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Psoralen-thymine C_4-cycloadduct formed in vitro in the photoinactivation with psoralen of Ehrlich ascites tumor cells

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Chimica. — *Psoralen–thymine* C_4*–cycloadduct formed* in vitro *in the photoinactivation with psoralen of Ehrlich ascites tumor cells* (*). Nota di LUIGI MUSAJO, FRANCO BORDIN, FRANCAROSA BACCICHETTI e RITA BEVILACQUA, presentata (**) dal Corrisp. L. MUSAJO.

RIASSUNTO. — Gli Autori hanno irradiato *in vitro* a 3.655 Å sospensioni di cellule del carcinoma ascitico di Ehrlich del topo in presenza di psoralene; in queste condizioni, come è stato dimostrato precedentemente, viene distrutta la capacità di trasmettere il tumore.

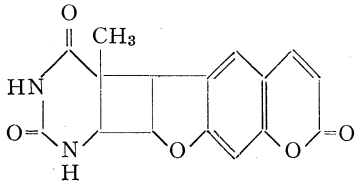
Il DNA cellulare è stato poi estratto e idrolizzato con acido formico all'88%: tra i prodotti di idrolisi è stato isolato un C_4–cicloaddotto fluorescente psoralene–timina identico al fotocomposto già ottenuto per irradiazione di timina e psoralene.

Questi risultati sono stati confermati irradiando sospensioni cellulari contenenti psoralene uniformemente marcato con tritio.

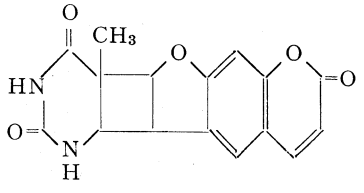
La perdita della capacità di trasmettere il tumore da parte del liquido ascitico, irradiato *in vitro* in presenza di psoralene, appare legata alla fotoreazione tra la furocumarina e le basi pirimidiniche del DNA cellulare.

In continuing our investigation of the mechanism of the action of skin-photosensitizing furocoumarins [1] we have identified and studied the photoreaction which takes place when a DNA solution containing a skin-photosensitizing furocoumarin is irradiated at 3.655 Å [2, 3, 4]. This photoreaction has the result of forming a stable chemical linkage between the macromolecule and the furocoumarin [3]. Following this we have been able to establish that two different types of photoadducts between furocoumarin and the pyrimidine bases of DNA are formed [4].

a) C_4–photocycloadducts in positions 4′–5′ of furocoumarin and 5–6 of the pyrimidine base; these substances are fluorescent in u.v. light and stable in an acid medium. For the photoadduct psoralen-thymine we have suggested structures corresponding to formulas I or II [5].



I



II

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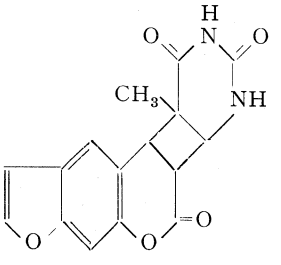
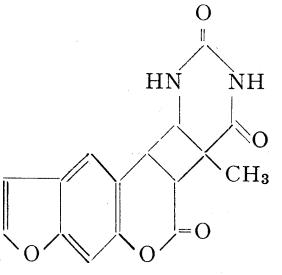
(**) Nella seduta del 9 dicembre 1967.

b) C_4–photocycloadducts in which is involved the double bond in position 3–4 of the pyronic ring of furocoumarin; these substances do not possess fluorescence and are unstable in an acid medium. For the photoadduct psoralen–thymine belonging to this class we have suggested the structures III or IV [6].



III



IV

All these substances, by irradiation at 2.537 Å, break up again yielding furocoumarin and the initial pyrimidine base [5, 6].

These photoreactions, in our opinion, are responsible for the biological effects of furocoumarins upon different substrates [1, 7, 8, 9, 10, 11, 12, 13].

Recently we have been able to establish that the ascitic exudate of the Ehrlich tumor, after irradiation at 3.655 Å (inactive by itself) in the presence of extremely small amounts of skin-photosensitizing furocoumarins such as psoralen or xanthotoxin, or bergapten, loses the ability to transmit the tumor to mice [14].

In order to ascertain whether this photoinactivation is connected with the formation of cycloadducts similar to those previously mentioned, we have extracted and hydrolysed the DNA of Ehrlich ascitic tumor cells after irradiation in the presence of psoralen.

The following experiments have been carried out:

A) A suspension of Ehrlich ascitic tumor cells of mice, cultured in our laboratories by transplantation in albino Swiss mice, diluted with physiological saline containing psoralen and kept in contact with crushed ice, has been irradiated, in conditions of sterility, with a Philips HPW 125 lamp (3.655 Å; distance 15 cm; intensity of irradiation 4.2×10^{15} quanta/sec/cm^2).

We have used suspensions containing 4×10^6 cells per 0.1 ml and with a final concentration of psoralen of 15 μg/ml.

After two hours irradiation the cells have been collected by centrifugation, washed many times with physiological saline by suspension and following centrifugation. The cell DNA have then been extracted by E.R.M. Kay's method, using saline 1 M and sodium dodecil-sulphate [15, 16].

The DNA solutions thus obtained have resulted fluorescent with a spectrum (Aminco Bowman spectrophotofluorimeter with Aminco XY recorder) showing a maximum at 400 mμ (exciting wavelength 330 mμ) similar to that of

solutions containing fluorescent photoadduct obtained from psoralen and pyrimidine bases [5] or DNA samples irradiated in the presence of psoralen.

The cell DNA has then been hydrolysed with 88 % formic acid at 175° for half an hour and the products obtained have been separated by paper chromatography (Wathman No. 1, solvent *n*–butanol–acetic acid–water 4 : 1 : 5). By examining the chromatogram in u.v. light at 3.655 Å a spot with violet fluorescence has appeared with R_F 0.71, the same as that of the fluorescent photoadduct obtained from psoralen and thymine (formulas I–II).

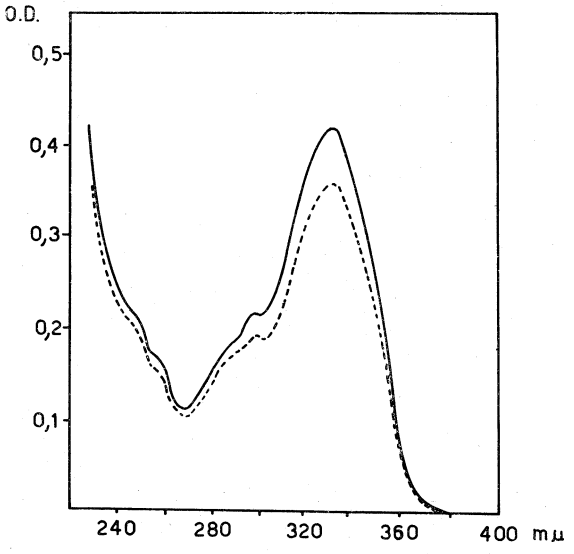


Fig. 1. – U.V. spectra of the photoadduct with violet fluorescence, prepared from psoralen and thymine ——, and of the substance isolated from the Ehrlich ascitic tumor cells after irradiation at 3.655 Å *in vitro*, in presence of psoralen – – – –. Beckman DB Spectrophotometer with Sargent recorder.

The isolated substance has also exhibited the same chromatographic behaviour of synthetic cycloadduct using other solvents (water) or other adsorbents (thin-layer of MN 300 G cellulose powder, Macherey Nagel). The same results have been obtained by hydrolysing DNA with other methods (70 % perchloric acid or 0.4 N hydrochloric acid in a sealed tube [4] heating in both cases at 100° for an hour).

This fluorescent substance has been isolated from the hydrolisates by preparative paper chromatography: its u.v. spectrum (see fig. 1) has proved identical with that of the synthetic fluorescent photoadduct and like this, upon irradiation at 2.537 Å (Philips TUV 15 W lamp) in glacial acetic acid, has reverted to the original monomers, psoralen and thymine, identified by chromatographic test [5].

B) These results have been confirmed by irradiating for two hours at 3.655 Å several cell suspensions containing uniformly tritium-labelled pso-

ralen ($1.9 \cdot 10^8$ cpm. mM); as control a part of every suspension has been kept, on the contrary, in the dark for two hours. The DNA extracted from the various samples has then been hydrolysed with formic acid.

All these operations have been performed according to the afore-mentioned methods.

After evaporation of the hydrolysates in vacuum, the residues thus obtained have been taken up with equal volumes of absolute ethanol; different aliquots of these solutions, after dilution to 3 ml with absolute ethanol, have been mixed with 3 ml of a toluene solution of scintillator (5 g of 2–5–diphenyloxazol and 0.5 g of 2–2′–p–phenylen–bis–5–phenyloxazol in 1000 ml).

The radioactivity has been determined with a liquid scintillator counter SELO, Milan. No radioactivity was found in the controls kept in the dark, whereas the DNA obtained from the irradiated cell suspensions has proved radioactive.

TABLE I.

No. of cells per 0,1 ml	Psoralen concentration $\mu g/10^6$ cells	Samples of extracted DNA No.	Psoralen linked to DNA $\mu g/10$ mg
$8.1 \cdot 10^6$	0.21	1	0.94
$8.1 \cdot 10^6$	0.21	2	1.04
$8.1 \cdot 10^6$	0.21	3	1.12
$6 \cdot 10^6$	0.33	4	0.89
$6 \cdot 10^6$	0.33	5	1.85

Irradiations (3.655 Å) of Ehrlich ascitic tumor cell suspensions in the presence of uniformly tritium-labelled psoralen. After two hours irradiation ($4.2 \cdot 10^{15}$ quanta/cm²/sec.) the cell DNA has been extracted and hydrolysed with 88% formic acid; by measured radioactivity of hydrolisates the amount of psoralen linked to DNA has been determined.

On the basis of the performed measurements (see Table I) it resulted that in five samples the psoralen amount linked to cell DNA was about 1 $\mu g/10$ mg. The hydrolysate of one of these DNA samples was applied as a continuous line upon Wathman No. 1 chromatographic paper, developing with *n*–butanol–acetic acid-water 4 : 1 : 5; the chromatogram was cut out in nine equal bands that were eluted with the same volume of absolute ethanol. For each eluate the fluorescence (λ max 400 mμ, λ exc. 330 mμ) and the radioactivity (3 ml eluate mixed with 3 ml of toluene solution of scintillator) were determined.

At R_F 0.70, corresponding to fluorescent adduct psoralen-thymine, both radioactivity and fluorescence maxima have resulted (see fig. 2).

The different course of the two diagrams at R_F values > 0.70 could be explained by the presence of non-fluorescent photocompounds (see formulas III or IV) or of their decomposition products by formic acid action.

To summarise, by irradiation *in vitro* at 3.655 Å of Ehrlich ascitic cells in the presence of psoralen (in these conditions the cells lose their tumor-producing capacity) a photoreaction between furocoumarin and the cells DNA occurs, giving a fluorescent C_4–cycloadduct, that was isolated and identified.

The experiments performed by labelled-psoralen, besides proving these results, show also the possible formation of non-fluorescent compounds.

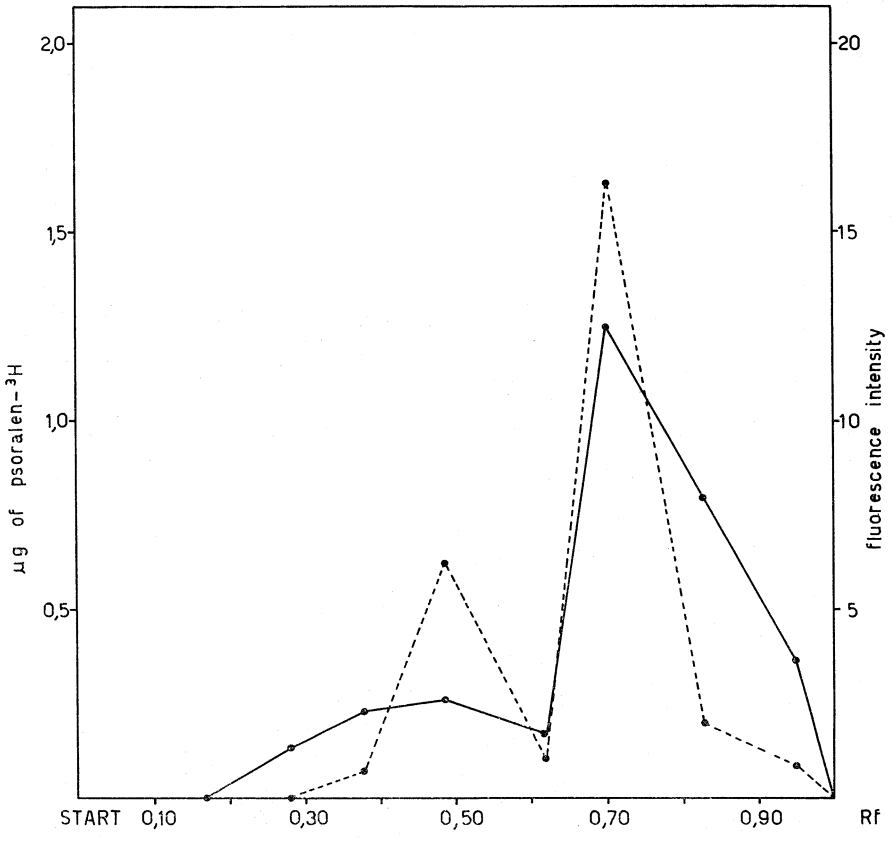


Fig. 2. – Paper chromatography (Wathman No. 1, solvent *n*–butanol–acetic acid–water 4 : 1 : 5) of one DNA sample hydrolisate, extracted from Ehrlich ascitic tumor cells irradiated at 3.655 Å in the presence of uniformly tritium–labelled psoralen.

——— μg of psoralen calculated on the basis of the radioactivity measurements, and – – – – fluorescence at 400 mμ, λ exc. 330 mμ (arbitrary units) determined as indicated in the text.

Quantitative ratio between different photoadducts is at present the object of our work.

In conclusion we believe that the destruction of tumor-producing capacity of Ehrlich ascitic cells in mice due to irradiation *in vitro* in the presence of skin-photosensitizing furocoumarins [14] rests upon a precise chemical fact, that is the photoreaction between furocoumarins and the pyrimidine bases of cell DNA.

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