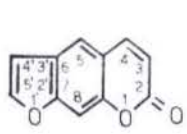
Biological consequences of the photobinding of furocoumarin molecules with nucleic acids

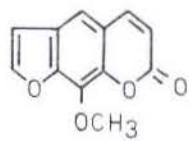
L. MUSAJO

Institute of Pharmaceutical Chemistry, University of Padua, Italy

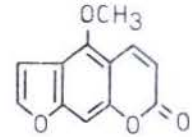
Furocoumarins are a well-known group of natural or synthetic substances, some of which [for instance psoralen (I), xanthotoxin (II), bergapten (III), 8-methylpsoralen (IV)] exert strong photosensitizing properties on



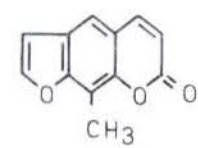
I



II



III



IV

various biological substrates by irradiation at 365 nm (MUSAJO, RODIGHIERO & CAPORALE, 1954; MUSAJO *et al.*, 1958; MUSAJO & RODIGHIERO, 1962; PATHAK & FITZPATRICK, 1959; PATHAK, FELLMAN & KAUFMAN, 1960; PATHAK, WORDEN & KAUFMAN, 1967). As their most studied effect is skin-photosensitization, the active substances were defined by us as « skin-photosensitizing » (MUSAJO & RODIGHIERO, 1962). Beside the active substances, many furocoumarin derivatives exist which are inactive.

The biological photosensitizing properties of furocoumarins may be explained by a photoreaction between these substances and nucleic acids, which was revealed for the first time in our laboratory five years ago (MUSAJO, RODIGHIERO & DALL'ACQUA, 1965) and which takes place when an aqueous solution of nucleic acids and photosensitizing furocoumarins is irradiated with long wavelength ultraviolet radiation.

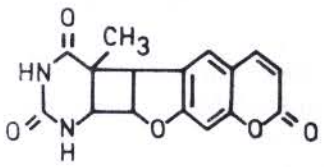
As a result of this photoreaction, some furocoumarin molecules are covalently linked to the macromolecule (MUSAJO *et al.*, 1966), without breakages of the internucleotide bonds (DALL'ACQUA, TERBOJEVICH & BENVE-

NUTO, 1968). After the photoreaction Tm value of DNA is increased (DALL'ACQUA & RODIGHIERO, 1966). Moreover, the photoreaction does not require the presence of oxygen (MUSAJO *et al.*, 1966).

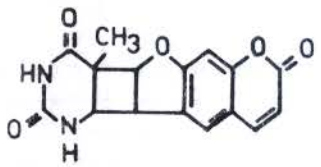
With the aim of clarifying the reactive sites of DNA we have examined the behaviour of the simple components of nucleic acids by irradiating at 365 nm aqueous solutions of purine and pyrimidine bases, nucleosides and nucleotides in the presence of skin-photosensitizing furocoumarins. Only with pyrimidine derivatives did we observe the formation of new compounds (MUSAJO, RODIGHIERO & DALL'ACQUA, 1965 ; MUSAJO & RODIGHIERO, 1965).

A C_4-cyclo-addition reaction takes place between pyrimidines and furocoumarines with the formation of a new cyclobutane ring. Pyrimidine bases always photoreact with their 5-6 double bond. Furocoumarins on the contrary can photoreact either *a*) with their 4′-5′ double bond or *b*) with their 3-4 double bond. (MUSAJO *et al.*, 1967*b*; MUSAJO, BORDIN & BEVILACQUA, 1967 ; KRAUCH, KRÄMER & WACKER, 1967). Therefore origin may be given to two types of compounds.

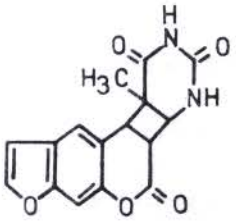
As an example of the structures of these photoadducts, we may consider those obtained from psoralen (I) and thymine, they are indicated by the formulas V and VI for the type *a*), VII and VIII for the type *b*). The two



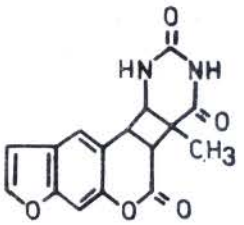
V



VI



VII



VIII

forms for each type derive from the double possibility of addition of the substances. The photocompounds of type *a*) have a violet fluorescence when observed in long wavelength ultraviolet light, while those of type *b*) are not fluorescent.

Also in native DNA pyrimidine bases are the reactive sites, giving a photo-cyclo-addition reaction analogous to that which occurs with the simple compounds.

In fact among the products of hydrolysis of a sample of DNA irradiated in the presence of psoralen and then hydrolyzed by heating in acidic medium we have isolated two fluorescent substances identical to the 4', 5'-photoadducts of psoralen with thymine and cytosine and a non-fluorescent one, identical with the 3,4-photoadduct of psoralen with thymine, all already previously obtained by irradiation of the simple compounds (MUSAJO *et al.*, 1967*c*).

The amount of the fluorescent photoadducts so obtained was nearly three times greater than that of the non-fluorescent one (BORDIN, MUSAJO & BEVILACQUA, 1969).

Let us consider now whether the well known biological effects which are produced by the « skin-photosensitizing » furocoumarins after irradiation at 365 nm can be explained on the bases of the photoreaction with nucleic acids. In a second time we shall consider some new biological consequences of this photoreaction.

I. — As I have already said, the more known and studied biological effect of furocoumarins is skin-photosensitization.

Concerning this effect, we have determined the action spectrum of xanthotoxin and bergapten for the photoreaction with native DNA, obtaining results which were in agreement with those obtained by BUCK, MAGNUS & PORTER (1960) and by PATHAK (1961) in studying the action spectrum of xanthotoxin for the production of erythema on human or guinea pig skin : in both cases the more effective radiations were those lying in the long ultraviolet region (DALL'ACQUA, MARCIANI & RODIGHIERO, 1969).

After this, we tried to ascertain whether a parallelism exists between the ability of the furocoumarins to photoreact *in vitro* with the nucleic acids and the ability of the same substances to photosensitize human or guinea-pig skin.

I recall that from the beginning of our research on the photosensitizing properties of these substances, we have tried to obtain a quantitative evaluation of this property. We achieved it by determining for each substance the minimum irradiation time necessary to obtain the outcome of erythema, operating of course in standard conditions.

Our early test (MUSAJO, RODIGHIERO & CAPORALE, 1954 ; MUSAJO & RODIGHIERO, 1962) consisted in placing 5 μg of substance for cm^2 of skin (the backs of human volunteers were used), in irradiating at 365 nm and in determining the minimum irradiation time necessary for the appearance of erythema. Considering the activity of psoralen (the parent compound

in this group) as equal to 100, the relative activities of the other substances were calculated.

Recently we have modified our previous test making it more suitable for assaying the very active methyl-derivatives of psoralen (CAPORALE *et al.*, 1967); guinea-pig skin was used, reducing the quantity of substance applied (2.5 $\mu g/cm^2$) but always determining the minimum irradiation time

TABLE 1.

Relative photoreactivity (365 nm) with native DNA and with yeast RNA and relative skin-photosensitizing activity (365 nm) of some furocoumarins.

Furocoumarins	Native DNA (*)		Yeast RNA (**)		Relative skin-photosensitizing activity on guinea-pig (psoralen = 100)
	Irradiation time necessary for a 20 % linkage of furocoumarin to DNA (seconds)	Relative photoreactivity (psoralen = 100)	Irradiation time necessary for a 10 % linkage of furocoumarin to RNA (seconds)	Relative photoreactivity (psoralen = 100	
8-methylpsoralen	90	373	126	190.4	540
5-methylpsoralen	144	233	360	66.6	450
Psoralen	336	100	240	100.0	100
Xanthotoxin . . . . . (8-methoxy-psoralen)	558	60	3740	6.4	71
Bergapten . . . . . (5-methoxy-psoralen)	1026	33	4440	5.4	61
Angelicin. . . . . (isopsoralen)	2160	15	1800	13.3	12 (***)
Xanthotoxol . . . . . (8-hydroxy-psoralen)	—	—	—	—	inactive

(*) Aqueous 0.1 % solutions of native calf-thymus DNA containing 10 $\mu g/ml$ of ^3H-furocoumarin were irradiated at 22°C (The incident radiation on 2 ml of solution was equivalent to 2.9×10^{16} quanta/sec.).

(**) Aqueous 0.1 % solutions of yeast RNA containing 3 $\mu g/ml$ of ^3H-furocoumarin were irradiated at 22°C. Incident radiation as in (*).

(***) The low skin-photosensitizing activity of angelicin did not allow a correct determination on guinea-pig skin, which has a lesser sensitivity than human skin (CAPORALE *et al.*, 1967). Therefore this datum is referred to the test on human skin (MUSAJO, RODIGHIERO & CAPORALE, 1954).

(in standard conditions) necessary to obtain erythema. The relative skin-photosensitizing activities of the various furocoumarins reported in Table 1 are obtained in these last conditions.

In order to test the connection between this property and the photoreaction with nucleic acids, we have chosen a number of furocoumarin derivatives from among those which have either a very high activity or only a moderate activity or from among those which are inactive. All of them were labelled with tritium and their photoreactivity was evaluated studying the rates of photoreactions with native DNA by irradiating at 365 nm aqueous solutions of the substances for different periods, operating always in the same conditions, and determining after each period of irradiation the amounts of furocoumarins linked to DNA (RODIGHIERO *et al.*, 1969).

The results so obtained are reported in Fig. 1. It is evident that the various furocoumarins have a very different photoreactivity with DNA.

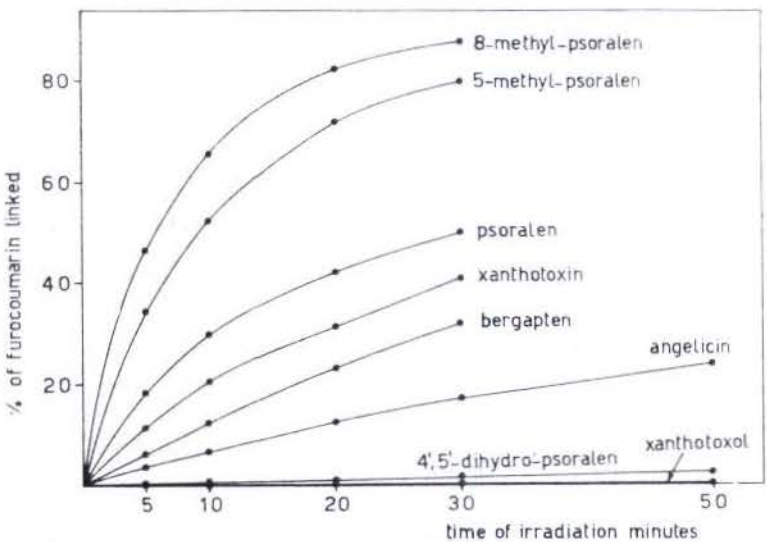


Fig. 1. — Photoreactions between some furocoumarins and native DNA by irradiation at 365 nm. Percentages of furocoumarins (referred to the amount initially present) linked to DNA as a function of the period of irradiation are reported.

In order to define in numbers the photoreactivity of each compounds we have calculated from the data obtained the time of irradiation which was necessary to give a linkage to DNA corresponding to 20 % of the amount of furocoumarin initially present. On the basis of the data so obtained, considering the photoreactivity of psoralen as equal to 100 the relative photoreactivities of the compounds have been calculated.

The results are reported in Table 1 together with the relative skin-photosensitizing activity of the various substances obtained in the test on guinea-pig skin. As it appears, the two activities, *in vivo* and *in vitro*, are almost parallel.

Considering that furocoumarins may photoreact also with RNA, the experiments were repeated with this type of nucleic acid. The results are reported also in Table 1. In this case we can see much less agreement between the *in vitro* photoreactivity and the *in vivo* skin-photosensitizing properties.

I think that the results now presented offer a clear support for a close correlation between the capacity of furocoumarins to photoreact with nucleic acids and that of provoking skin-erythema. DNA seems to be more directly involved than RNA.

But always concerning this topic, I recall another study worked out very recently by PATHAK and coworkers (personal communication). After having applied a tritiated furocoumarin to the skin of a guinea-pig and irradiated at 365 nm, the Authors observed that both DNA and RNA extracted from the treated portion of skin showed an evident radioactivity, while no trace of radioactivity was shown by various protein fractions, also extracted from the same portion of skin.

We may conclude that the photoreaction between furocoumarins and nucleic acids may take place not only *in vitro* but also *in vivo* and, more generally, that it is on the basis of the effects produced on the skin.

II. — Furocoumarins may also produce other biological effects. Among these I recall the lethal effects produced on bacteria cultures after irradiation at 365 nm studied by OGINSKY *et al.* (1959) and the mutagenic effects on *Sarcina lutea* cultures observed by MATHEWS (1963) and referred to a damage to DNA.

The photosensitized mutagenic effects of furocoumarins were studied also on *Drosophila melanogaster* by NICOLETTI & TRIPPA (1967). Moreover, COLOMBO & LEVIS (1965) observed the formation of giant cells after irradiation of mammalian cells *in vitro* grown in the presence of psoralen. COLOMBO (1968) also studied the effects of the irradiation of sea-urchin sperm in the presence of xanthotoxin on the development of sea-urchin eggs fertilized by the irradiated sperm : anomalies were observed that might be clearly attributed to a damage to DNA.

III. — Moreover we have studied the effects on viruses, observing the complete inactivation of some DNA-viruses after irradiation in the presence of psoralen, while some RNA viruses were more resistant (MUSAJO *et al.*, 1965).

Recently two DNA-viruses were studied more in detail, that is an *adenovirus*, which is lacking lipoproteic envelope, and *pseudorabies virus* belonging to the herpetic group, which has such an envelope (MUSAJO, PETEK & BACCICHETTI, unpublished results).

The suspensions of these two viruses lost completely their infectivity after irradiation for few minutes with long ultraviolet in the presence of 8-methyl-psoralen at a concentration of 10 μg/ml.

The *adenovirus* (2×10^7 infectious units per ml) irradiated in these conditions for 60' exerted antigenic activity, that is it was able to produce antibodies at high titer in rabbits.

Pseudorabies herpetic virus (5×10^6 infectious units per ml) irradiated also for 60' in the same conditions and inoculated in mice produced an antibody response of moderate intensity. However, this is a noteworthy result, considering the weak immunising power of herpetic vaccines inactivated with other methods.

IV. — Furthermore I shall report on our studies on mouse Ehrlich ascites tumor cells : the effect which was obtained by irradiation at 365 nm of a suspension of these cells in the presence of very small amounts of skin-photosensitizing furocoumarins was the loss of the transmitting tumor capacity of the cells, when they were injected in mice (Musajo *et al.*, 1967*d*). See Table 2.

For having a direct confirm that this biological effect is due to a linkage of the furocoumarin to DNA, we have extracted DNA from a pool of Ehrlich ascites tumor cells previously irradiated in the presence of tritiated psoralen. We have found at first that after irradiation, and only in this condition, psoralen was linked to DNA ; moreover, after hydrolysis of the extracted DNA, we have identified a substance which was identical to the fluorescent photocompound already obtained from psoralen and thymine (Musajo *et al.*, 1967*a*).

The photoinactivated by psoralen Ehrlich ascites tumor cells seemed to behave as the untreated tumor cells towards the Wright's liquid, trypan blue and in the Warburg's apparatus.

V. — Another investigation was carried out with the aim to ascertain whether the Ehrlich ascites tumor cells photoinactivated with skin-photosensitizing furocoumarins had the capacity to protect the animals against this tumor. Numerous experiments have been performed by us in the past four years using cells inactivated by irradiation in the presence of various compounds (bergapten, xanthotoxin, psoralen, 8-methyl-psoralen, and 4, 4', 8-trimethylpsoralen). The following results, obtained using psoralen and 8-methylpsoralen, are reported as the most significant ones (Musajo, Visentini & Baccichetti, unpublished results).

The cell suspensions were prepared by diluting the ascites fluid in such a manner that each ml contained 2×10^7 cells, adding 10 μg-ml of psoralen or 8-methyl-psoralen and irradiating it for 30 minutes, as previously described (Musajo *et al.*, 1967*d*). The inactivated cells were injected into the peritoneum in doses of approximately 10^7 cells/mouse : *a*) as a single injection,

TABLE 2.

Influence of irradiation (30 min at 365 nm, using a Philips HPW 125 lamp at a distance of 15 cm; irradiation intensity: 4.2 x 10^{15} hν/sec/cm^2) in the presence of furocoumarins on the tumor-producing capacity of Ehrlich ascites tumor cells. After irradiation, the cells were intraperitoneally transplanted in mice.

Furocoumarin concentration μg/16^6 cells	No. of treated mice	Mortality	
		No.	%
Psoralen (skin-active):			
— (controls)	168	168	100
0.0005	8	6	75
0.0025	8	3	37
0.005	8	0	0
0.025	8	0	0
0.05	28	0	0
0.25	110	0	0
0.5	10	0	0
1	60	0	0
Xanthotoxin (skin-active):			
— (controls)	50	50	100
0.0025	8	8	100
0.005	8	2	75
0.025	8	0	0
0.05	8	0	0
0.15	24	0	0
0.25	8	0	0
Bergapten (skin-active):			
— (controls):	50	50	100
0.0025	8	8	100
0.005	8	8	100
0.025	8	1	12
0.05	20	7	35
0.15	24	0	0
0.20	8	0	0
Xanthotoxol (skin-inactive):			
— (controls)	10	10	100
0.25	10	10	100

b) as two injections, the second two weeks after the first, *c*) as four injections, passed at weekly intervals. The challenge was performed by injecting 2×10^3 untreated tumor cells in each mouse. This dose caused death in about 70 % of controls and in most of the cases it occurred within 60 days from the tran-

splant. The challenge was achieved : in the cases *a*) and *b*) three weeks, and in the case *c*) four weeks after the first injection of inactivated cells. In all experiments the mortality rate was calculated at the end of 2 months observation from the inoculation.

The data of 5 experiments, worked out simultaneously with the relative controls, are summarized in Table 3.

TABLE 3.

Mortality observed after challenge with 2 x 10^4 untreated Ehrlich ascites tumor cells in control mice and in mice previously injected with Ehrlich ascites tumor cells photoinactivated in the presence of furocoumarins.

Experiment No.	Furocoumarin	Mice strain	No. of injections of inativated cells	Dead mice No. / Used mice No.	Total mortality %	P value (*)
1	Psoralen	NCL	0	27/40	67	
			1	32/38	84	> 0.1
2	Psoralen	NCL	0	59/80	73	
			4	37/127	29	< 0.001
3	8-methylpsoralen.	NCL	0	35/50	70	
			4	12/50	24	< 0.001
4	Psoralen	Swiss	0	46/50	92	
			1	29/55	52	< 0.001
5	Psoralen	Swiss	0	28/39	71	
			2	16/39	41	< 0.02

(*) P values calculated by Chi square.

The present results seem to indicate that cells photoinactivated in the presence of psoralen and 8-methyl-psoralen by long wavelenth ultraviolet irradiation induce a protection against tumor in most of the treated mice.

I am very grateful to all my co-workers, in particular to Prof. G. Rodighiero.

REFERENCES

BORDIN, F., L. MUSAJO & R. BEVILACQUA, 1969. *Z. Naturforsch.*, **24** *b*, 691.
BUCK, H. W., I. A. MAGNUS & A. D. PORTER, 1960. *Brit. J. Dermatol.*, **72**, 249.
CAPORALE, G., L. MUSAJO, G. RODIGHIERO & F. BACCICHETTI, 1967. *Experientia*, **23**, 985.
COLOMBO, G., 1968. *Exptl. Cell. Res.*, **48**, 167.

COLOMBO, G. & A. G. LEVIS, 1965. *Progr. Biochem. Pharmacol.*, **1**, 392.
DALL'ACQUA, F., S. MARCIANI & G. RODIGHIERO, 1969. *Z. Naturforsch.*, **24** *b*, 667.
DALL'ACQUA, F. & G. RODIGHIERO, 1966. *Rend. Accad. Nazl. Lincei*, **40**, 595.
DALL'ACQUA, F., M. TERBOJEVICH & F. BENVENUTO, 1968. *Z. Naturforsch.*, **23** *b*, 943.
KRAUCH, C. H., D. M. KRAMER & A. WACKER, 1967. *Photochem. Photobiol.*, **6**, 341.
MATHEWS, M. M., 1963. *J. Bacteriol.*, **85**, 322.
MUSAJO, L., F. BORDIN, F. BACCICHETTI & R. BEVILACQUA, 1967*a*. *Rend. Accad. Nazl. Lincei*, **43**, 442.
MUSAJO, L., F. BORDIN & R. BEVILACQUA, 1967. *Photochem. Photobiol.*, **6**, 927.
MUSAJO, L., F. BORDIN, G. CAPORALE, S. MARCIANI & G. RIGATTI, 1967*b*. *Photochem. Photobiol.*, **6**, 711.
MUSAJO, L. & G. RODIGHIERO, 1962. *Experientia*, **18**, 153.
MUSAJO, L. & G. RODIGHIERO, 1965. *Rend. Accad. Nazl. Lincei*, **38**, 591.
MUSAJO, L., G. RODIGHIERO, A. BRECCIA, F. DALL'ACQUA & G. MALESANI, 1966. *Photochem. Photobiol.*, **5**, 739.
MUSAJO, L., G. RODIGHIERO & G. CAPORALE, 1954. *Bull. Soc. Chim. Biol.*, **36**, 1213.
MUSAJO, L., G. RODIGHIERO, G. CAPORALE & C. ANTONELLO, 1958. *Farmaco, Ed. Sci.*, **13**, 355.
MUSAJO, L., G. RODIGHIERO, G. COLOMBO, V. TORLONE & F. DALL'ACQUA, 1965. *Experientia*, **21**, 22.
MUSAJO, L., G. RODIGHIERO & F. DALL'ACQUA, 1965. *Experientia*, **21**, 24.
MUSAJO, L., G. RODIGHIERO, F. DALL'ACQUA, F. BORDIN, S. MARCIANI & R. BEVILACQUA, 1967*c*. *Rend. Accad. Nazl. Lincei*, **42**, 457.
MUSAJO, L., P. VISENTINI, F. BACCICHETTI & M. A. RAZZI, 1967*d*. *Experientia*, **23**, 335.
NICOLETTI, B. & G. TRIPPA, 1967. *Rend. Accad. Nazl. Lincei*, **43**, 259.
OGINSKY, E. L., G. S. GREEN, D. G. GRIFFITH & W. L. FOWLKS, 1959. *J. Bacteriol.*, **78**, 821.
PATHAK, M. A., 1961. *J. Invest. Dermatol.*, **37**, 397.
PATHAK, M. A., J. H. FELLMAN & K. D. KAUFMAN, 1960. *J. Invest. Dermatol.*, **35**, 165.
PATHAK, M. A. & T. B. FITZPATRICK, 1959. *J. Invest. Dermatol.*, **32**, 255, 509.
PATHAK, M. A., R. L. WORDEN & K. D. KAUFMAN, 1967. *J. Invest. Dermatol.*, **48**, 103.
RODIGHIERO, G., L. MUSAJO, F. DALL'ACQUA, S. MARCIANI, G. CAPORALE & L. CIAVATTA, 1969. *Experientia*, **25**, 479.