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Effect of Selected Induction of Microsomal and Nuclear Aryl Hydrocarbon Monooxygenase and Epoxide Hydrolase as well as Cytoplasmic Glutathione S-Epoxide Transferase on the Covalent Binding of the Carcinogen Benzo(a)pyrene to Rat Liver DNA in Vivo

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Summary. Groups of four adult male rats [ZUR:SIV-Z] were pretreated with corn oil (control; 2 ml/kg/day i.p. for 3 days), *trans*-stilbene-oxide (SO; 200 mg/kg/day i.p. for 2 days), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 10 μg/kg i.p. once, 4 days before killing), phenobarbital (PB; 1 g/liter in the drinking water for 8 days), and dieldrin (20 mg/kg/day i.p. for 3 or 9 days). They received an injection of [G-^{3}H]benzo(*a*)pyrene (BaP, 31 μg/kg, $7.4 \cdot 10^9$ dpm/kg; i.v.) 16 h before killing. In the liver of each rat, five enzymatic activities and the covalent binding of BaP to DNA have been determined. The microsomal aryl hydrocarbon monooxygenase activity (AHM) ranged from 75% of control (SO) to 356% (TCDD), the nuclear AHM from 63% (SO) to 333% (TCDD). Microsomal epoxide hydrolase activity (EH) was induced up to 238% (PB), nuclear EH ranged from 86% (TCDD) to 218% (PB). A different extent of induction was observed in the two compartments. Highest induction of glutathione *S*-epoxide transferase activity (GST) was found with PB (202%). The DNA binding of BaP was modulated within 79% (dieldrin, 9 days) and 238% of control (TCDD). An enzyme digest of control DNA was analysed by Sephadex LH-20 chromatography. Multiple linear regression analysis with all data expressed as % of control yielded the following equation: DNA Binding = $1.49 \cdot$ Microsomal AHM $- 1.07 \cdot$ Nuclear AHM $+ 0.33 \cdot$ Microsomal EH $- 0.52 \cdot$ Nuclear EH $+ 0.11 \cdot$ Cytoplasmic GST $+ 58.2$. From this analysis it is concluded that (1) AHM located in the endoplasmic reticulum is most important in the formation of DNA-binding metabolites, (2) EH in the same compartment is not determinative in this respect nor has it a protective effect, (3) both membrane-bound enzyme activities located in the nucleus may inactivate potential ultimate carcinogens, and (4) cytoplasmic GST probably cannot reduce DNA binding due to its subcellular localization.

Key words: Carcinogen – Benzo(*a*)pyrene – DNA binding – Enzyme induction – Aryl hydrocarbon monooxygenase – Epoxide hydrolase – Glutathione S-transferase

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Benzo(*a*)pyrene (BaP) is the most extensively studied chemical carcinogen of the PAH class. The chemically unreactive molecule is believed to exert its toxic, mutagenic, and carcinogenic effects only after enzymatic conversion to one of a number of electrophilic metabolites that bind covalently to cellular components (Miller 1970; Sims 1976; Sims and Grover 1974; Wood et al. 1976).

Metabolism of PAH requires the enzymatic activity of the monooxygenase system, a membrane-bound enzyme complex able to oxidize various substrates. With PAH as substrates, it is named AHM (EC 1.14.14.1.) and oxidizes BaP to at least three epoxides, four phenols, and three quinones (review by Sims and Grover 1974; Yang et al. 1975).

All three epoxides can be further metabolised by the membrane-bound EH (EC 3.3.2.3.) to vicinal *trans*-dihydrodiols (Jerina et al. 1977; Oesch 1976), and the AHM has been shown to act again on the 7,8-diol (Sims 1976, Sims and Grover 1974). Among the highly reactive derivatives formed, the anti-diolepoxide is of particular interest since it is widely believed to be the most important ultimate carcinogen (King et al 1979, Sims 1976; Sims et al. 1974; Weinstein et al. 1976, Wood et al. 1976). There is, however, also evidence of DNA binding of one-step epoxides, phenolic epoxides, or radical derivatives (Glatt and Oesch 1977; Jernström et al. 1978; Kahl et al. 1979; Lesko et al. 1975; Owens et al. 1979; Rogan et al. 1978).

The liver has the highest capacity to metabolize BaP. Membrane-bound enzymes are located predominantly in the ER, less being found in the nuclear membrane (Jernström et al. 1976; Khandwala and Kasper 1973; Mukhtar et al. 1978, 1979; Pezzuto et al. 1977; Wishart and Fry 1977). They are inducible to higher activities by various compounds (review in: Estabrook and Lindenlaub 1979), and a different extent of enzyme induction in microsomes and nuclear membranes was found for the AHM after short-term administration of inducers (Jernström et al. 1976; Khandwala and Kasper 1973; Rogan et al. 1976; Viviani et al. 1978).

In a previous report (Viviani and Lutz 1978) we have shown that the induction of microsomal AHM increases the covalent binding of BaP to rat liver DNA in vivo, but that this binding is reduced after induction of the nuclear AHM. The present communication additionally deals with the influence of microsomal and nuclear EH and cytoplasmic GST on the binding, because the role of EH in the process leading to DNA-binding metabolites is controversial (Oesch 1976; Sims 1976; Sims and Grover 1974) and because the importance of conjugating enzymes has often been emphasized without appropriate in vivo data (Benson et al. 1978; Burke et al. 1977; Jones et al. 1978; Mulder and Scholtens 1977; Smith and Bend 1979). Various enzyme induction patterns have been obtained with the use of inducers of different specificity and with selected treatment schedules. The influence of each distinct enzyme activity on the binding of BaP to liver DNA and the importance of the nuclear membrane could be evaluated by a combination of enzyme induction and the phenomenon of individual variation.

Material and Methods

Chemicals

Reagents were purchased from reliable sources and were of the highest grade commercially available. BaP and dieldrin were obtained from Fluka AG, Buchs, Switzerland. SO was from Aldrich-Europe, Beerse, Belgium. TCDD was generously supplied by Givaudan SA, Dübendorf, Switzerland, and

sodium PB was from the Kantonsapotheke, Zurich, Switzerland. [G-^{3}H]BaP with a specific activity of 27 Ci/mmol and [7,10-^{14}C]BaP (61 mCi/mmol) were from The Radiochemical Centre, Amersham, England. BaP was purified further by column chromatography in hexane on silica gel to a radiochemical purity of >99%, and dieldrin was purified by recrystallization. The [7,10-^{14}C]BaP-4,5-epoxide was synthesized according to approved methods (Baran 1960; Dansette and Jerina 1974). An aliquot of *cis*-4,5-dihydroxy-4,5-dihydroBaP was saved during this synthesis.

Animals

Adult male rats (Sprague-Dawley derived ZUR:SIV-Z), weighing 270–300 g on the day of killing were used. They were housed two per cage on hardwood chips in clear Macrolone cages and left untreated for at least 1 week after receipt. Nafag laboratory chow (Pellets No. 890, Nafag AG, Gossau, Switzerland) and tap water were provided ad libitum. Rats in the same cage were given the same treatment.

Enzyme Induction

Four animals were used in each group of treatment. Dieldrin (20 mg/kg/day for 3 or 9 days) and SO (200 mg/kg/day for 2 days) were dissolved in corn oil ("Mazola", Maizena GmbH, Hamburg, FRG), and 2 ml/kg were injected i. p. Control rats received equivalent doses of oil only. A solution of TCDD in oil was prepared as described earlier (Viviani et al. 1978), and a single i. p. dose of 10 µg/kg in oil (2 ml/kg) was administered 4 days before killing. Sodium PB was given for 8 days in the drinking water. The concentration of 1 g/liter corresponded to about 60 mg/kg/day.

Administration of Tritiated BaP

Purified [G-^{3}H]BaP was dissolved in toluene and dispersed as a fine film in a roundbottomed flask by rotary evaporation of the solvent. Serum of the same rat strain was added, and the flask was vigorously shaken at ambient temperature in the dark under nitrogen for 18 h. The BaP was completely dissolved after this treatment. The solution was centrifuged for 20 min at 105,000 $\times g$, and the supernatant was stored at 4°. Sixteen hours before killing, 1.2 ml/kg of the solution (31 µg/kg; $7.4 \cdot 10^9$ dpm/kg) was injected into the penis vein under local anesthesia with Novesin (Wander AG, Berne, Switzerland). This dose of BaP is far below what was found to induce the AHM in the same rat strain (Lutz et al. 1978).

Preparation of Subcellular Components

Rats were killed by stunning and cervical dislocation at 09.00 h. As described previously (Viviani et al. 1978), liver microsomes and nuclei were isolated by calcium precipitation and sucrose density centrifugation, respectively, and protein measurements were made with the Folin reagent. During the preparation of microsomes, an aliquot of the 12,000 $\times g$-supernatant was diluted 10-fold with Hepes-buffer (see below) and was centrifuged for 30 min at 105,000 $\times g$. The supernatant cytoplasm was used for the GST assay.

Enzyme Assays

All enzyme assays were performed under subdued light. The substrate used for the radioassay of EH and particularly of GST had to be ^{14}-C-labeled because DNA binding was determined with high activities of tritiated BaP in the same animal. Enzyme assay mixtures therefore contained appriciable amounts of tritium which were easily separated from the ^{14}C counts by appropriate setting of the energy discriminators of the liquid scintillation counter (BF-5000; Lab. Berthold, Wildbad, FRG).

AHM was determined with several modifications (Viviani et al. 1978), using the fluorimetric procedure of Nebert and Gelboin (1968). Activities are expressed as fluorescence of alkali-extractable products formed in 1 min per mg protein whereby 1 U of fluorescence is brought about by 1 pmol of the synthetic 3-phenol.

EH was assayed using a modification of the thin-layer procedure of Jerina et al. (1977). Fifty microliters of a Tris-HCl buffer 0.4 M, pH 9.0 at 37°, and 100 µl of an accurately diluted microsomal or nuclear preparation in water were placed into a small tube and preincubated at 37° for 2 min. The reaction was started by adding, and rapidly mixing on a vortex shaker, 5 µl (10 nmol) of a solution of [^{14}C]4,5-epoxide (458 dpm/nmol) in acetonitrile containing 0.2% of saturated aqueous ammonia. Ten

minutes later, the reaction was stopped by the addition of 50 μl of tetrahydrofuran and vortexing, and the tube was put on ice. An aliquot of the reaction mixture (40 μl) was spotted onto a silica gel thin-layer plate on alumina foil (Kieselgel 60; Merck AG, Darmstadt, FRG), and the plate was developed in benzene:ethanol 10:1 for 30 min. Products were localized by their fluorescence under UV light. The 4,5-diol was identified by previously spotted standard 4,5-diol and had an R_f of 0.37. This spot was cut off, the 1 cm^2 chip was placed into a scintillation vial and shaken with 1 ml of dimethylsulfoxide for 10 min. Radioactivity was determined after the addition of 10 ml of toluene containing 7g/liter butyl-PBD (2-(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole; Ciba-Geigy AG, Basel, Switzerland). Results expressed as nmol 4,5-diol formed in 1 min per mg protein were calculated from duplicate determinations with a 0-min incubation as a blank. Product formation was found to be linear up to enzyme concentrations able to convert 50% of the added substrate.

GST was assayed according to James et al. (1976), with some modifications concerning the volumes used and with the 4,5-epoxide as a substrate. Five hundred microliters of Hepes-buffer (*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 20 m*M*, pH 7.8 at 37°), 50 μl of 80 m*M* reduced glutathione (freshly prepared in buffer), and 200 μl of a diluted cytoplasmic preparation in water were preincubated at 37° for 2 min. After the addition of the substrate, 4,5-epoxide (same amount of the solution described above), the reaction was run for 1 min and then stopped by vigorous vortexing with 2 ml of ethyl acetate. This extraction step was repeated twice, whereby all manipulations were standardized to get reproducible results. Ten milliliters of Insta-Gel (Packard Instruments Co., Downers Grove, IL 60515, USA) was added to the aqueous phase, and the radioactivity was determined after shaking and transfer of the mixture to scintillation vials. Enzyme activity was expressed as nmol conjugate formed in 1 min per mg protein and was calculated on the basis of the sum of the 1-min incubation and a blank (without enzyme) run for 0 min, minus the sum of a 0-min incubation of the same sample and a blank run für 1 min. Each determination was carried out in quadruplicate. Glutathione concentration was checked to be saturating, and the assay had a linear slope up to enzyme concentrations able to metabolise 10% of the 4,5-epoxide present.

Measurement of Radioactivity Bound to DNA

An aliquot of the minced liver was frozen immediately after excision and stored at −20° for 7 h. The DNA was then isolated according to previously published methods (Viviani and Lutz 1978). With L-[^{35}S]methionine, administered per os to a rat, and subsequent determination of the specific activity of chromatin protein and DNA (with contaminating chromatin protein) it was found that DNA, isolated as described, contains 0.5% protein. One to 4 mg dry DNA were dissolved in 4 ml of 14 m*M* sodium phosphate buffer, pH 6.8, and counted after the addition of 10 ml Insta-Gel. The net counts ranged from 220 to 1,300 cpm which were processed to yield the specific activity of the DNA on the basis of a counting efficiency of 0.28 and an absorbance at 260 nm of 6.18 for 1 μmol DNA nucleotides per ml. Effectiveness of DNA binding was calculated in "CBI" units (μmol BaP/mol DNA nucleotides)/(mmol BaP/kg b.w.) (Lutz 1979).

For the chromatography of hydrolyzed DNA, one male rat weighing 298 g was treated i.p. with 1.5 ml/kg of a solution of purified [*G*-^{3}H]BaP in corn oil ($9.0 \cdot 10^9$ dpm/kg; 43 μg/kg).

Twenty hours after the application, the animal was killed and liver DNA was isolated. An aliquot containing 575 μg purified DNA with 646 dpm was enzyme-digested to deoxyribonucleosides according to King et al. (1979). One half volume of methanol was added to the hydrolyzate which was then centrifuged for 1 min at 1,000 g. The supernatant was given onto a 1.0 × 30 cm column of Sephadex LH-20 and chromatographed with a 150-ml gradient of 30 to 100% methanol in water with a flow rate of 0.3 ml/min. The absorbance at 260 nm was recorded and 5-ml fractions were collected. Radioactivity was determined after the addition of 10 ml Insta-Gel. To the 12 early eluting fractions 2 ml of methanol were added. The limit of detection of radioactivity in any one fraction was determined to be 1.4 cpm (2 S.D.s), due to counting error and variation from vial to vial.

Calculations

One animal of the PB group and one of the dieldrin-9-day group exhibited a DNA-binding value which was 8 and 3.5 S.E. outwith the estimate, obtained by using the multiple linear regression analysis described below. Data from these two animals were not included.

The two-sided Student's t-test was used for the comparison of means, with corrections for data not similar in their S.D. (Johnson and Leone 1964). A p of 0.05 was chosen as the limit of significance.

Correlations between different data groups were determined using multiple linear regression (Method of least squares).

Results

Table 1 compiles the liver enzyme activities determined after pretreatment of the rats with several inducers, together with the observed covalent binding of BaP to DNA.

Effect of Specific Inducers on Enzyme Activities

Pretreatment of adult male rats with SO lowered the AHM (significantly only in the nuclei) but induced EH and GST. The finding of EH induction is in agreement with 2 other reports (Mukhtar et al. 1978; Schmassmann et al. 1978). The observed effects on AHM and GST, however, are not shared by Mukhtar et al. (1978), who reported an induction of AHM but no effect on the GST.

TCDD is a well-known inducer of monooxygenase activity (Aitio and Parkki 1978; Haugen and Coon 1976; Lucier et al. 1973). With our rats, the observed effect on AHM is not as pronounced as can be found with younger rats or other animal species (Lucier et al. 1973; Poland and Kende 1976). No effect of TCDD on the EH was evident after 4 days, although a higher dose and a longer waiting period resulted in a slight induction of the EH in the Wistar strain (Aitio and Parkki 1978). The low but significant increase in GST has been previously observed (Aitio and Parkki 1978; Baars et al. 1978).

As found previously in our (Viviani and Lutz 1978) and other laboratories (Khandwala and Kasper 1973; Rogan et al. 1976), PB significantly induced the microsomal, but barely the nuclear AHM. It is noteworthy that PB causes liver growth and proliferation of the ER (Lake et al. 1976) but no enlargement of the outer nuclear membrane (Hooper and Dick 1976). PB was found to be a potent inducer of microsomal and nuclear EH, in agreement with other reports for microsomes (Jerina et al. 1977; Oesch 1976) and, recently, for nuclei (Mukhtar et al. 1979). Moreover, the GST was doubled, showing a better inducibility than otherwise reported (Baars et al. 1978; Mukhtar and Bresnick 1976).

Dieldrin is an interesting enzyme-inducing compound since, when administered to rats, it showed unusual and time-dependent phenomena, which was shown for the AHM in our previous reports (Viviani and Lutz 1978; Viviani et al. 1978). Pretreatment for 3 days induced the AHM only in the nuclei and the EH only in the ER, whereas after 9 days, the extent of induction was generally higher and indistinguishable in the two subcellular compartments. Our results on microsomal activities are in agreement with other reports (Schmassmann et al. 1978; Vainio and Parkki 1976). GST was also induced only after prolonged treatment.

Covalent Binding of BaP to Liver DNA

Pretreatment of the rats with TCDD and PB resulted in a doubling of the binding of BaP to DNA. Nine-day treatment with dieldrin reduced this binding whereas SO or 3-day-dieldrin treatment did not affect it.

Table 1. Effect of pretreatment of adult male rats with various inducers on liver microsomal and nuclear AHM and EH, on cytoplasmic GST, as well as on the covalent binding of BaP to liver DNA. Animals were treated with corn oil (control; 2 ml/kg/day i.p.), SO (200 mg/kg/day i.p.), and dieldrin (20 mg/kg/day i.p.) during the time period indicated. TCDD (10 μg/kg i.p.) was administered as a single dose on day 0 of 4 days. Sodium PB was given in the drinking water (1 g/liter). Sixteen hours before killing, the rats were treated with [G-^{3}H]BaP (31 μg/kg). Units of enzyme activities and of DNA binding are given in "Material and Methods". Numbers on each line are from one single animal, numbers in parentheses give the percentage from the mean of the control group

	AHM		EH		GST	Binding
	Microsomal	Nuclear	Microsomal	Nuclear	Cytoplasmic	to DNA
	x_1[a]	x_2	x_3	x_4	x_5	x_0
	675 (84)	25 (101)	10.4 (87)	0.58 (104)	6.6 (96)	20.9 (94)
	1000 (124)	28 (115)	12.8 (107)	0.51 (90)	7.0 (101)	21.7 (98)
	830 (103)	24 (98)	14.3 (119)	0.64 (113)	7.3 (106)	24.0 (108)
	715 (89)	21 (86)	10.6 (88)	0.53 (94)	6.7 (97)	22.1 (100)
Control, 3 days	805 ± 145[b] (100)	24 ± 3 (100)	12.0 ± 1.9 (100)	0.56 ± 0.06 (100)	6.9 ± 0.3 (100)	22.1 ± 1.3 (100)
	700 (87)	29 (119)	20.3 (169)	0.78 (139)	11.9 (172)	17.4 (79)
	670 (83)	7 (30)	19.7 (164)	0.87 (155)	11.4 (165)	20.2 (91)
	500 (62)	11 (45)	16.4 (136)	0.68 (120)	15.5 (223)	19.8 (89)
	530 (66)	14 (59)	18.4 (153)	0.83 (147)	10.0 (144)	15.5 (70)
SO, 2 days	600 ± 100 (75)[e]	15 ± 5 (63)[c, e]	18.7 ± 1.7 (156)[c, e]	0.79 ± 0.08 (141)[c, e]	12.2 ± 2.3 (176)[c]	18.2 ± 2.2 (82)
	2665 (331)	72 (295)	14.0 (117)	0.52 (92)	10.1 (146)	68.5 (309)
	3570 (444)	96 (393)	11.0 (92)	0.47 (84)	8.0 (115)	60.4 (273)
	2525 (314)	76 (315)	11.7 (97)	0.52 (92)	10.5 (152)	43.8 (198)
	2685 (334)	82 (337)	12.1 (101)	0.41 (74)	9.7 (140)	37.4 (169)
TCDD, 4 days	2860 ± 480 (356)[c, e]	81 ± 10 (333)[c, e]	12.2 ± 1.3 (102)[e]	0.48 ± 0.05 (86)[e]	9.6 ± 1.1 (138)[c]	52.5 ± 14.4 (238)[c]
	1230 (153)	25 (103)	30.8 (256)	1.2 (214)	15.1 (219)	31.6 (143)
	1440 (179)	35 (144)	26.3 (219)	1.3 (239)	15.0 (217)	45.7 (207)
	1355 (169)	28 (114)	31.2 (260)	1.1 (193)	14.4 (208)	57.6 (260)
PB, 8 days	1260 ± 180 (157)[c, d, e]	27 ± 6 (111)[d, e]	28.5 ± 2.8 (238)[c, e]	1.2 ± 0.1 (218)[c, e]	14.0 ± 1.7 (202)[c]	45.0 ± 13.0 (204)[c]
	1050 (130)	42 (171)	15.0 (125)	0.54 (96)	8.0 (115)	14.9 (67)
	945 (118)	37 (153)	16.1 (134)	0.47 (84)	7.3 (105)	24.5 (111)
	975 (121)	41 (169)	16.3 (136)	0.61 (110)	8.0 (116)	16.8 (76)
	870 (108)	34 (141)	17.8 (148)	0.54 (96)	9.8 (141)	16.4 (74)
Dieldrin, 3 days	960 ± 75 (119)[d, e]	39 ± 4 (160)[c, d, e]	16.3 ± 1.1 (136)[c, d, e]	0.54 + 0.06 (96)[d, e]	8.3 ± 1.1 (119)	18.1 ± 4.3 (82)
	1560 (194)	47 (194)	28.8 (240)	1.4 (240)	14.6 (211)	18.3 (83)
	1510 (188)	46 (189)	24.2 (202)	0.93 (166)	8.4 (122)	19.8 (90)
	1115 (138)	37 (152)	24.5 (204)	1.0 (178)	14.0 (202)	17.8 (81)
Dieldrin, 9 days	1550 ± 360 (193)[c]	46 ± 7 (189)[c]	25.5 ± 2.2 (213)[c]	1.1 ± 0.2 (189)[c]	12.7 ± 2.8 (184)[c]	17.4 ± 2.6 (79)[c]

[a] Parameter designation as used for the mathematical analysis in Table 2
[b] Mean ± S.D.
[c] Significantly different from control ($p < 0.05$; Student's t-test)
[d] Significantly different extent of induction in microsomes and nuclei
[e] Significantly different extent of induction of AHM and EH in either microsomes or nuclei

By the use of data sets from each individual rat and with multiple regression analysis, it was possible to assign to each enzymatic activity a mathematical coefficient reflecting its influence upon DNA binding. The parenthesized figures in Table 1 were therefore analyzed according to the formula.

$$\begin{aligned}\text{DNA Binding} = \quad & a_1 \cdot \text{microsomal AHM} \\ & + a_2 \cdot \text{nuclear AHM} \\ & + a_3 \cdot \text{microsomal EH} \\ & + a_4 \cdot \text{nuclear EH} \\ & + a_5 \cdot \text{cytoplasmic GST} \\ & + a_0\end{aligned}$$

DNA binding and enzyme induction were expressed as percentages of their controls to allow a direct comparison of the coefficients without distortion by the enzymes' differing activities. The sign of each coefficient a_1 to a_5 reflects whether the corresponding enzyme activity will be proposed to have an increasing (+) or decreasing (−) effect on the DNA binding, the absolute number reveals the effectiveness of this effect.

Table 2 compiles the results of various regression analyses. The first line gives the coefficients when all parameters of the 22 rats are evaluated. It is apparent that both microsomal and nuclear AHM are strongly involved in DNA binding of BaP.

The influence of the other three parameters is not as clear. Their significance can be checked if a regression analysis is performed without the parameter under investigation. If S. E. and correlation coefficient r are worse than with the complete set of data, this will mean that this specific parameter contributes significantly to the improvement of the multiple correlation and, therefore, to the binding of BaP to DNA. From line 3 of Table 2 it is apparent that this is the case for the EH in the nuclear membrane. If the correlation is improved, the parameter under investigation deterioriates the original regression analysis. This is seen with GST in line 4 of Table 2.

When the microsomal EH is subjected to this test, the analysis reveals a slightly improved S. E. but a slightly lower r. This is a boderline case where an effect on the DNA binding cannot be clearly evaluated.

Table 2. Mathematical approach to the influence of different enzyme activities (x_i) on the covalent binding of benzo(a)pyrene to DNA (x_0). Calculated coefficients a_i, S.E., and correlation coefficient r obtained from multiple linear regression analysis of the data from 22 rats given in Table 1 to satisfy the formula

$$x_0 = a_1 \cdot x_1 + a_2 \cdot x_2 + a_3 \cdot x_3 + a_4 \cdot x_4 + a_5 \cdot x_5 + a_0$$

are tabulated. —, associated parameter excluded from the regression analysis

AHM		EH		GST cytoplasm			
ER	Nuclei	ER	Nuclei				
a_1	a_2	a_3	a_4	a_5	a_0	S.E.	r
1.49	−1.07	0.33	−0.52	0.11	58.2	40	0.880
1.45	−1.03	—	−0.23	0.18	60.2	39	0.875
1.42	−0.97	−0.04	—	0.03	55.1	40	0.870
1.51	−1.09	0.37	−0.49	—	64.1	39	0.880
1.41	−0.96	—	—	—	52.2	38	0.869

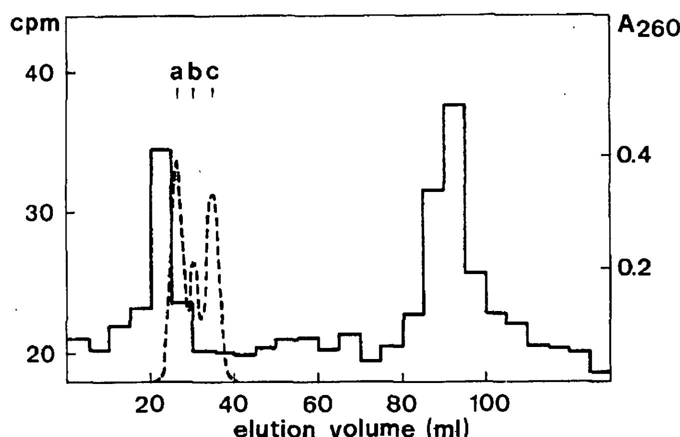

Fig. 1. Sephadex LH-20 column chromatography elution profile of enzyme-digested liver DNA from an adult male rat treated with [*G*-^{3}H]BaP (43 µg/kg, 9.0 · 10^9 dpm/kg; i.p.), 20 h before killing, without pretreatment with enzyme-inducing agents. ———, gross radioactivity, background level 20.4 ± 1.4 cpm; ----, absorbance at 260 nm. Deoxyribonucleosides eluted in the following order: *a* deoxyribopyrimidines; *b* deoxyguanosine; *c* deoxyadenosine

In a previous report (Viviani and Lutz 1978), the AHM was found to strongly influence the activation/inactivation pathways in the metabolism of BaP. If only this activity is taken for a regression analysis, there is still a good correlation (line 5 of Table 2) indicating the major importance of the AHM in the two compartments.

This analysis has shown that (*a*) the AHM in the ER is most important in the formation of DNA-binding metabolites derived from BaP, (*b*) the EH in the ER probably is not determinative in this process (if at all, its increase would raise the binding), (*c*) the outer nuclear membrane contains enzyme activities which can inactivate metabolites formed in the ER, with AHM being more effective than EH, and (*d*) there is no indication that the cytoplasmic GST can prevent the DNA from its interaction with a reactive metabolite. This supposed limited effectiveness of GST in the inactivation of BaP metabolites is probably due to its localization, on observation which was also made in vitro (Glatt and Oesch 1977).

Nucleoside Analysis of DNA to Show that the Radioactivity on DNA is Due to BaP Adducts

Many studies on Sephadex LH-20 column chromatography of enzymatically digested DNA altered by tritiated BaP in vivo revealed a fair amount of radioactivity in the early eluting fractions, co-chromatographing with unchanged deoxyribonucleosides. Only a minor part of total radioactivity was eluted in the region where less hydrophilic BaP-nucleoside adducts appear (Eastman et al. 1978; Kahl et al. 1979; Sims et al. 1974). If this early eluting radioactivity represents unchanged nucleosides, either due to non-enzymatic exchange or to biosynthetic incorporation of the label, the measurement of total radioactivity on the DNA could lead to false interpretations. We therefore performed a nucleoside analysis of a control DNA. Figure 1 shows the elution profile of a Sephadex LH-20 column of a hydrolysate of liver DNA from an animal treated with 43 µg/kg BaP (a similarly low dose as

used for the enzymatic studies). About 60% of the eluted radioactivity was found in the region where BaP-nucleoside adducts appear. Within our limit of detection, no radioactivity was found in the region of unaltered deoxyadenosine and deoxyguanosine, indicating that practically no biosynthetic incorporation of labeled BaP fragments into DNA occured and that tritium exchange was completely reversible. An appreciable amount of radioactivity eluted in front of the two purine nucleosides. There is evidence that incompletely digested oligonucleotides or other DNA-fragments of high molecular weight are present in this fraction (Eastman et al. 1978). It is conceivable that specific inhibition of the digesting enzymes occurs at the site of the lesion produced by the binding of BaP derivatives. It would then be expected that a DNA with more dense BaP-DNA adducts would give rise to a higher percentage of early eluting radioactivity. Such a tendency can indeed be observed: the higher BaP dosage used in other reports (Eastman et al. 1978; Kahl et al. 1979; King et al. 1979; Owens et al. 1979; Sims et al. 1974) was accompanied by a higher portion of radioactivity eluting with the early fractions.

Discussion

The multiple linear regression used for the analysis of our data was the simplest mathematical model available. It is possible that a more complex function would have yielded a better fit but the activation of BaP to the various DNA-binding metabolites is so diverse that we did not attempt to make further reaching assumptions. A mathematical approach is needed to elucidate direction and relative weight of the enzymes' influence on DNA binding. Our coefficients are derived from one experimental set and are only used for an indication of the order of magnitude of a certain influence. It is also to be noted that no difference can be made between endogenous and induced enzyme activities in our assays. The six-dimensional fitting to a plain can therefore not be extrapolated towards zero enzyme activity = zero binding, and the discussion of the influence of each enzymatic activity must be restricted to the range covered by this study.

Effect of Enzyme Activities on DNA Binding

In an earlier paper (Viviani and Lutz 1978) we have shown that the microsomal AHM is an important factor in the activation of BaP to DNA-binding metabolites whereas the AHM located in the outer nuclear membrane inactivates such electrophilic derivatives. The importance of the AHM is further strengthened by the present report where no indication was found supporting a major influence of an induction of the additionally determined EH and cytoplasmic GST. Pyerin and Hecker (1979) also found that mouse skin tumor initiation by 7,12-dimethylbenz(*a*)anthracene is positively correlated with the epidermal AHM activity but not with the EH activity in the target tissue. All these findings were not expected in the light of the inactivating and possibly detoxifying activity of EH and GST. Still, a minor influence of EH was concluded from our analysis and it is possible that EH is more important with carcinogens that go through simpler activation pathways than BaP or other PAH, e.g., ethylene derivatives or aflatoxins. BaP might represent a special situation in as much as EH degrades BaP-epoxides to vicinal *trans*-dihydrodiols (Jerina et al. 1977; Oesch 1976). Some of these metabolites can be fur-

ther oxidized by AHM and give rise to diolepoxides of which the anti-diolepoxide is a probable ultimate carcinogen. This three-step activation of BaP might be the reason for the ambiguous influence of EH on DNA binding. The situation is all the more delicate as this anti-diolepoxide is a very poor substrate for EH (Wood et al. 1976).

The possible significance of *conjugating* activities in the process leading to DNA binding has often been discussed (see, e.g., Benson et al. 1978; Burke et al. 1977, Jones et al. 1978; Mulder and Scholtens 1977; Smith and Bend 1979). In those reports, a decreasing influence on the binding was postulated although no in vivo data could be presented to support this hypothesis. In this paper, an increase of the cytoplasmic GST was shown not to affect the DNA binding of BaP. This lack of effectiveness of GST may be due to its subcellular localization in the cytoplasm, where this enzyme is separated from the proposed ultimate carcinogens of BaP or their precursors, which are still lipophilic and are believed to migrate within the membrane structures of the cell toward the necleus. An inducible GST activity distinct from that found in the cytoplasm has recently been established (Morgenstern et al. 1979). It is possible that this membrane-bound activity is more important in the inactivation processes with BaP.

In addition to GST, there are other enzyme activities able to conjugate BaP derivatives with hydrophilic groups. One of these, *PAPS sulfotransferase*, probably cannot either protect the DNA from binding with BaP derivatives because of its localization in the cytoplasm. Sulfate conjugates were found only with phenols, but not with diols (resulting from conjugation with epoxides) after the metabolism of BaP in the perfused rat liver or with hepatocyte (Jones et al. 1978; Smith and Bend 1979) but, interstingly, the enzyme has a high affinity for the 7,8-epoxide when tested in vitro in the absence of membrane structures (Nemoto et al. 1978). An induction of PAPS sulfotransferase has to our knowledge never been reported. Its influence on the activation/inactivation pathways leading to DNA-binding metabolites of carcinogens could nevertheless be studied by using 2,6-dichloro-4-nitrophenol, a specific inhibitor of PAPS sulfotransferase found to be applicable in vivo (Mulder and Scholtens 1977).

The membrane-bound *UDP-glucuronyl transferase* is inducible and is located in the ER and in the outer nuclear membrane (Duttón et al. 1977; Lake et al. 1976; Lucier et al. 1973; Mulder and Scholtens 1977; Wishart and Fry 1977). Its intracellular localization in hydrophobic environment might render this enzyme activity more important than the cytoplasmic enzymes discussed above. Almost all BaP metabolites can be conjugated with glucuronic acid (Nemoto and Gelboin 1976), but there are substantial quantitative differences. Best substrates are phenols, followed by the epoxides of which the 4,5-epoxide is the poorest one. The activity of this enzyme, as compared with EH[1], is about 10% with the 3-phenol or the 7,8-epoxide as substrates and 2% with the 4,5-epoxide. UDP-glucuronyl transferase activity therefore does not contribute much to the metabolism of the epoxides, a conclusion which is supported by the finding that the ratio glutathione:glucuronic acid conjugates of the 4,5-epoxide was about 10:1 in the perfused rat liver (Smith and Bend 1979). It is important to note that conjugation of BaP metabolites does not neces-

1 This is based on specific activities for the EH as measured in this study with the benzo(*a*) pyrene-4,5-epoxide as substrate and for UDP-glucuronyl transferase as determined by Nemoto and Gelboin (1975), both expressed in the same units (nmol product formed in 1 min per mg protein)

sarily lead to irreversible inactivation of reactive metabolites. It was shown recently that during the enzymatic hydrolysis of the BaP-3-glucuronide with -glucuronidase a BaP derivative was formed which bound to DNA to a far greater extent than either the 3-phenol or the glucuronide without the enzyme present (Kinoshita and Gelboin 1978). Such a conversion of conjugates to reactive and possibly carcinogenic molecules can occur in organs distant from the site of oxygenation and conjugation.

Formation and Consequences of BaP-DNA Adducts

The complex activation/inactivation processes with BaP lead to more than only one reactive metabolite. There is evidence that the anti-diolepoxide is one of these derivatives, but recent work would also support other metabolites like epoxidized phenols, primary epoxides and radicals (Glatt an Oesch 1977; Jernström et al. 1978; Kahl et al. 1979; Lesko et al. 1975; Owens et al. 1979; Rogan et al. 1978). Enzyme induction can alter the profile of reactive metabolites and may lead to different DNA adducts. Indeed, Kahl et al. (1979) found two separate DNA adducts in the perfused liver after pretreatment of rats with MC or PB and it has also been shown by Cohen et al. (1979) that pretreatment of mice with TCDD resulted in a different pattern of BaP-skin DNA adducts than found with the control animals. DNA could therefore be altered in a different manner after pretreatment of the animals and subsequent BaP exposure as compared to uninduced animals. Different reactive derivatives of BaP bind to quite different sites in DNA in vitro (Jernström et al. 1978) and the biological consequences of a DNA damage could depend on the exact site of binding (Cohen et al. 1979). Our study is based upon the measurement of total DNA adducts and therefore provides only an overall estimate of the DNA damage. Still, it was found in a number of studies that total DNA binding of a carcinogen and tumor incidence are modulated in parallel if animals are pretreated with a variety of substances (reviewed by Lutz 1979). It would therefore be tempting to use DNA binding as an early indicator for the influence of a pretreatment on tumor incidence. It cannot yet be concluded whether this correlation also holds for BaP-DNA binding under diverse conditions of enzyme induction and it would be premature to draw further reaching conclusions on tumor incidences.

It follows from the above discussion that more data are required before an influence of the induction of any enzyme activity on *tumor incidence* from a carcinogen can be predicted on the basis of DNA binding. Not only must the intracellular distribution of all enzyme activities required for the activation of the carcinogen under investigation be known in the target organ but it will also be necessary to analyze the carcinogen-DNA adducts from individual animals. Our conclusions drawn are valid for total binding of BaP to male rat liver DNA and will only then be more generally applicable when more data have revealed some unifying trends.

Abbreviations

BaP = benzo(*a*)-pyrene; *x*-phenol = *x*-hydroxybenzo(*a*)pyrene; *x,y*-epoxide = benzo(*a*)pyrene-*x,y*-epoxide; *x,y*-diol = (+)-*trans*-*x,y*-dihydroxy-*x,y*-dihydrobenzo(*a*)pyrene; anti-diolepoxide = (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; EH = epoxide hydrolase activity (other names used are epoxide hydratase and epoxide hydrase); AHM = aryl hydrocarbon monooxygenase activity; GST = glutathione *S*-epoxide transferase activity; ER = endoplasmic reticulum; PAH = polycyclic aromatic hydrocarbons; PAPS = 3′-phosphoadenosine 5′-phosphosulfate; PB = phenobarbital; SO = *trans*-stilbene oxide; TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; MC = 3-methylcholanthrene

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