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A Fluorometric Study of DNA-Bound Benzo[*a*]pyrene†

Masahiko Kodama* and Chikayoshi Nagata

ABSTRACT: Comparisons were made among the fluorescence spectra of DNA-bound benzo[*a*]pyrenes which were produced in vivo and in vitro. DNA from mouse skin treated with benzo[*a*]pyrene had a maximum emission beyond 400 nm, which was clearly distinguished from that of DNA-bound benzo[*a*]pyrene 4,5-oxide. The emission spectra from mouse skin were classified into two groups, type I and type II. The former was similar to the spectrum of benzo[*a*]pyrene, although the two maxima were shifted to longer wavelengths (410 and 435 nm). Type II was characterized by a broad peak around 430 nm. Type I and type II were obtained from different fractions of hydroxylapatite chromatography, but type I was changed into type II during storage. This suggests that type II is a modified product of type I. The emission spectra of both groups also were detected in in vitro activating systems, including photoirradiation, iodine treatment, and hydrogen peroxide treatment. Treatment of *Escherichia coli* with benzo[*a*]pyrene during culture produced only fluorescence of type I. Although the relationship between types I and II remains to be established, both types of fluorescence evidently indicate that the conjugated ring structure of the parent compound, benzo[*a*]pyrene, is preserved intact in DNA-bound benzo[*a*]pyrene. Several lines of evidence suggest that the proximate (active) form is an unidentified hydroxylated product including an oxy radical, but a cation radical cannot be completely excluded.

Polynuclear aromatic hydrocarbons represented by benzo[*a*]pyrene (B[*a*]P)[1] are important carcinogens in our environment. However, the active forms of their metabolites are still obscure in spite of many investigations. The candidates for the proximate (active) form of aromatic hydrocarbons are cation radicals, carbonium ions, epoxides, and oxy radicals. Cation radicals have never been detected among metabolites but in an in vitro model system they are able to be bound to nucleic acids (Hoffmann et al., 1970). Carbonium ions have been discussed only from a theoretical point of view (Dipple et al., 1968; Jeftic and Adams, 1970). K-Region epoxides were recently confirmed as metabolites of several aromatic hydrocarbons (Grover et al., 1971, 1972) and it was discovered that the 6-oxy radical was important in the metabolism of B[*a*]P (Nagata et al., 1968).

There are a number of activating model systems which include one or two active forms. Iodine produces cation radicals, while hydrogen peroxide, especially in combination with ferrous chloride, results in hydroxylation at several sites (Ioki, Kodama and Nagata, to be published), and photoirradiation forms the 6-oxy radical in the case of B[*a*]P (Inomata and Nagata, 1972). A model system which includes epoxide formation is not known at present, but organic synthesis of K-region and non-K-region epoxides can substitute for it (Sims, 1971, 1972; Waterfall and Sims, 1972; Dansette and Jerina, 1974). These in vitro model systems are superior to the enzymatic system using microsome preparation, because they simplify the analysis of the mechanism of activation and they provide marker materials for the analysis of carcinogen-bound macromolecules in vivo.

For characterizing the nature of the conjugates, the chromatographic analysis of radioactive hydrolysates is a useful tool (Baird and Brookes, 1973); however, more direct information on molecular structure is provided by the fluorometric method. In this paper the authors wish to describe the characteristic fluorescences of DNA-bound B[*a*]P which were obtained in vivo as well as in vitro.

Materials and Methods

B[*a*]P was purchased from the Nutritional Biochemical Corp. and purified by chromatography over an alumina column. 6-Hydroxy-B[*a*]P was prepared according to the method of Fieser and Hershberg (1938). B[*a*]P 4,5-oxide was a generous gift from Professor Okamoto of The University of Tokyo. [^{3}H]B[*a*]P (3.0 Ci/mmol, Radiochemical Center) was used for acid hydrolysis of B[*a*]P-bound DNA.

Highly polymerized calf-thymus DNA from Sigma

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[1] Abbreviation used is: B[*a*]P, benzo[*a*]pyrene.

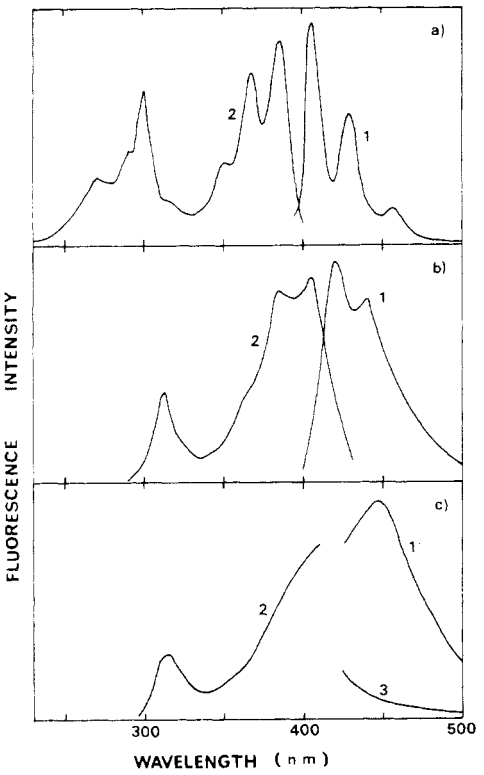

FIGURE 1: Fluorescence of DNA-bound B[*a*]P produced by photoirradiation: (a) B[*a*]P in ethanol; (b) DNA-bound B[*a*]P produced by photoirradiation in 100% ethanol; (c) DNA-bound B[*a*]P produced by photoirradiation in 33% ethanol; (curve 1) emission spectra; (curve 2) excitation spectra of free or DNA-bound B[*a*]P; (curve 3) emission spectra of control DNA without B[*a*]P treatment. A similar designation is applied to Figures 2–6.

Chemical Co. was used. Ethanol-soluble DNA was prepared according to the method of Weil et al. (1961). Apurinic and apyrimidinic acids were obtained according to Tamm et al. (1952) and Takemura (1959), respectively.

Photoirradiation. (a) Fifteen milliliters of an ethanol solution of 3 m*M* DNA (cetyltrimethylammonium salt) and 0.4 m*M* B[*a*]P was irradiated for 6 hr by the visible light of a high-pressure mercury lamp (200 V, 1.5 A), as described in a previous paper (Kodama and Nagata, 1969/70).

(b) Fifty milliliters of a 33% ethanol solution of 1.2 m*M* DNA–0.1 m*M* B[*a*]P–5 m*M* phosphate buffer (pH 6.8) was irradiated for 12 hr as above.

Treatment with Iodine. (a) Seventy-five milliliters of a 33% ethanol solution of 0.5 m*M* heat-denatured DNA, 0.1 m*M* B[*a*]P, 5 m*M* iodine, and 5 m*M* phosphate buffer (pH 6.8) was stirred in a light-shielded flask at room temperature for 2 hr (Hoffmann et al., 1970). (b) Seventy-five milliliters of an ethanol solution of 0.7 m*M* DNA, 0.1 m*M* B[*a*]P, and 5 m*M* iodine was stirred for 24 hr as above.

Treatment with Hydrogen Peroxide. (a) Seventy-five milliliters of a 33% ethanol solution of 0.5 m*M* heat-denatured DNA, 0.1 m*M* B[*a*]P, 15 m*M* hydrogen peroxide, and 10 m*M* sodium citrate buffer (pH 7.0) was incubated at 37° for 24 hr (Hoffmann et al., 1970). (b) Seventy-five milliliters of an ethanol solution of 0.7 m*M* DNA, 0.1 m*M* B[*a*]P, and 20 m*M* hydrogen peroxide was incubated at 37° for 24 hr.

Binding of B[a]P 4,5-Oxide to DNA. Twelve milliliters of an ethanol solution of 2 m*M* DNA and 0.1 m*M* B[*a*]P 4,5-oxide was stirred at 4° for 48 hr.

Binding of 6-Hydroxy-B[a]P to DNA. (a) Seventy-five milliliters of a 33% ethanol solution of 0.5 m*M* heat-denatured DNA, 0.03 m*M* [^{3}H]-6-hydroxy-B[*a*]P (7.5 mCi/mmol), and 5 m*M* phosphate buffer (pH 6.8) was stirred at room temperature for 24 hr. (b) Forty milliliters of an ethanol solution of 2 m*M* DNA and 0.3 m*M* 6-hydroxy-B[*a*]P was stirred for 24 hr.

B[a]P-Bound DNA from Escherichia coli. E. coli B was cultured in 1 l. of M-9 medium containing 5 g of casa amino acids, until OD_{590} (optical density) reached 0.7. Then B[*a*]P in dimethyl sulfoxide (10 mg/10 ml) was added, followed by a further 2-hr incubation (Brookes and Heidelberger, 1969). The DNA was isolated from the collected cells according to the modified procedure of Kirby (1956) (6% *p*-aminosalicylic acid (sodium salt); 1% sodium dodecyl sulfate–90% phenol, 1:1 v/v).

B[a]P-Bound DNA from Mice. The procedure was that described by Goshman and Heidelberger (1967). B[*a*]P in benzene (120 mg/9 ml) was applied in doses of 1.5 mg/mouse under dim light on the fur-plucked backs of 75 mice (female ddN, 20 g body weight), which were sacrificed 24 hr later. The skins (ca. 60 g) were removed and pulverized in liquid nitrogen, treated with hyaluronidase and Pronase, and homogenized with a Waring Blendor. The DNA was isolated as described by Kirby (1956). Experiments using a similar number of mice were repeated three times in total, and in the third experiment, the epidermis (5 g) and corium (40 g) of the skin removed were separated by scraping the skin with a blade onto a plastic plate. Both fractions were chopped finely with scissors and homogenized in 4–6 vol of 2% dodecyl sulfate by polytron (Kinematika, Switzerland, 115 V, 5 A), for 1 to 3 min at maximum velocity. Then, equal volumes of 12% *p*-aminosalicylic acid (sodium salt) and double volumes of 90% phenol were added successively. The DNA was successfully isolated by this simplified procedure with the same result.

Removal of Free and Physically Bound B[a]P. Both the DNA which was precipitated from the in vitro reaction mixture by ethanol or by 3 *M* NaCl (in the case of ethanol-soluble DNA) and the DNA which was isolated from either *E. coli* or the mouse skin were dissolved in water and extracted with chloroform 3 times, with ethyl acetate 3 times, and with ether once, followed by ethanol precipitation. In the case of DNA-bound 6-hydroxy-B[*a*]P, extraction with 80% phenol (twice) was substituted for the extraction with ethyl acetate. Except in this last case, a further extraction with 80% phenol did not change the results and 80% of the radioactivity of the DNA-bound [^{3}H]B[*a*]P was recovered after phenol extraction. The DNA was usually dissolved in 4 ml of water for measuring the fluorescence (OD_{260}/ml ranging from 20 to 50; in the mouse DNA it was 200).

Hydroxylapatite Chromatography. Purification and fractionation of mouse DNA and *E. coli* DNA were performed with a hydroxylapatite column on a linear gradient from 5 to 500 m*M* potassium phosphate buffer (pH 6.8). DNA was eluted between 40 and 100 m*M* phosphate buffer.

Hydrolysis of [^{3}H]B[a]P-Bound DNA. Acid hydrolysis was performed by incubating the DNA sample in 66% formic acid (1 mg/ml) at 30° for 19 hr according to the procedure of Burton and Petersen (1957). Alternatively, the DNA was heated in 70% perchloric acid at 100° for 60 min according to Marshak and Vogel (1951). The hydrolysate was condensed to dryness and extracted with methanol–water (5:1), with paper chromatography following. The radioactive and fluorescent spots were removed and eluted

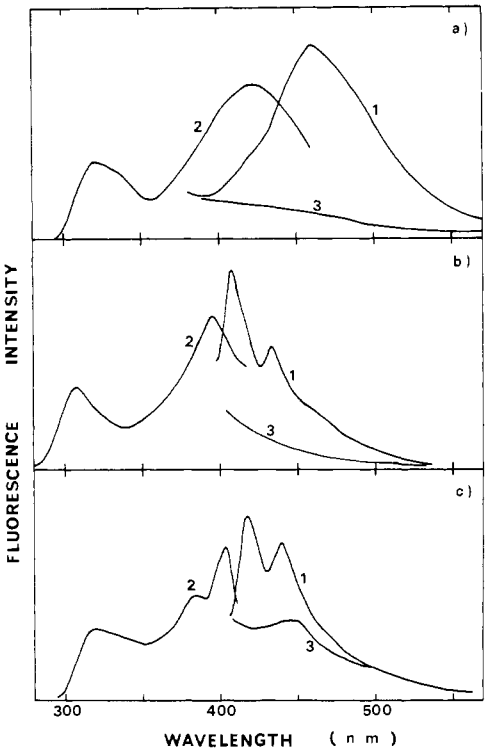

FIGURE 2: Fluorescence of DNA-bound B[*a*]P produced by iodine: (a) DNA-bound B[*a*]P produced in 33% ethanol; (b) DNA-bound B[*a*]P produced in 100% ethanol; (c) guanylic acid bound B[*a*]P produced in 33% ethanol.

with methanol-water (5:1).

Measurement of Fluorescence. The fluorescence of the neutral aqueous solution of DNA was measured at room temperature, by using an Aminco-Bowman spectrophotofluorometer with a Xenon lamp. The maximum sensitivity of this instrument was 10^{-8}–5×10^{-9} *M* B[*a*]P. The fluorescence of a B[*a*]P-treated DNA was consistently compared with that of the control DNA of the same concentration. The latter was not treated with B[*a*]P, but other than this it received the same treatment. Emission spectra were obtained by excitation at two excitation maxima, e.g. 300 and 380 nm, but the spectra were also checked for by excitation at other wavelengths. The same procedure was applied to the excitation spectra. The spectral data in the figures are presented without correction.

Results

Photoirradiation. In a previous paper (Kodama and Nagata, 1969/70), the present authors reported on the absorption spectra of DNA-bound B[*a*]P, which was obtained by photoirradiation with visible light in ethanolic solutions of B[*a*]P and ethanol-soluble DNA (cetyltrimethylammonium salt). The fluorescence spectrum of a similarly prepared sample is shown in Figure 1b, in comparison with that of free B[*a*]P (Figure 1a). The main peaks of the emission spectrum are shifted 10 to 15 nm to longer wavelengths compared with the ones of free B[*a*]P. To allow for a comparison with iodine or hydrogen peroxide treatment, the solvent for photoirradiation was changed from 100% ethanol to 33% ethanol. A quite different emission spectrum was then observed; it showed a single broad peak, devoid of fine structure in the longer wavelength band (Figure 1c). This kind of fluorescence will be designated as type II in contrast to type I of Figure 1b.

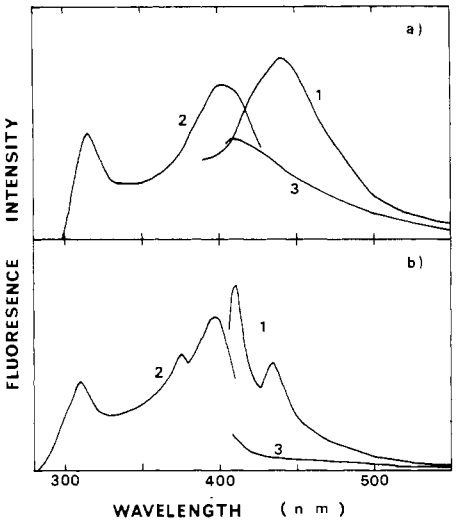

FIGURE 3: Fluorescence of DNA-bound B[*a*]P produced by hydrogen peroxide: (a) DNA-bound B[*a*]P produced in 33% ethanol; (b) DNA-bound B[*a*]P produced in 100% ethanol.

Chemical Linkage Induced by Iodine. According to the procedure of Hoffmann et al. (1970), DNA-bound B[*a*]P was prepared in 33% ethanol solution and checked for the fluorescence spectrum (Figure 2a), which apparently belonged to type II of the above classification. Although the fluorescence intensity was not so strong, the difference was remarkable and also reproducible between B[*a*]P plus iodine-treated samples and samples treated with iodine alone. The presence of two excitation peaks at 320 and 420 nm is characteristic of the B[*a*]P moiety, but not of alkylated DNA residues which have a single excitation maximum around 300 nm (Leng et al., 1968). Apyrimidinic acid was more effective in producing this fluorescence than apurinic acid (ratio of fluorescence intensity, 43:12), which finding is in agreement with the radioassay data of Hoffmann et al. (1970). The substitution of 100% ethanol for 33% ethanol in the reaction mixture by using ethanol-soluble DNA resulted in an emission spectrum of type I (Figure 2b). The replacement of DNA by GMP in the 33% ethanol solution also caused the same effect (Figure 2c).

Chemical Linkage Induced by Hydrogen Peroxide. Two kinds of fluorescence, type I and type II, were also observed in this system. The DNA sample which was treated with B[*a*]P plus H_2O_2 in 33% ethanol solution showed the emission spectrum of type II (Figure 3a), while the spectrum of type I was observed for the DNA sample which was treated in 100% ethanol (Figure 3b).

B[a]P 4,5-Oxide Bound to DNA. B[*a*]P 4,5-oxide (K-region epoxide) was reported to bind covalently with DNA without further activation (Grover et al., 1972; Wang et al., 1972). The binding experiment was tried in 100% ethanol and the treated DNA solution was checked on for the fluorescence spectrum. The emission spectrum (Figure 4c) was quite different from any of the above complexes but resembled greatly free B[*a*]P 4,5-oxide (Figure 4a).

6-Hydroxy-B[a]P Bound to DNA. The covalent linkage of [^{3}H]-6-hydroxy-B[*a*]P to DNA was reported by Ts'o et al. (1974) as well as by Nagata et al. (1974). From a time course study and from the effects of either activators or inhibitors, the conversion of 6-hydroxy-B[*a*]P to the 6-oxy-B[*a*]P radical is deemed to be responsible for the binding to DNA. However, the complex thus obtained showed no fluorescence spectra within the limit of sensitivity, while in-

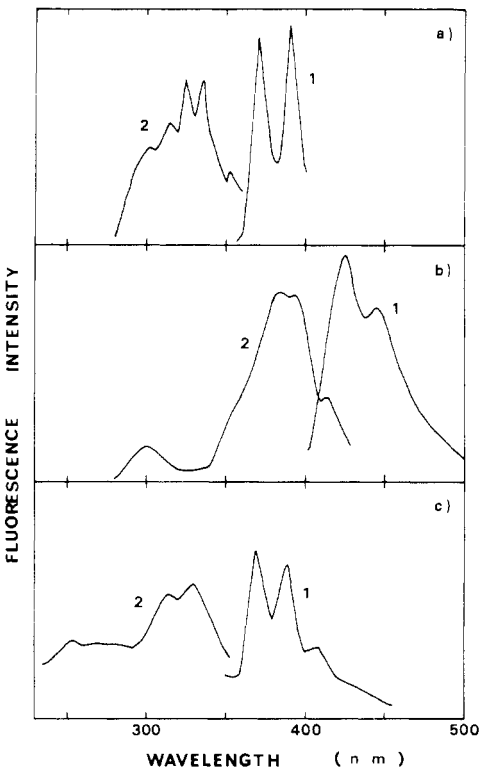

FIGURE 4: Fluorescence of DNA-bound B[*a*]P 4,5-oxide: (a) B[*a*]P 4,5-oxide in ethanol; (b) phenol(s) from B[*a*]P 4,5-oxide in ethanol; (c) DNA-bound B[*a*]P 4,5-oxide.

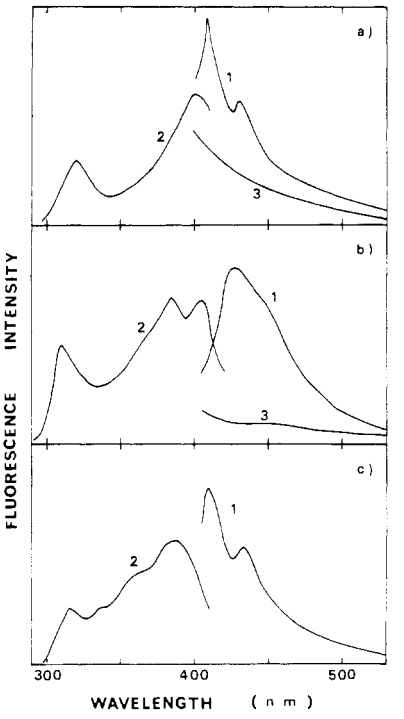

FIGURE 5: Fluorescence of DNA-bound B[*a*]P from *E. coli* or mice: (a) DNA-bound B[*a*]P from *E. coli;* (b) DNA-bound B[*a*]P from mice, hydroxylapatite fraction I; (c) DNA-bound B[*a*]P from mice, hydroxylapatite fraction V.

creased binding by several activators always accompanied the characteristic absorption spectra (maximum at 410 and 550 nm). One possible explanation for this is that DNA-bound 6-hydroxy-B[*a*]P does not resemble the phenols but the quinones which show no fluorescence.

DNA-Bound B[a]P from E. coli. According to the procedure of Brookes and Heidelberger (1969), DNA was isolated from B[*a*]P-treated *E. coli* and nontreated *E. coli* and checked on for the fluorescence spectra. Only DNA from B[*a*]P-treated *E. coli* showed the characteristic emission spectrum of type I (Figure 5a). Further purification of DNA through hydroxylapatite chromatography did not change the result.

DNA-Bound B[a]P from Mice. DNA was isolated according to Goshman and Heidelberger (1967) from mouse skin which was painted with B[*a*]P in benzene. The control DNA was obtained from mouse skin which was painted with benzene only. The emission spectrum showed the characteristics of type II (Figure 5b); however, the fractionation of DNA by hydroxylapatite chromatography disclosed the later eluted fractions (eluted between 60 and 100 m*M* potassium phosphate buffer) which possessed the type I fluorescence (Figure 5c). The other fractions remained as type II. During dialysis and following storage (at 4°) of the type I fractions, a conversion into type II was observed. Further purification of type II fractions did not change the result but proved that the emission is from B[*a*]P which covalently became bound with DNA (Table I).

Hydrolysis of B[a]P-Bound DNA. In order to clarify the relationship between type I and type II, the hydrolysis of B[*a*]P-bound DNA was attempted. A sample of type I was supplied by photoirradiation in 100% ethanol and a sample of type II was provided by iodine treatment in 33% ethanol, using [^{3}H]B[*a*]P. The lesser part of the hydrolysis was done by combining DNase and snake venom phosphodiesterase; the greater part of the hydrolysis, however, was done by depurination through formic acid, since in the case of photoirradiation and iodine, the dominant binding site is purine. The type I spectrum produced by photoirradiation was able to be detected after the final purification through paper chromatography (Figure 6a). In addition to this main fluorescence, however, another kind of fluorescence was sometimes observed. This latter might be classified as type II (Figure 6b). In the case of iodine-treated samples of type II, the same fluorescence as in Figure 6b was often observed in the final step. Nevertheless, the purification was not so complete as in the photoirradiated samples, partly because of the low intensity of the fluorescence in the starting materials.

Table I: Change of Fluorescence Intensity of DNA-Bound B[*a*]P from Mice (Type II) after Several Treatments.

Treatment	$E_{m,430}/OD_{260}$[a]	%
Before	0.90	100
Ethanol precipitation	0.78	87
Dodecyl sulfate–phenol	0.74	82
RNase–phenol	0.70	78
Pronase–phenol	1.04	115

[a] The contribution of the nontreated control DNA was subtracted from the fluorescence intensity of 430 nm in the B[*a*]P-treated mouse DNA.

Discussion

Data from Figures 1 to 5 are summarized in Table II. It is apparent that the fluorescence spectra of type II have a certain relationship with those of type I.

(1) Type I and type II are produced in two solvent conditions, 33 and 100% ethanol, respectively, by the same acti-

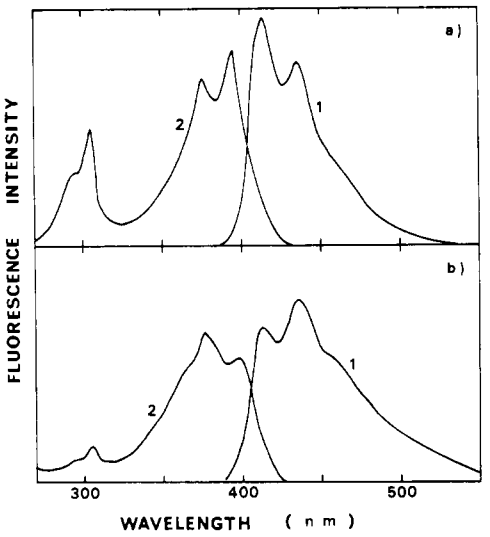

FIGURE 6: Fluorescence of purified acid hydrolysates of DNA-bound B[*a*]P, produced by photoirradiation in 100% ethanol: (a) formic acid (66%, 30°, 19 hr) hydrolysate; (b) perchloric acid (70%, 100°, 60 min) hydrolysate. R_f values of fluorescent products of a and b are 0.71 and 0.81, respectively, in the paper chromatography system (Whatman No. 1; 1 *M* ammonium acetate–ethanol, 3:7).

vating agents (photoirradiation, iodine, and hydrogen peroxide).

(2) The conversion of type I into type II was observed in the cases of (a) mouse DNA, (b) hydrolysates of photoirradiated DNA, and (c) iodine-treated GMP.

(3) The spectra of type II, once formed, remained unchanged by the substitution of the solvent from water into ethanol (from the sodium salt of DNA into the cetyltrimethylammonium salt).

In this connection, the observation of Girke and Wilk (1974) is worth noting. They obtained a B[*a*]P–purine product by mixing B[*a*]P in benzene with purine in trifluoroacetic acid. The primary product, a trifluoroacetate salt, was unstable and this decomposed into a secondary product as a neutral salt. The absorption spectrum of the secondary product was much broader and shifted to longer wavelength when compared with that of the primary product. Acidification of the secondary product by means of hydrochloric acid completely removed the fine structure of the spectrum, and resulted in a single broad peak. If the above data are applied to emission spectra, type II could be the secondary product of type I. The hydrolysis study on type II suggests the possibility that more than one component might be responsible for the spectra of type II. Although the excitation spectra of type II do have the characteristics of aromatic hydrocarbons, more precise discussion on the electronic structure of bound B[*a*]P should be limited to type I.

As far as the emission spectra of type II are concerned, the spectrum of mouse DNA resembled the spectrum occasioned by hydrogen peroxide rather than the spectrum occasioned by iodine. The type I emission spectra, however, are all alike among the several groups, except for a slight difference in the maximum peaks. The spectral data here obtained are not sufficient to deny the role of the cation radical in the in vivo activation system.

However, if the cation radical is formed in the process of the metabolism of B[*a*]P, it should be detected as B[*a*]P-dimer or it should be trapped as the B[*a*]P–pyridine complex, which the authors never succeeded in isolating. From the above considerations, hydroxylated products will then

Table II: Summary of Fluorometric Data of Figures 1–5.

	Maximum (nm) in	
	Emission Spectra	Excitation Spectra
Type I		
Photoirradiation	420, 440	313, (365), 385, 403
Iodine	410, 433	310, 395
Hydrogen peroxide	410, 435	310, 375, 395
E. coli	407, 430	320, 400
Mouse	410, 435	315, (360), 390
Type II		
Photoirradiation	445	315
Iodine	460	320, 420
Hydrogen peroxide	440	315, 405
Mouse	430, (450)	310, 385, 405
B[*a*]P 4,5-oxide (DNA bound)	370, 390, (410)	315, 330
B[*a*]P[a]	405, 428, 455	(270, 290), 300, 367, 385
3-Hydroxy-B[*a*]P	435, 460	(273, 298), 313,365,383, 403, 430
6-Hydroxy-B[*a*]P	440, 463	(272, 297), 305, 400
B[*a*]P 4,5-oxide	370, 390	313, 326, 335, 354
Phenol(s) from B[*a*]P 4,5-oxide	425, 445	300, 385, 395, 417
Pyrene	375, 387, 394	273, (310, 327), 333
Chrysene	365, 383, 403, 427	295, 305, 317, 330, 347 363
Benzo[*a*]anthracene	387, 410, 433	276, 286, 296, 333, 345, 362

[a] Fluorescences of free aromatic hydrocarbons were measured in ethanol. Figures in parentheses represent small peaks or shoulders.

be a more proximate form than the cation radical. The hydroxylation occurs by means of two routes: (a) the substitution reaction at the 6,3,1 region of B[*a*]P (Harper, 1958; Falk et al., 1962; Nagata et al., 1974) and (b) the epoxide formation at the 4,5, 7,8, and 9,10 regions of B[*a*]P (Sims et al., 1974). It had been recently reported that the epoxide hydrase inhibitor increased the binding of B[*a*]P to DNA in the liver microsome system (Bürki et al., 1974; Selkirk et al., 1974), and this report the present authors confirmed. Accordingly, epoxides (not necessarily the K-region epoxide) could be participants in the binding process in vivo but they are not the only factor involved. The fluorescence spectrum of mouse DNA is clearly distinguished from that of the K-region epoxide, and the same spectrum also indicated that the DNA-bound B[*a*]P possessed the intact conjugated ring structure of B[*a*]P. This means that the substitution reaction, and not the addition reaction, is responsible for a binding process of this kind. The 6 position of B[*a*]P is the most responsive site for the substitution reaction. The study of the tritium displacement by Blackburn et al. (1974) suggested this possibility in DNA binding in vivo. The fluorometric study of DNA-bound benz[*a*]anthracene recently reported by Daudel et al. (1974) also supported the possibility of the substitution reaction.

The fluorescence of the DNA-bound epoxide(s) was not detected in our usual preparations in vivo. However, the DNA sample from the mouse epidermis contained the satellite species of fluorescence which was clearly distinguishable from the main fluorescence of type II. The maximum peaks of emission for the former are at 385 and 405 nm when the excitation is between 330 and 350 nm. This latter kind of fluorescence is most likely derived from the DNA-bound epoxide(s), since the conjugated ring structure of B[*a*]P in this case is impaired.

The question may be asked, "Is the substitution reaction

of B[*a*]P limited in the binding process of nucleic acids?". Fluorometric studies on protein-bound B[*a*]P were started with the pioneer work of Miller (1951) and the emission spectra of bound B[*a*]P after hydrolysis with acid or alkali were investigated by several groups of workers. Most of the spectra reported up to now had an emission in a wavelength longer than 400 nm, and thus retained the intact conjugated structure of B[*a*]P (Moodie et al., 1954; Tarbell et al., 1956). On the other hand, Daudel et al. (1962) observed fluorescence of the chrysene type and the benz[*a*]anthracene type. Raha et al. (1973) also reported the production of chrysene from protein-bound B[*a*]P.

A separation of multiple forms of protein-bound aromatic hydrocarbons was recently reported by Corbett and Nettesheim (1974). Both the addition reaction through epoxides and the substitution reaction through hydroxylated hydrocarbons must be responsible for these multiple forms of conjugation.

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