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The dark-interaction between furocoumarins and nucleic acids

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Chimica. — *The dark-interaction between furocoumarins and nucleic acids* (*). Nota di FRANCESCO DALL'ACQUA e GIOVANNI RODIGHIERO, presentata (**) dal Corrisp. L. MUSAJO.

RIASSUNTO. — In relazione con le ricerche condotte da tempo in questo Istituto sulle fotoreazioni tra DNA e furocumarine fotosensibilizzatrici, gli AA. hanno studiato le interazioni che avvengono fra le stesse sostanze anche al di fuori di ogni irradiazione.

Tutte le furocumarine, sia quelle dotate di attività fotosensibilizzatrice che quelle inattive, formano complessi col DNA nativo. Infatti in presenza di DNA la loro solubilità in acqua è molto maggiore che in sua assenza e le loro proprietà spettrofotometriche subiscono delle variazioni. È stato inoltre osservato un leggero aumento del valore del T_m del DNA in presenza di una furocumarina (bergaptene).

La formazione del complesso viene inibita dalla presenza di formamide, da un'alta concentrazione salina e dalla denaturazione termica del DNA. Anche l' RNA ha una capacità molto ridotta di legare le furocumarine.

It is known that some furocoumarins are active photosensitizing agents, by irradiation at 3,655 Å, on human and guinea-pig skin [1, 2, 3] and on other biological substrates (microorganisms [4, 5, 6], mammalian cells [7], DNA–viruses [8]). Other furocoumarins are instead inactive. The relationships between biological activity and chemical structure have been well established for several years [1, 2, 3].

In recent papers [9, 10, 11] are reported the results of the research on the photochemical interaction between DNA and skin-photosensitizing furocoumarins which occurs when a solution of the substances is irradiated at 3,655 Å and which appears to be able to explain the mentioned biological photosensitizing effects.

A chemical stable linkage is formed by irradiation at 3,655 Å between photosensitizing furocoumarins and DNA [11]. The rate of the photoreaction appears to be affected by the possibility of the formation of a preliminary weak binding between DNA and furocoumarins, which occurs outside any irradiation. The possibility of such a dark-interaction had been already considered some years ago [12]. The increased viscosity of the DNA solutions, when they were added with furocoumarins, and the results obtained with the equilibrium dialysis method gave some indications on the ability of the furocoumarins to bind in the dark with DNA.

Now we have reinvestigated this possibility applying the procedure used by several AA. [13–19] for studying the interactions of aromatic polycyclic

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hydrocarbons with DNA. We communicate here our experimental results, which confirm the capacity of the furocoumarins to form complexes with native DNA and indicate the conditions which have influence on their formation [1].

MATERIALS AND METHODS.

Five samples of DNA were used:

(I) Calf thymus DNA, extracted with strong salt solution and deproteinized by saturation with sodium chloride [20]; T_m [2] = 83°.6;

(II) Calf thymus DNA, extracted with the aid of sodium dodecilsulfate [22]; T_m [2] = 89°;

(III) Calf thymus DNA, highly polymerized (Mann Research Laboratories, New York); T_m [2] = 87°;

(IV) Salmon sperm DNA, highly polymerized (Mann Research Laboratories, New York); T_m [2] = 86°;

(V) Salmon sperm DNA, highly polymerized (Calbiochem, Los Angeles, Cal.); T_m [2] = 86°.4.

The RNA used was prepared from yeast as described by Crestfield et al. [23]. A sample of yeast—RNA furnished by Sigma Chemical Co. was also used. The chromatic reaction [24] for DNA was negative for both RNA samples.

The furocoumarins used were prepared (by synthesis or by extraction from vegetable materials) in this Institute.

Solubilization procedure.—3–4 mg of bergapten, or other furocoumarins, were very finely ground and suspended in 10 ml of water or aqueous solutions of DNA or RNA. The suspensions were shaken for 4 hours [3] in a thermostatic bath at 20° C, filtered and centrifuged at 20,000 g for 1 hour at a constant temperature of 20° C, using a refrigerated centrifuge Phywe Eispirouette.

Spectrophotometric and fluorimetric measurements.—The concentration of furocoumarins in the solutions obtained as described above was determined by spectrophotometric method, operating at wavelengths longer than 310 mμ and using an Uvispeck Hilgher and Watts spectrophotometer. For the registration of U.V. spectra (290–360 mμ) a Beckman DB spectrophotometer was also used.

The aqueous solutions containing known amounts of nucleic acids and of furocoumarins, which were necessary for the determination of the ε–values of the furocoumarins in the presence of DNA or RNA, were prepared in two ways.

(1) Preliminary notices of this research were given in a previous communication [8].

(2) Determined as indicated by MARMUR and DOTY [21].

(3) Preliminary experiments worked out with bergapten and DNA solutions showed that the maximum of solubilization was reached after shaking for about 4 hours.

(1) To the solutions of DNA or RNA of known concentration were added the furocoumarins using concentrated solutions of these in methyl alcohol. As the amounts of furocoumarins to add were of the order of 0.03–0.1 mg, very small volumes of organic solvent were generally needed (about 1–1.5% of the total volume).

(2) Nearly saturated aqueous solutions of the furocoumarins were prepared by shaking for several hours fine suspensions in water and then carefully filtering; after the spectrophotometric determination of the concentration of the furocoumarins, DNA and RNA were added to measured volumes of the solutions, up to the stated concentrations.

The results obtained with the two methods were comparable.

For the fluorimetric determinations, made with a spectrophotofluorimeter Aminco-Bowman, were used solutions prepared as described in (2).

RESULTS.

Solubilization and modification of the optical properties of bergapten and other furocoumarins in DNA and RNA *solutions.*

(*a*) In Table I is reported the solubility of bergapten in solutions of DNA at various concentrations; there is a gradual increase of the amount dissolved with the increasing concentration of DNA. The molecular ratio between the amounts of bergapten solubilized more than in water and the nucleotides present in DNA is nearly constant at the various concentrations.

TABLE I.

Solubility of bergapten in aqueous solutions of DNA(II) *at various concentrations.*

Concentration of DNA		Bergapten solubilized more than in water (*)		$\frac{C_P}{C_{Berg}}$	λ max of bergapten in the solution		ε of bergapten at the λ max (*)
g.%	μMP/l (C_P)	μg/ml	μM/l (C_{Berg})		mμ	shift with respect to aqueous solution (*) mμ	
0.05	1349	8.04	37.22	36.24	322	+ 9	9940
0.1	2699	15.98	73.98	36.48	323	+10	9050
0.2	5398	29.66	137.31	39.31	324	+11	8570
0.3	8097	44.11	204.21	39.65	325	+12	8320

(*) The solubility of bergapten in water at 20° C is 5 μg/ml; the λ max in aqueous solution is 313 mμ and the ε_{313} value is 15250.

The formation of a binding is confirmed by the spectrophotometric properties of the solutions of DNA and bergapten: there is an evident shift of the λ max of the bergapten to a longer wavelength and a clear decrease of the absorbing properties of the bergapten (TableI).

The ε–value of bergapten at 313 mμ decreases by increasing the DNA concentration of the solutions. However at a constant concentration of DNA and in a limited range of bergapten concentrations, the ε–value remains practically constant. This fact makes possible the spectrophotometrical determination of the amount of bergapten solubilized.

Results very similar to those reported in Table I were obtained also with four other samples of DNA.

(*b*) On the contrary, the solubilizing power of RNA on the bergapten is very weak (see Table II). Moreover, there are no shifts of the λ max and the decrease in the ε–values are comparatively smaller.

TABLE II.

Solubilization of bergapten in aqueous solutions of RNA.

Concentration of RNA		Bergapten solubilized more than in water		$\frac{C_P}{C_{Berg}}$	ε_{313} of bergapten in the solutions (*)
g%	μMP/l (C_P)	μg/ml	μM/l (C_{Berg})		
0.05	1252	2.22	10.3	121.55	13320
0.1	2505	2.84	13.1	191.22	12530
0.2	5010	3.20	14.8	338.51	12160
0.3	7515	3.82	17.7	426.98	11840

(*) λ max of bergapten in all these solutions was rather the same as in aqueous solution.

(*c*) A behaviour analogous to that of bergapten is possessed also by other furocoumarins, i.e. psoralen, 4′–methylpsoralen, xanthotoxin, which are very active photosensitizing substances, angelicin, faintly active, bergaptol and imperatorin, which have no photosensitizing activity. All these substances showed a sharp solubilization in DNA solution and a much weaker one in the RNA solution (see Table III). Also the ε–values of the various furocoumarins showed a much stronger decrease in the presence of DNA than in the presence of RNA.

TABLE III.

Solubility of some furocoumarins in water and in 0.2 % DNA (I) *and* RNA *aqueous solutions.*

FUROCOUMARINS	SOLUBILITY μg/ml			SOLUBILITY RATIO		λ of measurement	ε		
	water	DNA 0.2 %	RNA 0.2 %	solub. in DNA / solub. in H_2O	solub. in RNA / solub. in H_2O		water	DNA 0.2 %	RNA 0.2 %
Psoralen	35.4	77.2	42.4	2.18	1.19	330	7730	5560	7140
Bergapten	5	28.4	8.2	5.68	1.64	313	15250	9435	12160
Xanthotoxin	36.05	151.5	36.3	4.20	1.01	310	11920	7095	9640
4'–Methyl–psoralen . .	5.4	16.9	5.4	3.12	1.00	335	6420	4580	6330
Angelicin	41.5	120.9	37.8	2.91	0.91	315	9190	5380	8455
Bergaptol	22.4	76.1	36.8	3.39	1.64	315	13040	7250	9285
Imperatorin	8.08	12.12	7.4	1.50	0.91	320	11550	9155	10360

TABLE IV.

Fluorescence intensities and spectra of some furocoumarins in water and in 0.2 % DNA *solutions.*

FUROCOUMARIN	Solution	Activating λ max mμ	Fluorescence λ max mμ	Relative intensity of fluorescence
Psoralen	water	340	450	100
	DNA(I)	340	445	54
Xanthotoxin	water	325	510	100
	DNA(I)	330	495	67
Bergapten	water	335	455	100
	DNA(I)	340	460	116

(*d*) The influence of DNA on the fluorescence of some furocoumarin solutions was also investigated. From the results obtained, reported in Table IV, the effect of DNA appears in general faint and inconstant. There are very small shifts of the activating and of the fluorescence λ max. The fluorescence intensity is slightly lowered in the psoralen and xanthotoxin solutions and barely enhanced in that of bergapten.

Influence of formamide on the DNA–*bergapten complex.*

We have tested the effect of formamide on the stability of the DNA–bergapten complex. Formamide is known to break the hydrogen-bonds; it is capable of provoking the denaturation of DNA, but, in aqueous solution, only at very high concentrations (more than 70 %) [27].

As fig. 1 shows, formamide added in low concentrations to solutions containing DNA and bergapten, produces a sharp increase in the optical density of the solutions at 313 mμ, whereas it does not exert a similar effect when added to a simple aqueous solution of bergapten.

This fact may be interpreted as a cleavage of the DNA–bergapten complex.

Influence of the ionic strength on the solubilization of bergapten in DNA.

In these experiments were used various aqueous solutions containing DNA(II) 0.15 % and different concentrations of sodium chloride or standard buffer (0.14M NaCl + 0.015M sodium citrate, pH 7.1). The results obtained in the solubilization tests are reported in Table V. They clearly

show that increasing the ionic strength of the solutions strongly decreases the capacity of DNA to solubilize the furocoumarin.

On the other hand, there is also a gradual increase in the ε-values at 313 mμ of the bergapten solubilized, which indicates a lowered binding. A linear relationship exists between the ε-values and the pNa of the solutions.

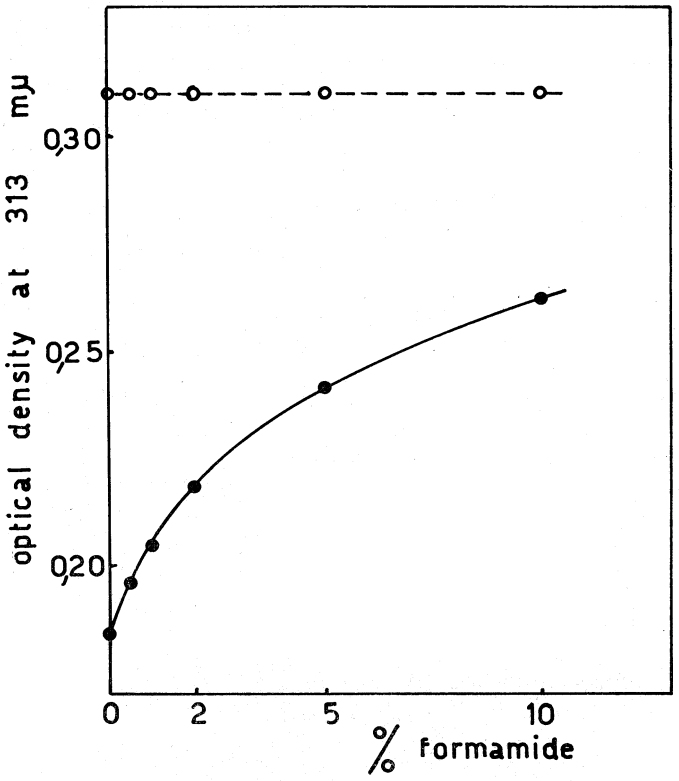

Fig. 1. – Action of formamide on bergapten and on DNA–bergapten complex.
Optical density at 313 mμ of various solutions of bergapten (4.4 μg/ml) -○- -○- and DNA(III) 0.05 % + bergapten 4.4 μg/ml -●——●- containing increasing amounts of formamide.

Influence of thermal denaturation on the solubilization power of DNA.

The solutions of various samples of DNA in distilled water were in part kept at the room temperature and in part warmed in a boiling water-bath for 10 minutes (temperature of the solution: 98° C) in small round flasks fitted with a reflux condenser. After rapid cooling, the solutions were immediately used for the solubilization tests, operating in parallel with the solutions kept at the room temperature.

The marked effect of the thermal denaturation appears from the results reported in Table VI: the samples of DNA after denaturation have lost almost completely the capacity to bind the furocoumarin: the quantities solubilized are very small and the decrease in the ε-value of bergapten is also very small.

TABLE V.

Solubilization of bergapten in aqueous 0.15 % DNA *solutions containing increasing amounts of sodium chloride or standard buffer.*

SALT CONCENTRATIONS	Bergapten solubilized more than in water		$\frac{C_P (*)}{C_{Berg}}$
	μg/ml	μM/l (C_{Berg})	
NaCl 0.002 M	16.72	77.40	52.29
NaCl 0.01 M	13.19	61.06	66.29
NaCl 0.05 M	8.72	40.3	100.44
NaCl 0.1 M	8.10	37.5	107.94
Standard buffer (**)	7.13	33.0	122.66

(*) C_P (μMP/l) is constant in all the solutions and is 4048.
(**) Composition of the standard buffer: 0.14 M NaCl and 0.015 M sodium citrate pH 7.1.

TABLE VI.

Solubility of bergapten in aqueous solutions of DNA, *before and after thermal denaturation.*

SAMPLES OF DNA AND CONCENTRATION OF SOLUTIONS	Bergapten solubilized more than in water				$\frac{C_P}{C_{Berg}}$		ε_{313} of bergapten in the solutions	
	native		denatured					
	μg/ml	μM/l (C_{Berg})	μg/ml	μM/l (C_{Berg})	native	denatured	native	denatured
DNA(I) 0.2 % C_P(μMP/l) = 4722 . . .	23	106.5	1.69	7.82	44.3	603.83	9435	14375
DNA(II) 0.2 % C_P(μMP/l) = 5398 . . .	29.66	137.31	1.45	6.71	39.31	804.47	8570	12250
DNA(III) 0.175 % C_P(μMP/l) = 3952 . . .	16.74	77.50	1.93	8.93	50.99	442.55	8625	12690
DNA(V) 0.1 % C_P(μMP/l) = 2828 . . .	14.03	64.95	0.675	3.125	43.54	904.96	9180	13921

Temperature profiles of the optical density of DNA *and* DNA + *bergapten solutions determined at* 260 *and* 313 mμ.

The temperature profiles of the optical density were determined in a way analogous (4) to those described by Marmur and Doty [21], using an Uvispeck Hilger and Watts spectrophotometer, equipped with the necessary attachment.

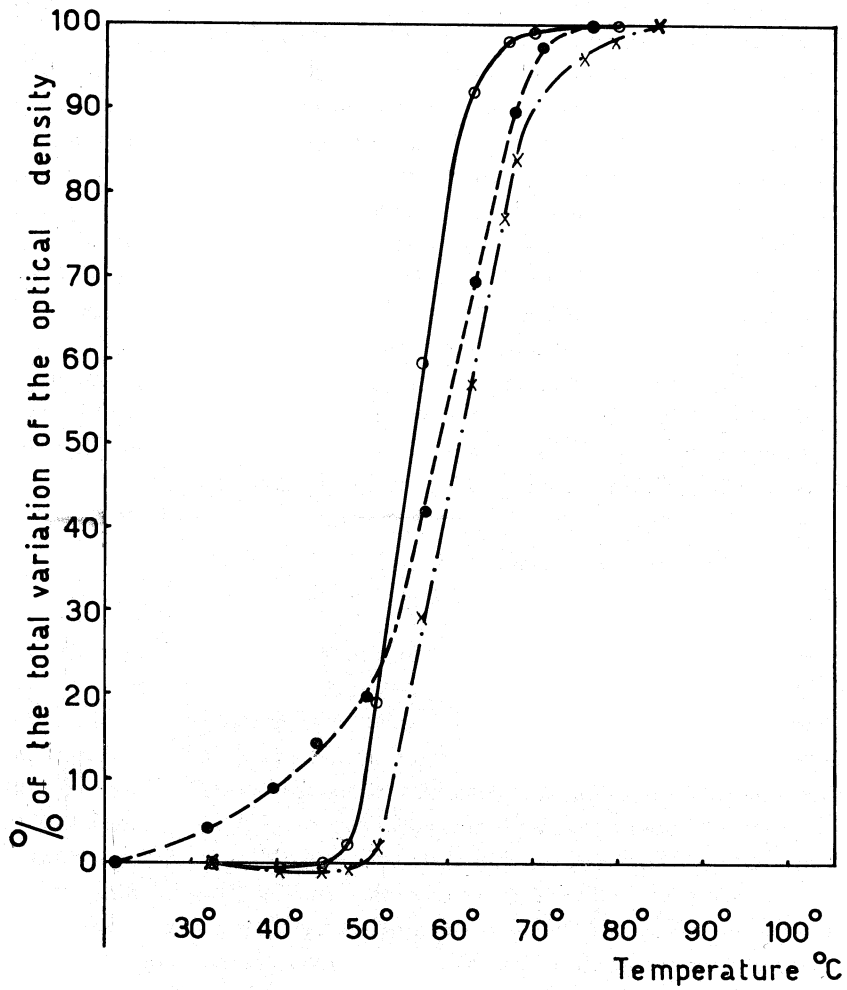

Fig. 2. – Temperature profiles of the optical density at 260 mμ and 313 mμ of aqueous solutions containing DNA and bergapten.

Solution containing DNA 0.064 %, O.D. at 260 mμ –○——○–; solution containing DNA 0.064 % and bergapten 9.8 μg/ml, O.D. at 260 mμ –×– · – – and at 313 mμ –●– —— –●– ——.

(4) The solutions were completely without salts, for excluding their negative influence on the " complexation " of bergapten, and they had a DNA concentration relatively higher (640 μg/ml) than those used by MARMUR and DOTY [21] (20 μg/ml), for preventing the denaturation caused by dilution [28].

Two solutions have been used: *a*) DNA 0.064 % in distilled water; *b*) the same solution containing 9.8 μg/ml of bergapten.

The solutions were placed (without dilution) in cuvettes having an optical path of 1 mm. The optical density of the solution *b*) at various temperatures was determined, not only at 260 mμ, using water as blank, but also at 313 mμ, using as blank the *a*) solution. As at this wavelength the absorption, due exclusively to bergapten, was very faint, the results obtained at this wavelength were confirmed by operating also with cuvettes having an optical path of 10 mm.

Fig. 2 reports the temperature profiles so obtained.

The presence of bergapten produces a slight enhancement of the T_m of DNA, providing another evidence of the interaction of DNA and bergapten.

Very interesting is the behaviour of the optical density at 313 mμ, that is the variations in the absorbing properties of bergapten. By increasing temperature of the solutions, there is an increase in the optical density, running fairly parallel to the increases observed, both in *a*) and in *b*) solutions, at 260 mμ, which are in relationship with the heat-denaturation of DNA.

This fact indicates that the release of bergapten, at first bound to DNA, occurs in a parallel way as the denaturation of the same DNA.

Only in the first tract the line of the optical density variations at 313 mμ shifts outside the other two lines. It is probable that this fact may be connected with analogous divergences observed in the submelting range between the variations in the viscosity and in the optical density [26].

Discussion.

The formation in the dark of a complex between the furocoumarins and native DNA is clearly shown by the results of the solubilization experiments and by the variations of the spectrophotometric properties of the furocoumarins solubilized (bathochromic shift of λ max and decrease of absorption power).

The results now presented confirm those previously obtained with the viscosity measurements and with the equilibrium dialysis method [12].

It appears evident that the helix structure of DNA is an important condition for the binding of the furocoumarins. In fact this binding capacity is almost completely lost after heat-denaturation by all the four samples of DNA examined. Moreover, we have observed that the bergapten bound to the native DNA is gradually released as the heat-denaturation of DNA takes place.

Even with yeast–RNA the binding of furocoumarins occurs to a very small extent. The linkage between furocoumarins and native DNA is undoubtedly weak: in fact, in a previous work [11] we have observed that by precipitation with ethanol of the DNA, from an aqueous solution, the complex breaks completely and in the DNA precipitated there is no trace of the furocoumarin.

On the other hand, the energy of this linkage is sufficient to produce a slight stabilization of the helix structure of DNA, as indicated by the slightly

higher T_m-value found in the DNA in the presence of bergapten. The same effect is shown also by other furocoumarins [27].

The cleaving effect of formamide may suggest that hydrogen-bonds are involved in the formation of these complexes.

We may find some analogies of behaviour between the furocoumarins and the polycyclic aromatic hydrocarbons in comparison with DNA. Similar, for example, is the influence of DNA on the spectrophotometric properties of the two groups of substances [13, 15, 19] and also the effect of the salts present in the solutions, which greatly lower, in both the cases, the solubilizing power of DNA [13, 15].

On the contrary, whereas the fluorescence of the aromatic polycyclic hydrocarbons is strongly quenched by DNA [13], that of the furocoumarins is only slightly affected.

As we have pointed out in other papers [8, 12], there is no relationship between the ability of the furocoumarins to bind in the dark with DNA and their photosensitizing properties.

Complexes are formed by both the active and inactive substances. But, when the complexes are irradiated (3,655 Å), a photoreaction takes place only in the presence of photosensitizing furocoumarins [9].

The weak preliminary binding appears to be a very favourable condition for these photoreactions. In fact, while the photoreaction occurs at a high rate with the native DNA, which has a high solubilizing power, it occurs, on the contrary, at an extremely reduced rate with the RNA and with the denatured DNA [10], which have a much smaller solubilizing power.

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