Isolation of a benz[a]pyrene-thymine photoadduct from DNA hydrolized after irradiation at 365 nm in the presence of benz[a]pyrene

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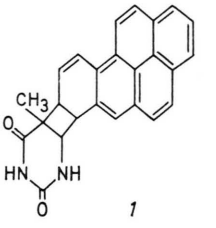
The photoreaction which takes place between benz[a]pyrene and DNA by irradiation at 365 nm leads to a covalent combination. To ascertain the reactive sites of DNA, a sample of the macromolecule has been precipitated after irradiation in the presence of benz[a]pyrene and hydrolized; among the products of hydrolysis four different fluorescent substances have been isolated, one of which was identical with the photoadduct benz[a]pyrene-thymine, already prepared by irradiation of the two substances at 365 nm. This photoadduct has been obtained in an amount corresponding to a molecular ratio 1 : 7000 in respect to the nucleotides (P atoms) present in the sample of DNA.

In recent times evidence has been obtained that benz[a]pyrene added to an aqueous solution of DNA and irradiated with long wavelength ultraviolet radiation forms a stable combination with the macromolecule. TS'O and LU [1] have found that DNA irradiated in the presence of tritiated benz[a]-pyrene and then precipitated from the solution retains some radioactivity. Very recently KODAMA and NAGATA [2] by irradiating an alcoholic solution of benz[a]pyrene and other aromatic polycyclic hydrocarbons in the presence of DNA as the cetyltrimethylammonium salt, in addition to a photooxidation of the guanine moieties, found a covalent linkage of the hydrocarbons to the DNA derivative.

On the other hand, RICE [3] has demonstrated the formation of photoadducts by irradiation of benz[a]pyrene in the presence of the purine and pyrimidine bases present in the nucleic acids. We, too, in a previous research [4] have confirmed the formation of photoadducts both with the pyrimidine and the purine bases. Moreover from the irradiated (365 nm) mixture of benz[a]pyrene and thymine we have isolated a photoadduct to which on the basis of the elemental analysis and of its ultraviolet spectrophotometric properties, we have attributed the structure **1** or **2**:



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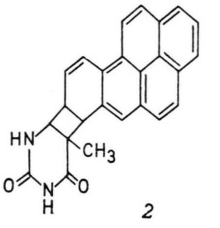

2

This compound derives therefore from a C_4-cycloaddition of benz[a]pyrene (engaging its 7,8 positions) to the 5,6 positions of thymine.

We recall that analogous photoadducts have recently been obtained [5–7] also by irradiating the pyrimidine bases of the nucleic acids in the presence of some furocoumarins; they have been isolated also from DNA hydrolized after irradiation in the presence of furocoumarins. However in these cases purine bases do not photoreact.

The aim of the present study has been to identify the reactive sites of DNA in the photoreaction with benz[a]pyrene, that is, to ascertain whether benz[a]pyrene could link to DNA by a mechanism similar to that already demonstrated with simple bases.

Materials and Methods

DNA from *salmon* sperm, natrium salt, highley polimerized, (Calbiochem, Los Angeles, California, U.S.A.) was used. Benz[a]pyrene was purchased from Fluka A.G., Buchs, Switzerland; m.p. 176 – 178°, λ_{max} 296 nm ($\varepsilon \geqslant 5.800$), λ_{min} ($\varepsilon \geqslant 2.800$). ^{3}H-benz[a]pyrene was obtained from the Radiochemical Centre, Amersham, England; it had a specific radioactivity of 8530 mCi/

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[1] Proc. nat. Acad. Sci. USA **51**, 272 [1964].
[2] Chem. biol. Interactions **1**, 99 [1969/70].
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mMol; for use in the experiment it was diluted with non radioactive benz[a]pyrene until a specific radioactivity of 0,86 mCi/mMol was reached.

Radioactivity measurements. These were performed with a liquid scintillation system, Beckman 150 SL, using for each counting 10 ml of the following solution: g 4 of 2,5-diphenyl-oxazole; g 0,075 of 2,2'-*para*-phenyl-bis(5-phenyloxazole); g 120 of naphthalene; dioxane up to 1000 ml of solution.

Irradiation. Aqueous 0,15% DNA solutions containing sodium chloride 2×10^{-3} M were added to the solution of benz[a]pyrene in a small volume of acetone, shaken for 2 hours at room temperature and, after standing for 48 hours in the dark, poured into Petri dishes and irradiated with Philips HPW 125 lamp (emission almost exclusively at 365 nm) provided with an aluminium conic reflector and placed at a distance of 15 cm (4.2×10^{15} quanta/cm^2/sec). During the irradiation the temperature of the solution was 37 – 38°.

Precipitation and hydrolysis of DNA. Sodium chloride was added to the irradiated solutions to a 1 M concentration and DNA was precipitated by adding 2 volumes of ethyl alcohol. After centrifugation, the collected samples of DNA were washed three times with ethyl alcohol 70%, kept in contact with dry acetone for 2 hours and then, after exposition to air, dried under vacuum.

The samples of DNA were hydrolysed by reflux heating with solution of 5 N hydrochloric acid (50 ml for 1 g DNA) for 90 minutes. After cooling the solutions were neutralized and then extracted with equal volumes of benzene (extractions were repeated five times). The combined benzene extracts, dried with anhydrous sodium sulphate, were concentrated under reduced pressure to very small volumes. The solutions obtained were used for the chromatographic separations.

Chromatography. In the column chromatography separations alumina (M. Woelm Eschwege – Germany, activity III according to Brockman) was used.

Thin-layer chromatography was performed using plates of cellulose powder MN 300 (Macherey, Nagel and Co., Düren, Germany) which were developed with acetone-water (1 : 1 (v/v)). To obtain more constant results, we have determined the R_B values, taking as reference benz[a]pyrene:

$$R_B = \frac{\text{distance of substance from origin}}{\text{distance of benz[a]pyrene from origin}}$$

Results

Isolation of a benz[a]pyrene-thymine photoadduct

670 ml of DNA solution, additioned with 15 mg of benz[a]pyrene dissolved in 5 ml of acetone, was poured into two Petri dishes with a diameter of 15 cm and irradiated for 8 hours. After precipitation and hydrolysis of DNA and extraction with benzene, the combined and concentrated extracts were chromatographed on a column of alumina (2×10 cm), using various solvents for the development of the column and the elution of the substances. These solvents and the results obtained are reported in Table I.

By means of this fractionation, four substances were separated in a pure state, as was indicated by their behaviour on chromatoplates. The UV absorption spectra of these four substances are almost identical. By contrast their chromatographic behaviour is sufficiently different to say that they are four different compounds. Among these, the substance contained in the fraction C and an R_B value of 3.17, identical to that of the photoadduct benz[a]pyrene-thymine previously obtained by irradiation of the two simple compounds [4]. The identity of these two substances was confirmed by a comparison in other chromatographic systems, as is

Solvent	Fractions obtained	UV spectrophotometric* and thin-layer chromatographic data
Benzene	A (unchanged benz[a]pyrene)	
Benzene–ethyl acetate 1:1	B Chromatographed again on a Al_2O_3 column (1×10 cm); elution with benzene: B_1	max: 226, 256, 266, 276, 288, 300, 340, 356, 375, 395, 406 nm $R_B = 2.35$
	elution with benzene–ethyl acetate: B_2	max: 226, 255, 265, 288, 300, 338, 355, 375, 395, 406 nm $R_B = 2.95$
Acetone–methanol	C For a purification, chromatographed again on a Al_2O_3 column (1×20 cm)	max: 226, 256, 265, 288, 299, 338, 355, 375, 395, 406 nm $R_B = 3.17$
Ethanol 95°	D For a purification, chromatographed again on a Al_2O_3 column (1×5 cm)	max: 258, 268, 288, 301, 340, 356, 375, 395, 408 nm $R_B = 4.10$

Table I. Column chromatographic fractionation of the benzene extract of the hydrolysis products of DNA irradiated at 365 nm in the presence of benz[a]pyrene. * UV absorption spectra were determined on methyl alcoholic solutions of the substances obtained after evaporation to dryness of the solution eluted from the chromatographic columns.

reported in Table II; UV spectra are reported in Fig. 1.

Plates	Solvent	Photo-adduct from DNA	Photoadduct benz[a]py-rene-thymine
MN 300 cellu-lose powder	acetone—water 1:1	R_B 3.17	R_B 3.17
Kieselgel Merck	benzene—acetone 1:1	R_F 0.80	R_F 0.81
Kieselgel Merck	benzene—methanol 9:1	R_F 0.74	R_F 0.74
Kieselgel Merck	Chloroform—acetone 7:3	R_F 0.59	R_F 0.59

Table II. Chromatographic behaviour of photoadduct isolated as fraction C (see table I) from DNA hydrolized after irradiation in the presence of benz[a]pyrene, and of photoadduct benz[a]pyrene-thymine [4].

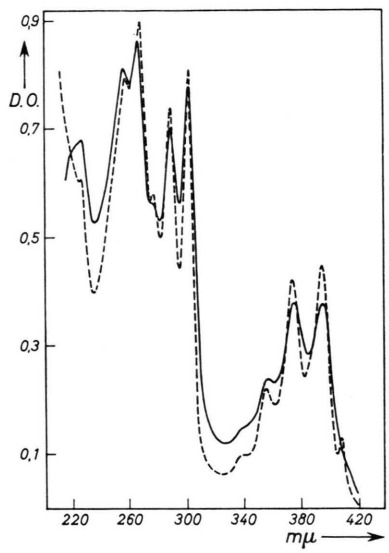

Fig. 1. UV absorption spectra determined in methyl-alcoholic solutions of photoadduct benz[a]pyrene-thymine — — — and of photoadduct obtained from hydrolysis of DNA after irradiation in the presence of benz[a]pyrene with R_B value 3.17 ———.

Hydrolysis of DNA irradiated in the presence of ^{3}H-benz[a]pyrene

35 ml of aqueous solution of DNA added to 3 mg of tritiated benz[a]pyrene (0,01 mCi) dissolved in 2 ml of acetone were irradiated for 2 hours. DNA was then precipitated, hydrolised and extracted with benzene as previously described. The concentrated benzene extract was chromatographed on a thin layer cellulose plate. After development, the plate was divided into 3 mm bands: the cellulose powder corresponding to each band was quantitatively withdrawn, added to 2 ml of absolute ethyl alcohol and heated for a few minutes on a water bath. The alcoholic solutions were then used for the radioactivity measurements: the results obtained are reported in Fig. 2. A maximum of radioactivity corresponds to

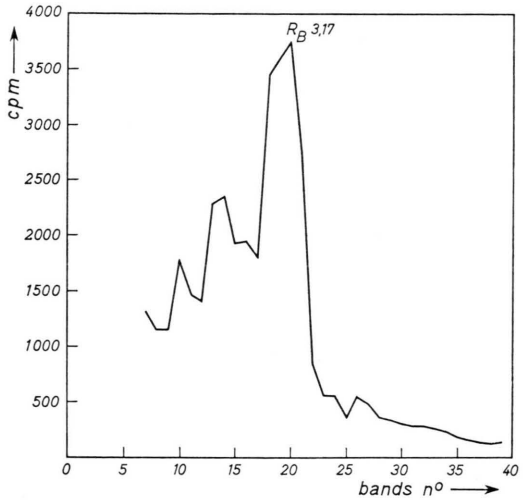

Fig. 2. Radioactivity values of 3mm bands obtained by subdivision of plate after chromatography.

an R_B value of 3.17, identical to that of the photoadduct benz[a]pyrene-thymine already isolated after irradiation of a mixture of thymine and benz[a]pyrene [4]. On the basis of the radioactivity of this peak, it was possible to calculate that this photoadduct was obtained with a molecular ratio of 1 : 7.000 in respect to the nucleotides present in the used DNA.

Conclusion

The results obtained show that by hydrolysis of DNA after irradiation at 365 nm in the presence of benz[a]pyrene, it has been possible to isolate a substance corresponding to the photoadduct benz[a]pyrene-thymine, already obtained by irradiation of these compounds. In this research only this photoadduct was definitely identified, but the isolation also of other quite similar compounds indicates that photoadducts with other bases can also be formed; however, identification of the bases of these photoadducts is difficult. As Fig. 1 shows, the peak of photoadduct benz[a]pyrene-thymine present in the chromatoplates is higher than all other peaks which are also present; this fact seems to indicate that among the bases of DNA thymine has the highest photoreactivity.

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