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COVALENT BINDING OF BENZO[a]PYRENE TO DNA IN FISH LIVER

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SUMMARY: Benzo[a]pyrene became bound to the hepatic DNA in juvenile English sole (Parophrys vetulus) force fed tritiated benzo[a]pyrene. No statistically significant change was observed in the level of the binding from 16 h to 2 wk after the single exposure. Specific activities of binding were similar for both DNA and protein. Moreover, a binding index was calculated to represent the number of benzo[a]pyrene molecules bound per 10^6 nucleotides after administration of a theoretical dose of 1 mmole of hydrocarbon per kg body weight. The value for English sole liver DNA was of the same order of magnitude as the values reported for mouse skin and mammary gland in which benzo[a]pyrene is carcinogenic.

INTRODUCTION: Bottom-dwelling fish, such as those belonging to the pleuronectid family, can encounter high levels of PAHs in their environment (1). English sole, a pleuronectid, sampled from chemically polluted areas exhibit a high frequency of liver neoplasms (2) indicating a possible association of pollutants with tumor development (1). English sole accumulate PAHs (e.g., BaP) from diet and sediment and metabolize them extensively (3-4). A number of BaP metabolites characterized as mutagens (5) and proximate carcinogens in mammals (6) are formed in liver of BaP-exposed English sole (3-4). The important question was whether reactive metabolites of BaP formed in fish liver have a sufficiently long half-life to interact with cellular macromolecules,

Abbreviations used: PAH, polynuclear aromatic hydrocarbon; BaP, benzo[a]pyrene; DMBA, 7,12-dimethylbenz[a]anthracene; SDS, sodium dodecylsulfate; 1xSSC, 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.4; CBI, covalent binding index; BPDE, (+)-7β , 8α -dihydroxy-9 , 10α -epoxy-7,8,9,10-tetrahydroxybenzo[a]pyrene.

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especially DNA, because it is believed that chemical carcinogens induce cancer through interaction with DNA. Studies with mammals (6-11) show that the magnitude of binding of carcinogens to DNA *in vivo* correlates roughly with the carcinogenic potential of the compound. Determination of *in vivo* binding of PAHs, such as BaP, should provide valuable comparative data to assess whether the extent of binding in fish is similar to that in mammals.

In this paper we provide information on the extent of covalent binding of BaP intermediate(s) to hepatic DNA and protein in English sole exposed to orally administered BaP. The results show that the extent of binding of BaP to DNA in fish liver was comparable to values reported for mammalian tissues susceptible to PAH-induced neoplasms and that the chemical modification of the hepatic DNA was observed for at least 2 wk after a single exposure to BaP.

MATERIALS AND METHODS: Sodium p-aminosalicylate, deproteinized salmon sperm DNA, SDS, ribonuclease-A type 1-A, and protease type V were purchased from Sigma Chemical Co., St. Louis, Missouri. The ribonuclease was dissolved in water (1 mg/ml) and heated at 80-85°C for 15 min to inactivate any contaminating deoxyribonucleases; the protease was also dissolved in water (1 mg/ml), self-digested for 1 h at 37°C, heated for 2 min at 80°C then quenched in ice. The ribonuclease and protease solutions were stored at -20°C. Generally labeled [^{3}H]-BaP was purchased from Amersham-Searle, Arlington Heights, Illinois. The [^{3}H]-BaP was purified as described previously (12). The m-cresol was distilled under reduced pressure. All other chemicals were of reagent or analytical grade and used without further purification.

Animals: English sole (118±19g), obtained by trawling from Puget Sound, were kept in flowing seawater at 11°C and fed a diet of minced clams for three weeks. The feeding was stopped two days prior to starting the experiment and resumed 48 h after initiation of exposure, with the fish being fed every other day. The fish were force-fed gelatin capsules (no. 3) containing 0.69 mCi of [^{3}H]-BaP (sp act 17.4 Ci/mmole) dissolved in 50 µl of corn oil. The average dose of BaP was 88±12 µg/kg body weight. Fish were sacrificed by a blow to the head at 16, 24, 96, 168 and 336 h after being fed BaP; the liver was removed and stored at -65°C.

Radioactivity in Liver: The concentration of radioactivity in liver was determined from a sample (0.1g) of liver solubilized in 1 ml of Soluene-350 (Packard Instrument Co.) and decolorized with 200 µl of both isopropanol and 30% hydrogen peroxide; 15 ml of Dimilume-30 (Packard Instrument Co.) was added and the radioactivity measured using a Packard 300C liquid scintillation spectrometer. The distribution of radioactivity

in liver as unconverted BaP, organic solvent-soluble BaP metabolites, and water-soluble BaP metabolites was determined by homogenization of liver and ethyl acetate extraction, followed by thin-layer chromatography of the organic phase (4). A 200 µl aliquot of the liver homogenate was diluted to 1 ml with water, and an aliquot removed for measurement of radioactivity. The remaining aqueous solution was mixed with 1 volume of N_2-saturated acetone, then extracted twice with 1 volume N_2-saturated ethyl acetate. The organic extract was dried over Na_2SO_4, an aliquot removed and radioactivity measured. The remaining extract was removed by suction, evaporated to dryness under a stream of nitrogen, then dissolved in 50-100 µl of ethyl acetate containing BaP and its oxygenated standards. The organic extract with added standards was spotted on the pre-adsorbent layer of a channeled thin-layer chromatographic plate (Whatman No. LK5D, Whatman Inc., Clifton, N.J.) and developed with hexane: ether (95:5, v/v). The adsorbent from the thin-layer chromatographic plate was removed in three fractions. The first fraction contained BaP metabolites (R_f = 0.1) and the pre-adsorbent pad; the second fraction was the adsorbent between R_f of 0.1 and 0.5. The third fraction of adsorbent contained unmetabolized BaP (R_f = 0.5). The radioactivity was measured in each fraction. All of these operations were performed under red light to minimize photooxidation.

Isolation of Liver DNA: Liver DNA was isolated according to parts of the methods by Kirby (13), Kirby and Cook (14) and Marmur (15): Liver tissue (1-2g) was minced in 5 volumes of sodium p-aminosalicylate, sec-butanol, tetrasodium EDTA dihydrate, water (1:1:0.35:143 w/w/w/v) adjusted to pH 8 with hydrochloric acid. SDS was added to a final concentration of 1% just prior to homogenization in a loose-fitting Teflon-glass homogenizer. The homogenization was performed at 0°C. The homogenate was gently shaken with an equal volume of phenol reagent (phenol, m-cresol, 8-hydroxyquinoline, water 100:14:0.1:11 w/w/w/w) for 45 min at 40°C. After centrifugation for 40 min at 4°C and 10,000xg the aqueous phase was removed by suction. The phenol reagent fraction was re-extracted with 0.5 volume of the sodium p-aminosalicylate mixture. NaCl was added to the combined aqueous phases to a final concentration of 3% (w/v) and shaken with 0.5 volume of phenol reagent for 10 min and centrifuged as described above. The phenol reagent fractions were combined and stored at -20°C for later protein isolation. The viscous aqueous phase was shaken for 5 min with 1 volume of chloroform-isoamyl alcohol (24:1 v/v) and centrifuged at 3000xg for 5 min. Crude DNA was precipitated by addition of 2 volumes of 2-ethoxyethanol (-20°C) and spooled onto a glass rod. The nucleic acids were dissolved overnight at 4°C in 5-10 ml of 0.1xSSC, and after dissolution the solution was adjusted to 1xSSC by adding 10xSSC. RNA contaminants were degraded by a 1 h incubation at 37°C with ribonuclease solution (0.1 ml, 1 mg/ml). Protein contaminants were removed by addition of protease (0.1 ml, 1 mg/ml) and 0.25 volumes of 20% SDS to bring the solution to 0.5% SDS, followed by overnight incubation at 37°C. SDS was added to a final concentration of 1%, and the DNA solution was extracted with 1 volume of chloroform-isoamyl alcohol; the DNA was precipitated with 2 volumes of ethanol (-20°C). The DNA was washed twice with ethanol, once with ether and dryed under a stream of N_2. The concentration of DNA, dissolved in 2 ml of deionized distilled water, was estimated from the absorbance at 260 nm using an extinction coefficient of 22.9 ml/mg-cm, which was determined for salmon sperm DNA. DNA isolated by this procedure had A_{260}:A_{280} and A_{260}:A_{230} values of 1.82±0.03 and 2.2±0.2, respectively (10). Prior to liquid scintillation counting the DNA solution was made 0.5N in perchloric acid using concentrated perchloric

TABLE 1. Distribution of BaP as parent hydrocarbon or metabolites and extent of covalent binding of metabolites to DNA and protein in liver of English sole after exposure to orally administered ^{3}H-BaP.

Exposure, h	% Administered Dose	Unconverted BaP	Organic Soluble Metabolites	Water Soluble Metabolites	Specific[a] Activity (Protein)	Specific[a] Activity	CBI[b]	Binding[c] Values
		% of Total Radioactivity[d]				DNA		
	(n=4)[e]				(n=3)	(n=3)	(n=3)	(n=3)
16	0.6±0.1[f]	0.8 1.2	16 15	83 84	101±28[f]	51±30[f]	41±25[f]	16± 9[f]
24	0.8±0.2	1.1 0.3	9.5 12	89 88	90±24	51±31	45±26	16±10
96	0.4±0.1	0.2 0.7	4.1 6.4	96 93	56±39	42±15	46±18	13± 5
168	0.2±0.1	0.4 0.7	4.8 7.6	95 92	14± 2	25±18	22±16	8± 6
336	0.3±0.2	0.7 0.4	11 4.3	86 95	31±18	50±32	46±28	16±10

[a] fmole BaP equivalents/mg DNA or protein.
[b] CBI_{DNA} = [(dpm/mgDNA)/(dpm administered/kg fish)]/3.24x10^{-9}=(μmole BP equivalents/ mole nucleotides)/(mmole BP administered/kg fish); using 309g DNA=1 mole of nucleotides.
[c] nmole BaP equivalents/mole nucleotides.
[d] Since only two fish were analyzed, individual values are given.
[e] Number of fish analyzed at each time point.
[f] Mean ± standard error.

acid and heated for 20 min at 100°C. Insta-gel (Packard Instrument Co.) was added and the radioactivity was measured.

Isolation of Liver Protein: The phenol reagent phases were treated essentially according to method (b) of Poland and Glover (16), except the protein was precipitated by the addition of two volumes of acetone followed by the use of 10% SDS in both dissolution-precipitation steps. The final precipitate was washed, in centrifuge tubes, twice with acetone, once with ether and dryed under a stream of nitrogen. An aliquot (20 mg) was first dissolved in 1 ml of Soluene-350 then 15 ml of Dimilume-30 was added and the radioactivity measured.

Statistical Analysis: All data were analyzed by one-way analysis of variance. The average of all time periods for specific activities for protein and DNA were tested for difference using Students t-test and the Mann-Whitney test, at a significance level of 0.05.

RESULTS: Radioactivity in liver represented 0.6% of the administered dose at 16 h (Table 1). The concentration of BaP-derived radioactivity in liver of English sole did not change significantly from 16 h through 336 h after the oral administration of BaP; greater than 98% of the total radioactivity in liver was in the form of the organic solvent-

and water-soluble metabolites (Table 1). Individual metabolites in the organic or aqueous phases were not characterized in this study because detailed characterization of nonconjugated and conjugated metabolites in English sole exposed to BaP via both diet or sediment has been published earlier (3-4).

The binding data (Table 1) for DNA are given as specific activities (fmole BaP equivalents/mg macromolecule), binding values (nmole BaP equivalent/mole of nucleotide) and covalent binding indices (μmole BaP/mole of nucleotides)/(mmole BaP administered/kg body weight), to permit comparison with published data for mammals where the data are usually expressed in one of these three modes.

Data in Table 1 show that detectable binding of BaP intermediates to DNA in liver was observed from 16 to 336 h after BaP-exposure. In fact, no statistically significant change was observed in the binding values for DNA during the 2 wk exposure (Table 1). As with DNA, the extent of binding of BaP to liver protein did not change significantly over the 2 wk period (Table 1). Moreover, the average of all the specific activity values for protein, 58±51, was not significantly different from the average value for DNA, 44±39; the values are expressed as the mean ± SD calculated from individual values for 15 fish analyzed during the entire experiment.

DISCUSSION: The results demonstrate for the first time the *in vivo* binding of BaP intermediate(s) to hepatic macromolecules, DNA and protein, in fish. Interaction of activated carcinogens with macromolecules, especially DNA, is strongly implicated in initiation of malignant lesions (6-11). The present findings showing chemical modification of liver DNA in English sole exposed to BaP is of considerable importance since these fish do encounter high levels of BaP and other carcinogenic PAHs (1-10 ppm) in their environment (1) and they are susceptible to liver neoplasia (2).

In a separate experiment (data to be published) fish were exposed simultaneously to [^{14}C] and [^{3}H]-labeled BaP via an intraperitoneal injection. The binding values for DNA were similar for both [^{14}C] and [^{3}H]-BaP indicating that the radioactivity associated with DNA using tritiated BaP was due to chemical modification of DNA by BaP intermediates and not due to tritium exchange with DNA. Viviani et al. (17) performed a nucleoside analysis of enzymatically digested liver DNA from a rat fed [^{3}H]-BaP, and found no tritium associated with unaltered deoxyadenosine or deoxyguanosine indicating practically no biosynthetic incorporation of labeled BaP fragments into DNA.

The binding potency expressed as CBI which shows how many molecules of a PAH are bound covalently per 10^6 DNA nucleotides after administration of a single dose of 1 mmole/kg body weight allows comparison of our data with those reported for mammalian tissues although the dose of PAH administered is different in various studies. Based on a compilation of data from studies with mammals, Lutz (10) calculated a CBI of 56 for mammary gland and 4 for liver of rats at 16 h after a single feeding of DMBA (18); a CBI of 24 for skin and 7 for liver were obtained when mice were exposed to BaP via oral route (19). Both skin and mammary gland are susceptible to PAH-induced neoplasia (7-11). DMBA binds to a greater extent to DNA from liver (CBI=37) of partially hepatectomized rats (20) than to the DNA from intact adult liver (CBI=4); rapidly proliferating liver tissue of partially hepatectomized rodents is susceptible to DMBA-induced neoplasms (21) while intact liver of rat is not. In the present study the average value for CBI (Table 1) at any time during the 2 wk was in the range of values for mammalian tissues susceptible to PAH-induced neoplasms. Thus, if a correlation between covalent binding of a PAH with DNA and the susceptibility of a tissue to the carcinogen is valid, then our results suggest that BaP is a potential carcinogen for English sole liver.

For at least 2 wk after the single exposure to BaP, DNA in sole liver remained chemically modified at a high level. Evidence from studies with mammals suggests that in addition to the magnitude of binding, the persistence of chemical modification of DNA in target tissues also provides information on susceptibility of the tissue to neoplastic lesions (22-25). Marguardt et al. (25) reported that DMBA-DNA complex in regenerating rat liver persists for at least 4 wk after the administration of DMBA, while such persistent binding was not observed in intact liver. Janss et al. (26) observed that DMBA-DNA complex within rat mammary parenchymal cells was maintained at a relatively constant level from 3 to 14 days after intragastric administration of the PAH. The persistence or maintenance of a PAH-DNA complex might be due to either a slow repair mechanism or the slow release of PAH or its primary metabolites from intracellular lipid (27), coupled with subsequent activation. Regardless of the cause of the persistence of binding, the observation that DNA in sole liver remained modified for a long period is important, because this interaction may adversely influence a number of critical events such as replication (28) and transcription (29). Stromberg et al. (30) observed significant chromosomal damage, indicated by increased frequency of sister chromatid exchange rates, in kidney cells isolated from BaP-exposed English sole.

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REFERENCES

1. Malins, D.C., McCain, B.B., Brown, D.W., Sparks, A.K. and Hodgins, H.O. (1980) NOAA Tech. Memo. OMPA-2, pp 1-295.
2. Pierce, K.V., McCain, B.B. and Wellings, S.R. (1978) J. Natl. Cancer Inst., 60, 1445-1453.
3. Varanasi, U. and Gmur, D.J. (1981) Chemical Analysis and Biological Fate: Polynuclear Aromatic Hydrocarbons (Cooke, M. and Dennis, A.J. eds), pp 367-376, Battelle Press, Columbus.
4. Varanasi, U. and Gmur, D.J. (1981) Aquatic Toxicol., 1, 49-67.

5. Nagao, M., and Sugimara, T. (1978) Polycyclic Hydrocarbons and Cancer, Vol. 2 (Gelboin, H.V., and Ts'O, P.O.P., eds), pp. 99-121, Academic Press, New York.
6. Kapitulnik, J., Levin, W., Conney, A.H., Yagi, H. and Jerina, D.M. (1977) Nature, 266, 378-380.
7. Brooks, P. and Lawley, P.D. (1964) Nature, 202, 781-784.
8. Miller, E.C. and Miller, J.A. (1974) The Molecular Biology of Cancer (Busch H, ed.), pp. 377-402, Academic Press, London.
9. Huberman, E. and Sachs, L. (1977) Int. J. Cancer, 19, 122-127.
10. Lutz, W.K. (1979) Mutation Res., 65, 289-356.
11. Phillips, D.H., Grover, P.L., and Sims, P. (1979) Int. J. Cancer, 23, 201-208.
12. Varanasi, U. and Gmur, D.J. (1980) Biochem. Pharmacol., 29, 753-761.
13. Kirby, K.S. (1965) Biochem. J., 96, 266-269.
14. Kirby, K.S. and Cook, E.A. (1967) Biochem. J., 104, 254-257.
15. Marmur, J. (1961) J. Mol. Biol., 3, 208-218.
16. Poland, A. and Glover, E. (1979) Cancer Res., 39, 3341-3344.
17. Viviani, A., von Daniken, A., Schlatter, Ch. and Lutz, W.K. (1980) J. Cancer Res. Clin. Oncol., 98, 139-152.
18. Janss, D.H. and Ben, T.L. (1978) J. Natl. Cancer Inst., 60, 173-177.
19. Suss, R. and Maurer, H.R. (1968) Nature, 217, 752-753.
20. Marquardt, H.A., Bendick, A., Philips, F.S. and Hoffmann (1971) Chem.-Biol. Interact., 3, 1-11.
21. Marquardt, H., Sternberg, S.S. and Philips, F.S. (1970) Chem.-Biol. Interact., 2, 401-403.
22. Lo, K. and Kakunaga, T. (1981) Biochem. Biophys. Res. Commun., 99, 820-829.
23. Margison, G.P. and Kleihaus, P. (1975) Biochem J., 148, 521-525.
24. Kleihaus, P. and Margison, G.P. (1974) J. Natl. Cancer Inst., 53, 1839-1841.
25. Marquardt, H.A., Bendick, A. and Philips, F.S. (1971) Proc. Am. Assoc. Cancer Res., 12, 15.
26. Janss, D.H., Moon R.C. and Irving, C.C. (1972) Cancer Res., 32, 254-258.
27. Wills, E.D. (1980) Microsomes, Drug Oxidations, and Chemical Carcinogenesis(Coon, M.J., Conney, A.H., Estabrook, R.W., Gelboin, H.V., Gillette, J.R. and O'Brien, P.J. eds), pp 545-548, Academic Press, New York.
28. Hsu, W.T., Harvey, R.G., Lin, E.J. and Weiss, S.B. (1977) Proc. Natl. Acad. Sci. U.S.A., 74, 1378-1382.
29. Leffler, S., Pulkrabek, P., Grunberger, D. and Weinstein, I.B. (1977) Biochemistry, 16, 3133-3136.
30. Stromberg, P.T., Landolt, M.L. and Kocan, R.M. (1981) NOAA Tech Memo. OMPA-10, pp 1-43.