3-METHYLCHOLANTHRENE-INDUCIBLE BINDING OF AROMATIC HYDROCARBONS TO DNA IN PURIFIED RAT LIVER NUCLEI

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Summary: Purified rat liver nuclei contain enzymes capable of activating polycyclic aromatic hydrocarbons which can then bind to nuclear DNA. This has been demonstrated by incubating nuclei with [^{14}C] hydrocarbon and purifying the nuclear DNA containing bound hydrocarbon. Intraperitoneal injection of the rats with 3-methylcholanthrene twenty-four hours before livers were removed raised the level of hydrocarbon binding to nuclear DNA four-fold. Omission of reduced nicotinamide adenine dinucleotide phosphate from incubation mixtures caused a four-fold decrease in the ability of 3-methylcholanthrene induced nuclei to bind hydrocarbon to nuclear DNA. The most potent carcinogens in a series of seven hydrocarbons displayed a relatively higher extent of binding to nuclear DNA. The data suggest that nuclear aryl hydroxylases effect covalent binding of hydrocarbons to DNA and presumably initiate the cancer process.

Microsomal hydroxylases have been shown to catalyze the formation of a covalent bond between polycyclic aromatic hydrocarbons and DNA (1,2), and the extent of binding of these compounds has been reported to correlate with the carcinogenic activity of the hydrocarbon (3-6). In particular, Kinoshita and Gelboin found a relationship in mouse skin between tumorigenesis and the degree of binding of hydrocarbons to DNA investigated under the same conditions (7).

Induction of BP[1] hydroxylase in rat liver microsomes by MCA

[1]Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide; DMBA, 7,12-dimethylbenz[a]anthracene; MCA, 3-methylcholanthrene; BP, benzo[a]pyrene; MBA, 7-methylbenz[a]anthracene; DBA, dibenz[a,h]anthracene; BA, benz[a]anthracene; A, anthracene; SSC, standard saline citrate.

and dependence of the reaction on NADPH was reported by Conney et al (8). Because hydroxylases requiring NADPH are mainly located in the endoplasmic reticulum of mammalian cells and covalent binding of hydrocarbons to DNA can be catalyzed in vitro in the presence of rat liver microsomes (1,2), it is generally accepted that microsomal enzymes induce the reactive species of hydrocarbons capable of binding to cellular constituents. However, aryl hydrocarbon hydroxylases recently have been shown to be induced by MCA ten to fifteen-fold in both rat liver microsomes and nuclei (9). We report here the MCA inducible capacity of purified rat liver nuclei to bind hydrocarbon to nuclear DNA in vitro and suggest that nuclear hydroxylases might be significant in the primary process of tumor induction.

Materials and Methods: Weanling (3 week old) male Sprague-Dawley rats (Sasco Inc., Omaha, Neb. or the Eppley Colony) were used. [^{14}C]DMBA, [^{14}C]BP, [^{14}C]BA, and [^{14}C]A were purchased from Amersham/Searle; [^{14}C]DBA was purchased from Mallinckrodt, and [^{14}C]MCA was purchased from New England Nuclear, while [^{14}C]MBA was prepared in our laboratory. Specific radioactivities ranged from 5-32 mCi/mmole. NADPH was obtained from Sigma Chemical Co.

When indicated, animals were injected intraperitoneally with 25 mg MCA per kg body weight 24 hours before killing. Animals were starved 20 hr before cervical dislocation and immediate removal of the liver. Nuclei were purified from rat liver according to Berezney, et al (10). Purity and integrity of nuclei were routinely established by electron microscopy. Concentration of nuclei in terms of protein was determined by the Lowry method (11).

To assay binding of hydrocarbon to nuclear DNA, fresh nuclei (3 mg protein) were incubated for 10' at 37° in 1 ml containing

0.05 M Tris-HCl, pH 7.5, and 0.2 mM [^{14}C]hydrocarbon. NADPH (0.5 μmole) was added after 1' of incubation. The reaction was stopped by addition of 0.1 ml 20 x SSC and 0.1 ml 10% sodium dodecyl sulfate. Purification of nuclear DNA was continued according to Church and McCarthy (12). The above mixture was shaken 10'; 1.2 ml water-saturated phenol, pH 8.0, was added and the mixture was shaken 10' and then centrifuged at 4° for 10', at 1000 x G. The aqueous layer was removed, made 1 M in $NaClO_4$, extracted twice by shaking with an equal volume of chloroform: octanol (10:1) and centrifuging as above. To the aqueous layer was added two volumes of ethanol, and DNA was wound out on a glass rod and washed with 95% ethanol. DNA was redissolved in 1 ml 0.1 x SSC, made 1 x SSC, precipitated again with ethanol, washed, and finally dissolved in 1 ml 0.1 x SSC. DNA was determined by absorbance at 259 nm ($\varepsilon = 6.6 \times 10^3$) and by the diphenylamine reaction (13). Bound [^{14}C]hydrocarbon was counted in Aquasol (New England Nuclear) with about 90% efficiency.

Purified rat liver microsomes with known aryl hydroxylase activity were a kind gift of Dr. Carter Grandjean.

Results and Discussion: Previous work has demonstrated that hydrocarbons require activation either by chemical or enzymic means to bind covalently to DNA (1,2,14). The ability of liver nuclei from rats induced with MCA to bind chemically a series of aromatic hydrocarbons to nuclear DNA is shown in Table I, indicating that hydrocarbons are activated and bound to nuclear DNA by nuclear enzymes just as microsomal hydroxylase catalyzes the binding of hydrocarbons to added DNA (1,2). While the relative levels of binding of the hydrocarbons in Table I do not agree exactly with those previously reported for a number of the same

Table I

Binding of Hydrocarbons to Nuclear DNA After Induction by MCA

[^{14}C]hydrocarbon	pmole hydrocarbon/μmole DNA	Iball's index of carcinogenic potency on mouse skin (15)
DMBA	14.5 ± 5.5	151
-NADPH	4.4 ± 3.0	
MCA	7.6 ± 3.0	101
BP	8.4 ± 5.2	80
-NADPH	2.4 ± 1.6	
MBA	5.4 ± 2.3	45
DBA	4.9 ± 0.5	26
BA	5.0 ± 3.2	-
A	2.5 ± 1.2	-

Binding of [^{14}C]hydrocarbons was measured in at least five preparations of nuclei. Nuclei (3 mg protein), 0.2 mM [^{14}C]hydrocarbon, and 0.5 mM NADPH (unless omitted as indicated) in 1 ml of 0.05 M Tris-HCl, pH 7.5, were incubated for 10' at 37° (NADPH was added after 1' of incubation). DNA was purified as described.

Table II

Levels of Binding Activity in Uninduced and MCA-induced Rat Liver Nuclei

[^{14}C]hydrocarbon	pmoles hydrocarbon bound/μmole nuclear DNA Nuclei from uninduced liver	induced liver
DMBA	3.8 ± 3.6	14.5 ± 5.5
BP	2.2 ± 0.9	8.4 ± 5.2

Binding of [^{14}C]hydrocarbon was measured as described in Table I.

hydrocarbons (4,6), the same general phenomenon of higher levels of binding with stronger carcinogens occurred. The binding we report can be attributed to nuclear aryl hydroxylase, recently reported inducible 15-fold in these rats by MCA (9). We saw (Table II) only a four-fold increase of binding with MCA induction, but this probably is not a true measure of hydroxylase activity in the nuclei. Binding of hydrocarbon to DNA is presumably influenced by permeability of nuclear membrane to the hydrocarbon, proximity of activated hydrocarbon to an appropriate binding site on the DNA, and steric hindrance to binding from other chromosomal material. These factors may reduce the level of hydroxylase activity indicated by binding when compared with activity measured by the benzpyrene hydroxylase assay (16).

Saturation curves for each [^{14}C]hydrocarbon indicated that hydrocarbon was in excess in the incubation mixtures. The chemical nature of the bond between hydrocarbon and DNA was demonstrated in several ways, especially the consistent level of bound hydrocarbon in samples of DNA precipitated repeatedly with ethanol. We were unable to detect protein in DNA purified from nuclei after incubation with hydrocarbon. The possibility of hydrocarbon actually binding to contaminating RNA instead of DNA was eliminated by the data in Table III, which indicate that neither the amount of DNA nor the level of binding of hydrocarbon to DNA was diminished by treatment with ribonuclease. Omission of NADPH from the incubation mixture resulted in a four-fold decrease in the level of binding of DMBA and BP to DNA (Table I). The residual activity when NADPH was not added is attributed to the presence of endogenous NADPH in the nuclei. These results suggest that the binding activity is dependent upon NADPH, also a characteristic of the microsomal enzyme (1,2).

Table III

Effect of Ribonuclease Treatment on Hydrocarbon Bound to DNA

	Treatment	
	+RNase	-RNase
µmole DNA precipitated after treatment	1.0	1.0
pmole bound [^{14}C]BP/µmole DNA	8.2	8.0

After standard incubation of nuclei with [^{14}C]BP, DNA was purified. The 2.1 µmoles of DNA obtained was split into 2 aliquots, which were incubated in 1 x SSC at 37° for 1 hr with or without 1 mg/ml boiled pancreatic ribonuclease. DNA was precipitated, washed with ethanol, redissolved in 0.1 x SSC, and counted. Concentration of DNA was determined by optical density.

Identification of the binding activity as a function of the nuclei rests on two lines of evidence. Purity and integrity of nuclei are shown in Figure 1, which demonstrates that preparations of nuclei contained few contaminating mitochondria and microsomes. The nuclear envelope was intact, the proportion of condensed and noncondensed chromatin was normal, and the nucleoli contained both fibrillar and granular components. The nuclei were discrete and well separated from other cellular components. To eliminate the possibility that binding activity actually resided in microsomal contaminants of the nuclei, purified microsomes (1 mg protein) with known aryl hydroxylase activity were added along with NADPH to a standard incubation mixture of uninduced nuclei and [^{14}C]BP or [^{14}C]DMBA. In the presence of added microsomes, the level of binding of both BP and DMBA was about 80% that of the values for uninduced nuclei. Thus, the ability of nuclei to activate hydrocarbons for covalent binding to nuclear DNA resides in a MCA-inducible, NADPH-dependent nuclear activity.

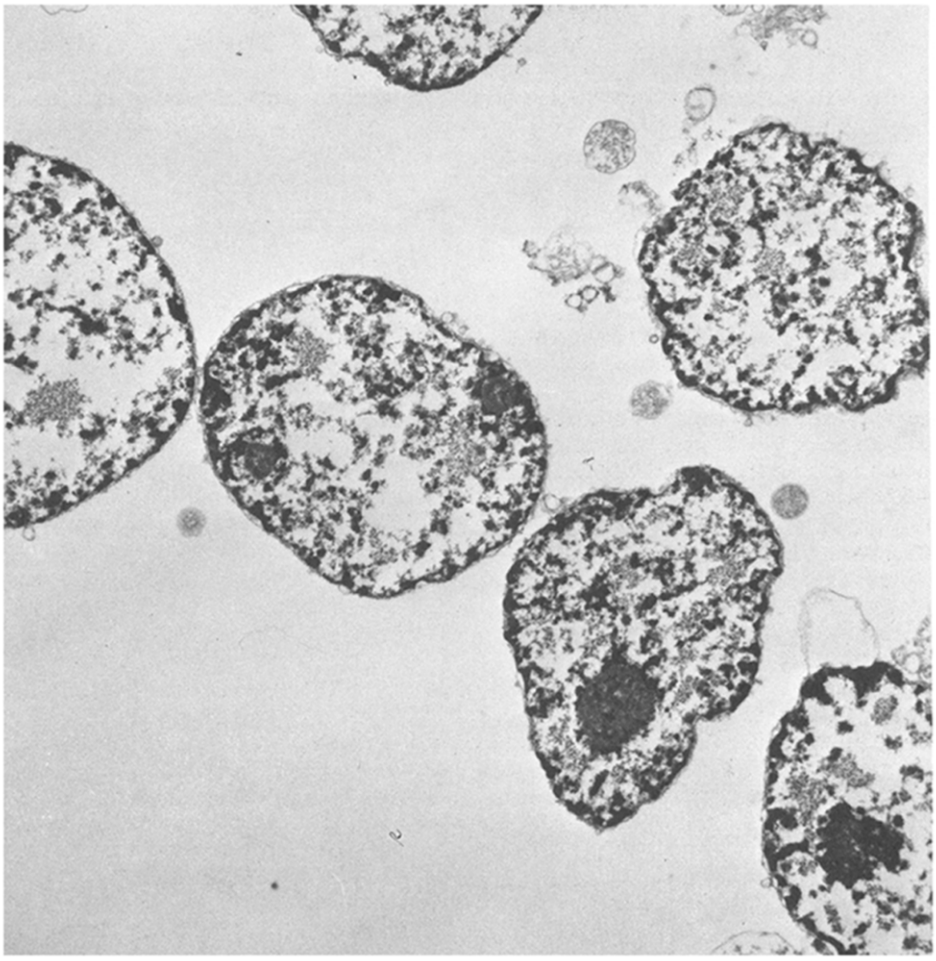

Figure 1

A Preparation of Rat Liver Nuclei

Purified nuclei were fixed in glutaraldehyde, postfixed in osmium tetroxide, imbedded, and viewed in a Siemens 1A electron microscope.

Presence of DNA binding activity and aryl hydroxylase (9) in the nucleus correlates with recent findings of other electron transport activities in the nucleus (10,17). We believe that the nuclear binding activity is of great importance in the carcinogenesis of hydrocarbons and other compounds requiring hydroxylase activation because of the proximity of the enzyme to nuclear DNA.

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1. Gelboin, H.V. (1969) Cancer Res. 29, 1272-1276.
2. Grover, P.L., and Sims, P. (1968) Biochem. J. 110, 159-160.
3. Brookes, P., and Lawley, P.D. (1964) Nature (London) 202, 781-784.
4. Brookes, P., and Lawley, P.D. (1964) J. Cell and Comp. Physiol. 64, Sup. 1, 111-128.
5. Goshman, L.M., and Heidelberger, C. (1967) Cancer Res. 27, 1678-1688.
6. Duncan, M., Brookes, P., and Dipple, A. (1969) Int. J. Cancer 4, 813-819.
7. Kinoshita, N., and Gelboin, H.V. (1972) Proc. Nat. Acad. Sci. 69, 824-828.
8. Conney, A.H., Miller, E.C., and Miller, J.A. (1957) J. Biol. Chem. 228, 753-766.
9. Khandwala, A.S., and Kasper, C.B. (1973) Biochem. Biophys. Res. Commun. 54, 1241-1246.
10. Berezney, R., Macaulay, L.K., and Crane, F.L. (1972) J. Biol. Chem. 247, 5549-5561.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Church, R.B., and McCarthy, B.J. (1968) Biochem. Genet. 2, 55-73.
13. Burton, K. (1956) Biochem. J. 62, 315-322.
14. Hoffman, H.D., Lesko, S.A., and Ts'o, P.O.P. (1970) Biochemistry 9, 2594-2604.
15. Iball, J. (1939) Am. J. Cancer 35, 188-190.
16. Nebert, D.W., and Gelboin, H.V. (1968) J. Biol. Chem. 243, 6242-6249.
17. Berezney, R., and Crane, F.L. (1972) J. Biol. Chem. 247, 5562-5568.