-called skin-photosensitizing furocoumarins) exert on various biological substrates [6].

It has been demonstrated that, without any irradiation, a complex is formed between native DNA and furocoumarins. Active and inactive substances in these conditions have the same behaviour [1, 3, 5,].

After irradiation at 3,655 Å of an aqueous solution containing native DNA and a photosensitizing furocoumarin, a photoreaction occurs with the formation of a stable linkage between the two substances. Inactive furocoumarins do not photoreact [2–4].

The first evidence of these photoreactions was obtained by examining the modifications of the fluorescence spectra of photosensitizing furocoumarins after irradiation in the presence of native DNA [2]. This was later confirmed and more extensively studied using a labeled furocoumarin [4].

We now report the results of our study on the influence of such a photoreaction on a characteristic property of native DNA, that is the melting temperature (Tm). We have found that the irradiation at 3,655 Å of a DNA solution containing a photosensitizing furocoumarin provokes an increase of the Tm–value.

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Materials and methods.

The sample of DNA used in the present experiments was extracted from calf thymus with the aid of sodium dodecilsufate [7]. Other samples of calf thymus DNA, highly polymerised, and of salmon sperm DNA, obtained from Mann Research Laboratories, New York, were also used in preliminary experiments. The three samples of DNA demonstrated the same behaviour.

The furocoumarins used have been prepared in our Laboratory, by synthesis or by extraction from vegetable materials.

The preparation of the solutions containing DNA and furocoumarins was made as described in a previous note [5]. Aqueous solutions containing 0.1 % DNA and 20–40 μg/ml of a furocoumarin and, for comparison, simple aqueous 0.1 % DNA solutions were always used.

For studying the influence of the simple addition (without irradiation) of furocoumarins on the Tm–value of DNA, 0.2 ml of these solutions were diluted with 9.8 ml of 3 mM phosphate buffer (pH 7.2) and then used for the spectrophotometric determination of Tm.

For studying the effect of the irradiation at 3,655 Å in the presence of furocoumarins, the same solutions, irradiated or not, after an addition of NaCl to a M concentration, were cooled and then added to two volumes of cold ethanol and centrifuged; the precipitated DNA was washed twice with cold 70 % aqueous ethanol and redissolved in the same volume as in the initial stage with 3 mM phosphate buffer pH 7.2. A small part of this solution (0.2 ml) was diluted with the same buffer (9.8 ml) and then used for the spectrophotometric determination of Tm.

The remaining concentrated solution was used for the fluorimetric determinations.

Tm was determined as described by Marmur and Doty [8], using an Uvispeck Hilgher and Watts spectrophotometer, equipped with the necessary attachement.

The fluorimetric determinations were made with an Aminco–Bowmann spectrophotofluorimeter.

For the irradiation, a Philips HPW 125 lamp, with emission at 3,655 Å, was used. The solutions, placed in Petri-disks 3 cm in diameter, were kept at a distance of 20 cm from the lamp. (Irradiation power 0.63 mW/cm^2).

Results and discussion.

The results obtained show that the simple addition of a furocoumarin (20–40 μg/mg DNA) to a DNA solution in 3 mM phosphate buffer (pH 7.2), without any irradiation, provokes a small but evident increase of the Tm–value (1.2–2.5°; see fig. 1 *a*). No difference was noted in this regard between the photosensitizing and the inactive substances.

After precipitation with ethanol from these solutions, DNA, centrifuged and redissolved in phosphate buffer 3 mM (pH 7.2) showed the same Tm–value as the original sample.

The small enhancement that resulted may be considered as a consequence of the complex-formation in the dark between DNA and furocoumarins [1, 5], complex which is completely broken by ethanolic precipitation of DNA [4].

More evident effects were obtained by irradiation at 3,655 Å of the solutions containing DNA and furocoumarins and examining the Tm of the DNA samples after ethanolic precipitation, centrifugation and redissolution in phosphate buffer at pH 7.2.

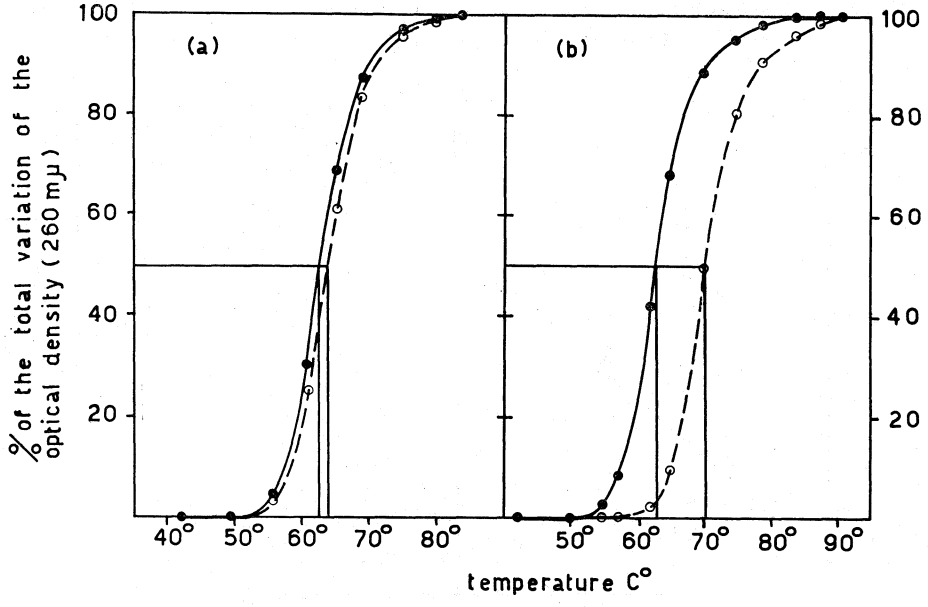

Fig. 1. – Influence of bergapten on the optical density-temperature profiles of DNA.

a) without irradiation: –●——●– solution 2 mg% of DNA in 3 mM phosphate buffer pH 7.2; –○– –○– the same solution containing 20 μg/mg DNA of bergapten.

b) after irradiation (2 hours at 3,655 Å; 0.63 mW/cm²): –●——●– DNA irradiated alone, precipitated with ethanol and redissolved in 3 mM phosphate buffer pH 7,2; –○– – –○– – DNA irradiated in the presence of bergapten (20 μg/mg DNA), precipitated with ethanol and redissolved in 3 mM phosphate buffer pH 7.2.

When DNA was irradiated in the presence of a photosensitizing furocoumarin (psoralen, xanthotoxin, bergapten), its Tm value showed a sharp increase and the same DNA had assumed a blue-violet fluorescence. Fig. 1*b* shows the denaturation profiles of DNA irradiated alone and in the presence of bergapten. By increasing the period of irradiation, there was a gradual increase both of the fluorescence intensity and of the Tm value, as indicated in fig. 2.

However, when DNA is irradiated alone (in the absence of furocoumarins) or in the presence of a skin-inactive furocoumarin, such as ber-

gaptol and isopimpinellin, after ethanolic precipitation no modification in the Tm–value and no fluorescence was observed.

The results obtained are summarized in Table I. They provide further evidence that the photoreaction with DNA occurs only with the photobiologically active furocoumarins, while the inactive ones do not photoreact.

In preceding experiments [4] the formation of a stable linkage between DNA and bergapten by irradiation at 3,655 Å was demonstrated using this furocoumarin ^{14}C labeled. After irradiation DNA, precipitated from the solution with ethanol, showed a radioactivity and a fluorescence with λ max 405 mμ, both increasing with the increase of the period of irradiation.

The fluorescence with λ max in the same spectral region now observed in DNA after irradiation in the presence of psoralen and xanthotoxin extends the same conclusion to these furocoumarins.

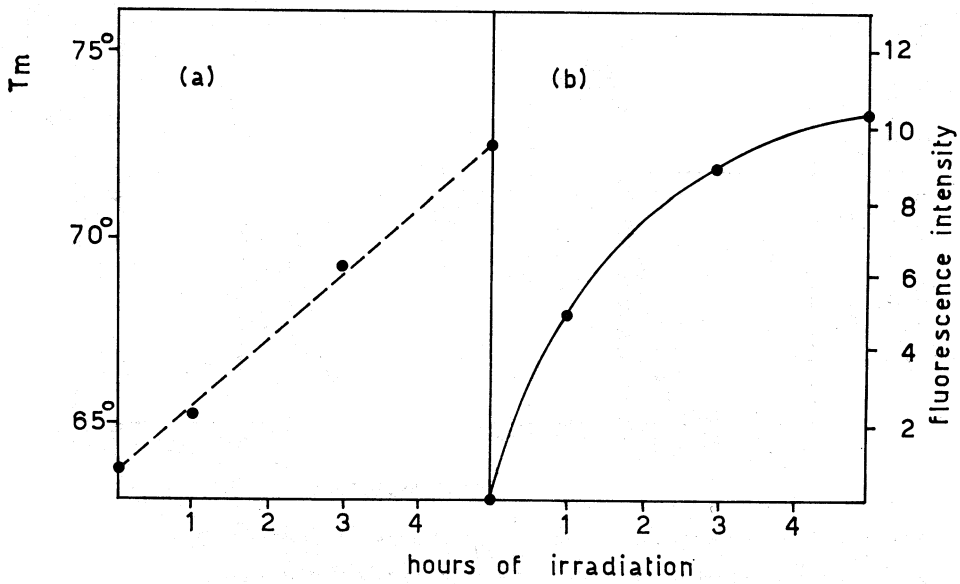

Fig. 2. – Increase of the Tm value *a*) and of the fluorescence at 400 mμ *b*) of DNA irradiated for increasing periods in the presence of psoralen (20 μg/mg DNA). After irradiation (see fig. 1 *b*) DNA was precipitated with ethanol and redissolved in 3mM phosphate buffer pH 7.2.

The increase of the Tm–value of DNA suggests that a stabilization of the helix structure of DNA is produced, as a consequence of the formation of the stable chemical linkage of the furocoumarins to DNA.

In this respect, the effect of the furocoumarins by irradiation is opposite to that of several other photodynamic substances, such as acridine-orange [9], methylene blue, rose bengal etc. [10, 11], which, by irradiation, lower the Tm–value of DNA.

It is known that the two groups of substances have a different behaviour under irradiation. The photodynamic dyes act on the substrates by a photo-

oxydative process; the irradiation of DNA in the presence of methylene blue [12] or lumichrome [13] provokes the selective photo-oxydation of the guanine moieties.

TABLE I.

Increases of the Tm*-value of* DNA *and fluorescence observed in* DNA *after irradiation at* 3,655 Å.

2 hours of irradiation in the presence of a furocoumarin, precipitation with ethanol and redissolution in 3 mM phosphate buffer (pH 7.2).

FUROCOUMARINS	Concentration (μg/mg DNA)	Increase of Tm	FLUORESCENCE	
			activating λ max	fluorescence λ max
None	0	0°	—	—
Psoralen	40	+ 7°	330	400
Xanthotoxin	45	+ 10°	340	425
Bergapten	20	+ 7,1°	335	405
Bergaptol	20	0°	—	—
Isopimpinellin	20	+ 1,3°	—	—

The photosensitizing furocoumarins on the contrary are completely lacking in photo-oxydative properties [14]. It has been ascertained that under irradiation at 3,655 Å they form a stable photo-adduct with DNA and it is very probable that the reactive sites of DNA are the pyrimidine bases. In fact, experimenting with the simple compounds, it has been found [2] that the photosensitizing furocoumarins photoreact with the pyrimidine bases (thymine, cytosine, uracil) forming new compounds, which consist of a pyrimidine and a furocoumarine moiety [15], but no photoreaction has been observed with the purine bases (guanine and adenine).

Aknowledgments.—The present investigation was carried out as part of the research program on the mechanism of action of the skin-photosensitizing furocoumarins, that Prof. Luigi Musajo and the AA. have been studying at the Institute of Pharmaceutical Chemistry of the University of Padova for several years.

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