Binding of Polycyclic Hydrocarbons to Rat Mammary Gland Cellular Macromolecules *in Vivo*[1] (35996)

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Cellular macromolecules have been generally considered as the targets of carcinogens since only these macromolecules seem capable of storing, replicating, and transferring information required in cell propagation and growth. A specific reaction between the cellular macromolecules and a carcinogen seems to be an essential step in the induction of a neoplasm. Binding of carcinogens to nucleic acids and protein has been extensively studied in the skin and the liver (1–4), but the nature of the receptor macromolecule(s) involved in this process is unknown. Chemical carcinogenesis in the mammary gland has been widely investigated during the last two decades (5), but data concerning the specific binding of chemical carcinogens to mammary gland tissue are not available. Although binding of 7, 12-dimethylbenz[*a*]anthracene (DMBA) to rat mammary gland DNA was reported recently (6), detailed data was not presented. We have studied the *in vivo* binding of DMBA to the DNA, RNA, and protein of rat mammary gland, as well as the nature of these bindings.

Materials and Methods. The inguinal mammary glands from 60- to 70-day-old, female Sprague-Dawley rats (from the Holtzman Co., Madison, Wisconsin) were used in all experiments. Rats were fed on a conventional ration (Rockland diet) and given water *ad libitum*. Binding studies were carried out with 3 polycyclic aromatic hydrocarbons: DMBA, a carcinogen; and two noncarcinogens, 1,2-benz[*a*]anthracene, and anthracene. Olive oil (0.25 ml) containing 1 mg of either DMBA, 1,2-benz[*a*]anthracene, or anthracene and 2 mCi of their respective ^3H hydrocarbons was injected directly into both right and left inguinal mammary glands of female rats. The rats were killed 20 hr later and both inguinal mammary glands were removed, dissected free of the lymph nodes, and then rinsed with 0.85% NACl solution. For each experiment, mammary glands from 5 to 8 rats were pooled. The glands were homogenized in a 0.25 *M* sucrose containing 0.05 *M* Tris HCl buffer (pH 7.4), 0.008 *M* $MgCl_2$, 0.01 *M* KCl, and 0.3 m*M* $CaCl_2$ in a Virtis homogenizer for 1 min and then for 3 min in a glass homogenizer fitted with a Teflon pestle. The homogenate was filtered through cheesecloth and the filtrate separated by differential centrifugation (7) into 3 fractions: cytoplasmic, microsomal, and nuclear. The cytoplasmic and nuclear preparations underwent further fractionation and each of these subfractions was examined for its capacity to bind carcinogens (Table I). The subfractions from the cytoplasm were prepared by the phenol extraction method of Muramatsu *et al.* (8). The preparation of the nuclear-soluble (nucleoplasm) and -insoluble (nucleoprotein and nucleolus) subfractions involved osmotic shock treatment, homogenization, and centrifugation, followed by phenol extraction to obtain the soluble nuclear fraction. The insoluble nuclear fraction, containing mainly DNA and protein, was separated into DNA and DNA-associated protein by the method of Marmur (9), modified slightly by addition of 0.1% sodium lauryl sulfate in the first deproteinization step.

All of the protein fractions were further purified by passing them through a short column (0.5 × 7 cm) of Sephadex G-75, followed by incubation with pancreatic

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TABLE I. Binding of Polycyclic Hydrocarbons to Different Fractions of Cellular Macromolecules of Rat Mammary Gland.

Data from two experiments.

Cellular macromolecule fraction	Amount of binding (pmole/mg)[a]		
	DMBA	Anthracene	1,2-Benzanthracene
DNA	12.1; 10.1	86.4; 125.0	11.2; 11.6
DAP[b]	62.4; 48.0	93.1; 97.6	18.9; 19.4
NRNA	11.7	5.1; 2.3	4.7; 3.0
NSP	13.0	39.7; 51.6	3.9; 9.4
CRNA	23.4; 22.0	57.0; 36.4	7.1; 4.5
CSP	202.0; 257.4	86.3; 25.6	3.1; 12.0
CIP	99.5; 92.8	51.1; 63.0	24.1; 7.3
rRNA	14.4; 12.1	3.6; 13.6	3.5
rSP	28.8; 29.2	29.5; 25.6	5.6; 5.6
rIP	11.6	54.5; 31.2	13.7; 6.5

[a] Protein was determined by the method of Lowry *et al.* (15) using bovine serum albumin as standard.

[b] DAP, DNA associated protein; NRNA, nucleoplasmic RNA; NSP, nucleoplasmic soluble protein; CRNA, cytoplasmic RNA (mainly tRNA); CSP, cytoplasmic soluble protein; CIP, cytoplasmic insoluble protein; rRNA, ribosomal RNA; rSP, ribosomal soluble protein; rIP, ribosomal insoluble protein.

RNase (in phosphate buffer (pH 7.4), 50 μg/mg of protein) at 37° for 2 hr, and then repeating the Sephadex column purification step. The RNA and DNA fractions obtained usually contained small amounts of protein after the final step of deproteinization. They were treated with pronase (in Tris buffer (pH 7.8), 50 μg/mg of RNA or DNA) at 37° for 16 hr and were passed through the Sephadex G-75 column.

DNA was determined quantitatively by the diphenylamine method (10), modified by adding 0.025 *M* $CdCl_2$ to the reaction mixture to increase the color intensity and to shorten the reaction time to 6 hr. RNA was determined by measuring the OD of the acid hydrolysate at 260 mμ. The DeDeken-Grenson and DeDeken (11) values for yeast RNA were used to calculate the specific activity of RNA. The radioactivity in each cellular component was estimated by dissolving 0.1 to 0.2 ml of the solution in 10 ml of scintillation fluid (1 liter contains 80 g of naphthalene; 4 g of 2,5-diphenyloxazol (PPO); 50 mg of 1,4-bis-2(5-phenyloxazolyl) benzene (POPOP); 400 ml of ethylene glycol monomethyl ether; and 600 ml of toluene) and was counted in a Nuclear Chicago liquid scintillation counter in the usual manner.

Results and Discussion. Under the experimental conditions described, cross contamination of the three classes of cellular constituents was not detected. The results in Table I show that the amount of DMBA (or its metabolites) bound to the cytoplasmic soluble protein was remarkably high in comparison to other cellular constituents. DMBA is bound minimally to RNA fractions as well as nuclear and ribosomal proteins. In contrast, the noncarcinogen anthracene is bound to a greater extent to DNA than to the other cellular constituents. Of the three polycyclic hydrocarbons studied, 1,2-benz[*a*]anthracene shows the least binding capacity to the cellular macromolecules.

Since the nature of the binding rather than the amount of the hydrocarbon bound may be importantly involved in its carcinogenicity, studies were carried out to estimate the amount of radioactivity in the isolated samples that was extractable by benzene under different conditions. The results are summarized in Table II. As shown, about 6% of DMBA bound to DNA occurred by hydrophobic or other noncovalent binding and 14% (20% when corrected for the 6% due to hydrophobic binding) was probably buried in the macromolecule matrix and became soluble after digestion with DNase. Apparently 80% of the DMBA which was bound was due to covalent binding; this fraction was resistant to benzene extraction either after DNase digestion or after heating to 80%. In contrast, about 80% of anthracene is bound to DNA by means of intercalation, and the remaining was hydrophobic (12%) and covalent (8%) binding. There is no significant difference in binding to the protein between DMBA and anthracene. About 80% of these bonds are covalent in nature and are resistant to benzene extraction even after digestion with pronase.

Most of the radioactive anthracene found

TABLE II. Polycyclic Hydrocarbon-Bound DNA and Protein Extracted by Benzene under Different Conditions.

Sample	% of radioactivity extracted by benzene			
	At 37°[a]	After pronase treatment[b]	After DNase digestion[c]	After heating to 80°[d]
DNA-DMBA-^{3}H	6	—	20	18
DNA-anthracene-^{3}H	12	—	80	81
CSP-DMBA-^{3}H	21	24.8	—	—
CSP-anthracene-^{3}H	28	28	—	—

[a] 0.1 ml of sample was mixed with 2 ml of benzene at room temperature and shaken at 37° for 10 min. The mixture was centrifuged at 2000*g* for 10 min, and the benzene phase was added to 10 ml of scintillation fluid.

[b] The protein fraction was incubated with pronase (50 μg/mg of protein) pH 7.8 at 37° overnight and followed by benzene extraction.

[c] The DNA-hydrocarbon preparations were digested with DNase, pH 9.0 at 37° overnight.

[d] The DNA-hydrocarbon preparations were heated to 80° for 10 min then followed by hot benzene extraction (boiling) for 5 min.

in the DNA dissolved in benzene when the DNA was heated to 80° suggesting that anthracene is bound to DNA by means of intercalation. The low intercalation ability of DMBA may explain why the total amount of DMBA bound to DNA was much less than that of the anthracene. This is consistent with the fact that the anthracene molecule is planar with a thickness of 3.7 Å, while DMBA molecule is nonplanar with a thickness of 4.95 Å (12).

It can be concluded that carcinogen DMBA and noncarcinogen anthracene differ markedly in the nature of their binding to the DNA. The magnitude of binding of DMBA to DNA, however, is significantly smaller than that to cytoplasmic soluble protein. Whether the preferential binding of DMBA to cytoplasmic protein is related to the process of carcinogenesis is not known. Findings in our present investigation are in agreement with previous work on skin carcinogenesis by Heidelberger (13) and by Goshman and Heidelberger (14), that carcinogenic polycyclic hydrocarbons preferentially bind to cytoplasmic protein than to either DNA or RNA.

Summary. Binding of polycyclic aromatic hydrocarbons to cellular macromolecules of the rat mammary gland *in vivo* was studied. The carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) binds covalently to both DNA and cytoplasmic soluble protein. The binding of DMBA to cytoplasmic protein, however, is significantly greater than to DNA. Binding of DMBA to other macromolecular fractions is minimal. The noncarcinogen anthracene binds covalently to protein but forms a hydrophobic bond with DNA.

1. Heidelberger, C., and Moldenhauer, M. G., Cancer Res. **16**, 442 (1956).

2. Brooks, P., and Lawley, P. D., Nature (London) **202**, 781 (1962).

3. Miller, E. C., and Miller, J. A., Cancer Res. **7**, 468 (1947).

4. Willard, R. F., and Irving, C. C., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **23**, 167 (1964).

5. Dao, T. L., Progr. Exp. Tumor Res. **11**, 235 (1968).

6. Janss, D. H., Moon, R. C., and Irving, C. C., Proc. Amer. Ass. Cancer Res. **12**, 64 (1971).

7. Shibko, C. A., Koivistoinen, P., Tratayek, C. A., Newhall, A. R., and Friedman, L., Anal. Biochem. **19**, 514 (1967).

8. Maramatsu, M., Hodnett, J. L., and Busch, H., J. Biol. Chem. **241**, 1544 (1966).

9. Marmur, J., J. Mol. Biol. **3**, 208 (1961).

10. Burton, K., Biochem. J. **62**, 315 (1956).

11. DeDeken-Grenson, M., and DeDeken, R. H., Biochim. Biophys. Acta **31**, 195 (1959).

12. Sayre, D., and Friendlander, P. H., Nature (London) **187**, 139 (1960).

13. Heidelberger, C., Cancer Res. **30**, 1949 (1970).

14. Boshman, L. M., and Heidelberger, C., Cancer Res. **27**, 1678 (1967).

15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

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