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A Microsome-dependent Binding of Benzo[a]pyrene to DNA[1]

Harry V. Gelboin

Chemistry Branch, National Cancer Institute, NIH, Bethesda, Maryland 20014

SUMMARY

Benzo[a]pyrene-^{3}H incubated in the presence of rat liver microsomes with calf thymus DNA binds covalently to the DNA. The reaction requires reduced nicotinamide adenine dinucleotide phosphate and is greater when the microsomes are obtained from rats pretreated with polycyclic hydrocarbons. The binding is due to the formation of unknown metabolite(s) which then may bind to the DNA in the absence of microsomes.

INTRODUCTION

Polycyclic hydrocarbons may interact with DNA through either weak physical interaction or covalent linkage. The physical interaction of polycyclic hydrocarbons to DNA has been demonstrated in several laboratories (2, 10, 18), and a chemical bonding of 3,4-benzpyrene to DNA is induced *in vitro* by photoradiation (19). The biologic significance of these interactions is not known. *In vivo* studies have shown that polycyclic hydrocarbons applied to the skin of mice become covalently bound to DNA (3) and to protein (9, 11). Further, DNA bound to polycyclic hydrocarbons can be isolated from mammalian cells grown *in vitro* in the presence of the isotopically labeled hydrocarbon (5). The covalent chemical bonding between carcinogen and cell macromolecules represent the keystone facts of two theories of chemical carcinogenesis. The binding of carcinogens to cell proteins support the protein deletion hypothesis of cancer (8, 12, 15), and the binding of polycyclic hydrocarbons to DNA supports the idea that carcinogenic transformation involves an alteration in the genetic composition of the cell.

Since the formation of a covalent link between polycyclic hydrocarbons and DNA requires the breaking and reforming of a chemical bond in the polycyclic hydrocarbon, as well as in the DNA, it is reasonable to assume an enzymatic basis for the chemical change and hence for the *in vivo* interactions of the hydrocarbon with DNA. The primary enzyme system metabolizing polycyclic hydrocarbons is the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-requiring microsomal hydroxylating system. This enzyme system may well be the catalytic agent causing the formation of DNA-polycyclic hydrocarbon complexes. The enzyme aryl hydroxylase is a NADPH-requiring microsomal enzyme that is found in numerous tissues in a variety of species (6, 20). The enzyme system converts polycyclic hydrocarbons to a large number of metabolites (17). This report shows that a NADPH-requiring microsome catalyzed reaction results in the formation of product that is bound covalently to DNA.

MATERIALS AND METHODS

Male Sprague-Dawley rats were obtained from the National Institutes of Health Animal Supply. 9,10-Dimethyl-1,2-benzanthracene-T and 3,4-benzpyrene-T were obtained from Nuclear-Chicago. Calf thymus DNA was obtained from Worthington Biochemicals. NADPH was obtained from Calbiochem.

Male Sprague-Dawley rats weighing 50–60 gm were injected with either 1 mg of 3-methylcholanthrene (MC) in 0.25 ml corn oil or with corn oil only. Twenty-four hours later the rats were sacrificed by decapitation and the livers removed. Two gm of liver were homogenized in 10 ml of 0.25 M sucrose and the microsomes prepared by a previously described procedure of differential centrifugation (7). Microsomes from one gram of liver were suspended in 1.4 ml of 0.25 M sucrose.

The Incubation of Hydrocarbons with Microsomes. Each flask contained in a total volume of 3.0 ml the following: 50 μmoles sodium phosphate buffer, pH 7.4; 100 μmoles ethylenediaminetetraacetate (EDTA); 2 mg NADPH; 2.0 mg DNA; 0.2 ml of microsomal suspension in 0.25 M sucrose; and 40 μg of ^{3}H-labeled polycyclic hydrocarbons in 0.1 ml of ethanol. The benzo(a)pyrene (BP)-^{3}H ranged from 5×10^5 to 10^6 dpm/min/μg. The specific activity of 7,12-dimethylbenz(a)anthracene (DMBA) was 10^7 dpm/min/μg. The flasks were incubated in air at 37°C for 14 minutes. Unless otherwise indicated, microsomes from MC-treated rats were used.

DNA Isolation by CsCl Sedimentation. Immediately after the incubation period, 1.0 ml of 0.4% sodium lauryl sulfate was added and the mixture homogenized in a Potter-Elvehjem homogenizer for five minutes at room temperature. One ml of CsCl in 0.1 M EDTA at a density of 1.8 was added and the mixture transferred to a centrifuge tube where additional CsCl was added to a final solution density of 1.65 gm/ml. The mixture was centrifuged for 66 hr at 180,000 × *g* at room temperature in an angle head rotor in either a Spinco or International centrifuge. Fractions were collected in 0.5 ml portions. Optical density determinations at 260 mμ were made on aliquots. Additional aliquots were removed, and 200 μg of carrier DNA were added to each tube. The acid-precipitable material was precipitated with 2.0 ml of 10% trichloracetic acid and the pellets extracted successively with hot methanol until no radioactivity was detectable in the extract. This usually required 7–10 extractions. The precipitate was then

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hydrolyzed in 1 ml hyamine and radioactivity determined in a toluene, 2,5-diphenyloxazole (PPO), 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) scintillator in a Packard scintillation counter.

Phenol Extraction of DNA. The extraction was performed by a modification of a procedure of Kirby (14). The reaction mixture was treated with 3 ml of a phenol reagent which consisted of 600 gm phenol in 55 ml H_2O, 75 ml *m*-cresol, and 0.500 gm 8-hydroxyquinoline. The mixture was shaken vigorously. The aqueous phase was removed and treated with an equal amount of ethoxyethanol. The precipitate formed was extracted successively with ethoxyethanol:water (1:1), twice with 100% ethanol, twice with hot ethanol at 70°C for 15 minutes. When radioactivity was detected in the extract, additional ethanol extractions were performed. In most of the experiments the DNA was redissolved in 1.0 ml of water and reprecipitated by the addition of 0.1 ml 2M $MgCl_2$ and an equal volume of a 1:1 mixture of ethoxyethanol:ethanol. The pellet was successively washed with 1:1 ethoxyethanol: ethanol, twice with ethanol, twice with ethanol at 70°C for 15 minutes, and 4 times with cold 95% ethanol. The pellet was then hydrolyzed for optical density and radioactivity determinations. Precipitates were hydrolyzed in 0.5 N perchloric acid (1.0 ml) at 90°C for 15 minutes, and the radioactivity of a 0.5 ml aliquot was determined in Bray's solution (4 gm PPO, 2 gm POPOP, 60 gm naphthalene, 100 ml methanol, 20 ml ethylene glycol, and dioxane to 1000 ml) in a Packard scintillation counter.

Phenol Extraction and CsCl Sedimentation. In one experiment, the DNA obtained by the phenol extraction was further purified by dissolving in H_2O and centrifuging in CsCl as previously described.

RESULTS

Chart 1 shows a cesium chloride sedimentation analysis of DNA incubated in the presence of rat liver microsomes and benzo[a]pyrene-^{3}H. The DNA bands sharply in 2 to 3 fractions. The radioactivity curve indicates the amount of nonextractable isotope in each fraction. Chart 1*C-E* shows the correspondence between nonextractable radioactivity from BP-^{3}H bound to the precipitate and the optical density peak of DNA. The amount of radioactivity sedimenting in this region is roughly dependent on the amount of microsomal enzyme added. At the very low level of enzyme of 0.025 ml (Chart 1*E*), very little radioactivity is observed in the peak DNA tube. With no DNA added (1*B*) no radioactivity sediments in the 1.7 density region, and with the unincubated preparation (1*A*) no radioactivity sediments with the DNA. Thus, the sedimentation of radioactivity from BP-^{3}H in the DNA density region is dependent on incubation in the presence of enzyme and DNA. This indicates that metabolic activation is required for the binding of radioactivity from BP-^{3}H to DNA. With no DNA added to the incubation, no peak of radioactivity is observed in the 1.7 density region, indicating that we are not dealing with the binding of a metabolite of BP-^{3}H to a macromolecule other than DNA that may have an unusual sedimentation character. Since the entire incubation mixture is centrifuged in this gradient and very little radioactivity sediments at the upper

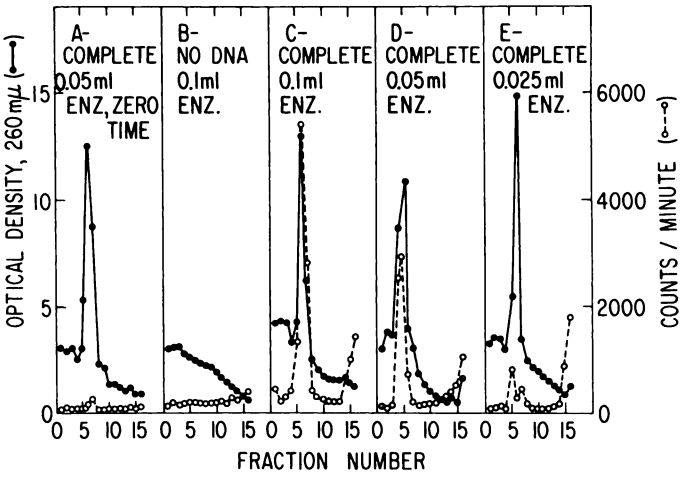

Chart 1. Cesium chloride sedimentation analysis of DNA incubated in the presence of rat liver microsomes and benzo[a]pyrene-^{3}H.

density where protein sediments, it is unlikely that the radioactivity that sediments with the DNA is largely due to protein contamination. Small amounts of protein, of course, may be entrapped in the DNA.

The question arises as to the type of binding between the DNA and the derivative of BP. The DNA can be extensively extracted with organic solvents, dissolved and reprecipitated in water, and further extracted so that no further radioactivity is extracted. The radioactive derivative of BP remains bound to the DNA fraction and is only released upon hydrolysis of the DNA. It thus seems that the binding of ^{3}H from BP-^{3}H is of a covalent nature. This indication, however, must be confirmed by characterization of the bound radioactive compound.

Chart 2 shows the further purification of the DNA bound to derivatives of BP-^{3}H. In this experiment the DNA was first isolated by the phenol procedure, precipitated by the ethoxyethanol method, and then further purified by sedimentation in CsCl. Essentially no radioactivity is detected in any region of the gradient other than in the region where the DNA sediments. Also, essentially no ultraviolet-absorbing material is detectable in regions other than in the DNA fractions, indicating a relatively high degree of DNA purification.

Effect of Pretreatment of the Rat with 3-Methylcholanthrene on Microsomal Enzyme Activity. Table 1 shows the binding of radioactivity from BP and DMBA catalyzed by different amounts of microsomes from control rats and rats pretreated 18 hours prior with MC. Doubling the amount of microsomes roughly doubles the amount of binding of BP-^{3}H to DNA and pretreatment of the rats with MC increases the activity of the microsomal preparation by about 2–4 fold. The binding of DMBA-^{3}H did not increase with enzyme amount, but preparations from MC-treated rats were about five times more active than control microsomes. The amount of binding is of the order of one BP per 50,000 nucleotides and of the order of one DMBA per 500,000 nucleotides.

Table 2 shows the requirements for binding of BP-^{3}H to DNA. There is an absolute requirement for incubation in the

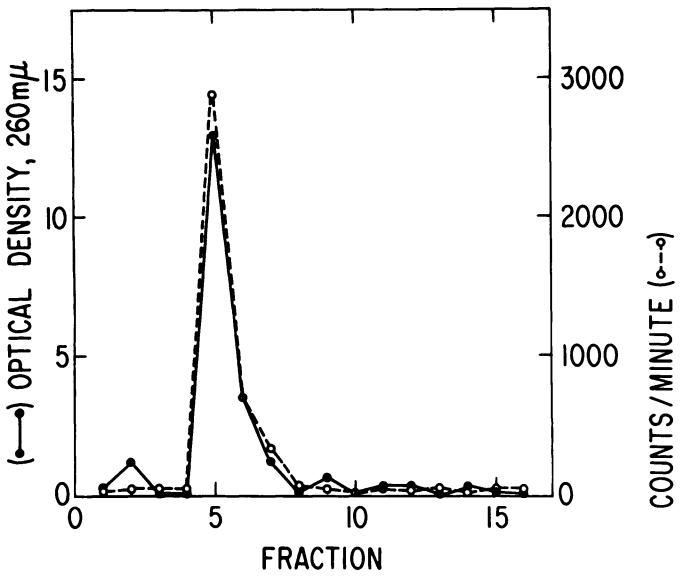

Chart 2. Purification by phenol extraction in cesium chloride sedimentation of DNA-BP-^{3}H complex; BP, benzo[a]pyrene.

Table 1

Microsomes	Amount (ml)	Substrate	cpm/mg DNA ($\times 10^{-2}$)
Control	0.05	BP-^{3}H	34
Control	0.10	BP-^{3}H	50
MC	0.05	BP-^{3}H	88
MC	0.10	BP-^{3}H	198
Control	0.05	DMBA-^{3}H	14
Control	0.10	DMBA-^{3}H	11
MC	0.05	DMBA-^{3}H	67
MC	0.10	DMBA-^{3}H	62

The effect of microsomes from control and MC-treated rats on BP-^{3}H and DMBA-^{3}H binding to DNA. The source of microsomes was from control or MC-treated rats. Their preparation is described in Methods. The protein content of the microsomal suspension from control and MC-treated rats was approximately the same. MC,-3-methylcholanthrene; BP, benzo[a]pyrene; DMBA, 7,12-dimethylbenz(a)anthracene.

Table 2

System	BP-^{3}H-to-DNA binding (% of control)
Complete system, control	100
Complete system, DNA after incubation	81–112
Complete system, BP-^{3}H after incubation	11
Complete system, no NADPH	3
Complete system, zero time	<1

Requirements for binding benzo[a]pyrene (BP)-^{3}H to DNA. The results of several different experiments. The amount of binding is compared to a control system utilizing microsomes from 3-methylcholanthrene-treated rats. Complete system described in Methods. The DNA was isolated by phenol extraction. When the DNA was added after the incubation, the second incubation time was 14 minutes at 37°C. NADPH, reduced nicotinamide adenine dinucleotide.

presence of NADPH suggesting the involvement of the O_2- and NADPH-requiring microsomal enzymes. Table 2 also shows that the binding is not due to some enzyme-catalyzed change in the DNA which yields a product that then reacts with BP-^{3}H. Thus, metabolic activation of BP-^{3}H is required. DNA, however, need not be present during the incubation. About the same level of DNA bound radioactivity is observed when the DNA is added either before or after the incubation. After incubation of the BP-^{3}H with microsomes, the DNA can be added either directly to the reaction mixture or the supernatant fluid of the reaction mixture after sedimentation at 100,000 × *g* for one hour. DNA added after incubation either to the reaction mixture or to the supernatant fluid of the reaction mixture results in the same degree of binding of ^{3}H to DNA as when the DNA is present during the primary incubation.

Table 3 shows that RNA can be substituted for the DNA, and approximately the same level of binding occurs with RNA when it is used as the macromolecule receptor. In other studies we found that the microsomes also catalyze the binding of metabolites of BP to protein, but we have no evidence as to the covalent nature of this binding.

Concentration of BP-^{3}H and DNA and Degree of DNA-BP-^{3}H Complex Formation (Table 4). The highest specific activity of counts/μg DNA is obtained with lower levels of DNA added to the incubation mix. At greater than 1.0 mg of added DNA the total amount of radioactivity increases but the specific activity of the DNA falls. Relatively large amounts of DNA are added to the incubation mix since lower levels of DNA are more susceptible to the nuclease activity present in microsomes and small amounts of DNA are more difficult to isolate. The EDTA is added during the incubation to inhibit nuclease activity and to maximize the amount of DNA recovered.

Table 5 shows that the maximum substrate concentration for BP-^{3}H binding to DNA is about 20 μg BP/flask. At greater substrate concentrations there appears to be an inhibition of the formation of the DNA-BP-^{3}H product.

DISCUSSION

The molecular mechanism of polycyclic hydrocarbon carcinogenesis is obscure. A reasonable hypothesis is that the carcinogen effects a heritable change by altering the structure of DNA. Brookes and Lawley (3) have demonstrated a correlation between the degree of binding of a series of polycyclic hydrocarbons to mouse skin DNA and their relative carcinogenic activity. The polycyclic hydrocarbons are relatively inert chemically, and covalent interactions between these molecules and DNA would seem to require a metabolic activation of the hydrocarbon. Sims has shown (17) that benzo[a]pyrene is converted *in vivo* to a large number of metabolites including various free and conjugated hydroxy and dihydroxy derivatives. The conversion of BP to hydroxylated products is catalyzed by the microsomal enzyme system originally described by Conney *et al.* (4). This enzyme system is inducible, and the present study shows that products of this reaction bind probably in a covalent fashion to DNA. We do not know the nature of the metabolite that binds to DNA or the nature of the

Table 3

System	cpm/mg RNA ($\times 10^{-2}$)
Complete system, + 2 mg RNA	325
Complete system, + 2 mg RNA zero time	7

Binding of benzo[a]pyrene to RNA. The complete system is described in Methods. Yeast RNA replaced DNA. The RNA was isolated from the reaction mixture by phenol extraction, precipitated with ethanol and washed exhaustively with the same procedure described for DNA.

Table 4

DNA added (mg)	cpm/mg DNA ($\times 10^{-2}$)
0.5	1545
1.0	508
1.5	374
2.0	361
4.0	368

Binding of benzo[a]pyrene-^{3}H to DNA and added DNA. The DNA was isolated by phenol extraction as described in Methods. The actual recovery of DNA varied but was usually about 40–70%. The inability to completely recover the added DNA may be due to DNase activity in the enzyme preparation. The inability to recover the DNA fully raises the question as to effective concentration of the DNA during the incubation.

Table 5

BP-^{3}H (μg/tube)	cpm/mg DNA ($\times 10^{-2}$)
10	71
20	400
40	294
80	171

The effect of various concentrations of benzo[a]pyrene (BP-^{3}H) on the binding of BP-^{3}H to DNA.

bound form. The level of binding is of the order of one hydrocarbon per 50,000 to 500,000 nucleotides. The low level of binding raises the question of the possibility of unique sites on the DNA that may be susceptible to interaction with derivatives of BP. The low level of binding observed *in vitro* is comparable to that reported for the *in vivo* studies on mouse skin (3). Salser and Balis (16) reported the presence of very small amounts of amino acids in highly purified *E. coli* DNA. It is conceivable that these amino acids might be the specific sites for binding. Another possibility is that only unique sequences of nucleotides can bind hydrocarbons. It is also possible that only a low level of reactive hydrocarbon metabolite is available to DNA *in vivo* because the enzyme system may be largely localized in sites outside of the nucleus and spatially removed from DNA. Microsome-like structures are in close approximation to the nuclear membrane, and Bach and Johnson (1) have reported microsome-like activity present in the nucleus. These may contain the enzymes required for the activation of the BP to a reactive form.

The enzyme system described may be responsible for the *in vivo* formation of covalently bound DNA polycyclic hydrocarbon complexes.

The relationship of the hydroxylase enzyme to carcinogenesis requires considerable clarification. The enzyme is quite ubiquitous. It has been shown to be present in a variety of tissues by histochemical technics (20), and we have examined the enzyme content of numerous tissues of at least four different species. The enzyme is present in 90% of the tissues we have examined. Furthermore, the enzyme is highly inducible and the inducibility varies in different species and in different tissues (D. W. Nebert and H. V. Gelboin, manuscript in preparation). The enzyme is present and inducible in a variety of cells grown in culture (13). The cells that contain the enzyme are generally sensitive to the toxic action of the polycyclic hydrocarbons. The enzyme is lacking in five cell lines grown in culture. These cells are resistant to the toxic action of the hydrocarbons. There thus seems to be a positive relationship between the toxic action of the polycyclic hydrocarbon and the presence of the enzyme system. This may be true for the carcinogenic activity as well. More needs to be known about the profile of metabolic products of the control and induced enzyme in each tissue and species and the nature of the carcinogenic intermediate and, if possible, to determine the characteristics of the enzyme with respect to each product formation. Furthermore, it seems in the realm of possibility that conditions could be developed that increase the ratio of nontoxic to toxic or carcinogenic metabolites and in this manner diminish the carcinogenic activity of the polycyclic hydrocarbons.

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REFERENCES

1. Bach, M. K., and Johnson, H. G. The Isolation and Characterization of a Novel Microsome Fraction from Washed and Detergent-Treated Nuclei of HeLa Cells by the Use of Solutions Containing Deoxyribonucleic Acid. Biochemistry, *6:* 1916–1933, 1967.
2. Boyland, E., and Green, B. The Interaction of Polycyclic Hydrocarbons and Nucleic Acids. Brit. J. Cancer, *16:* 507–517, 1962.
3. Brookes, P., and Lawley, P. D. Evidence for the Binding of Polynuclear Aromatic Hydrocarbons to the Nucleic Acids of Mouse Skin: Relation Between Carcinogenic Power of Hydrocarbons and Their Binding to DNA. Nature, *202:* 781–784, 1964.
4. Conney, A. H., Miller, E. C., and Miller, J. A. Substrate-Induced Synthesis and Other Properties of Benzpyrene Hydroxylase in Rat Liver. J. Biol. Chem., *228:* 753–766, 1957.
5. Diamond, L., Defendi, V., and Brookes, P. The Interaction of 7,-12-Dimethylbenz[a]anthracene with Cells Sensitive and Resistant to Toxicity Induced by This Carcinogen. Cancer Res., *27:* 890–896, 1967.
6. Gelboin, H. V., and Blackburn, N. R. The Stimulatory Effect of 3-Methylcholanthrene on Benzpyrene Hydroxylase Activity in Several Rat Tissues: Inhibition by Actinomycin D and Puromycin. Cancer Res., *24:* 356–360, 1964.
7. Gelboin, H. V., and Sokoloff, L. Effects of 3-Methylcholanthrene and Phenobarbital on Amino Acid Incorporation into Protein. Science, *134:* 611–612, 1961.

8. Heidelberger, C. Studies on the Molecular Mechanism of Hydrocarbon Carcinogenesis J. Cellular Comp. Physiol., *64* (Suppl. 1)*:* 129–148, 1964.
9. Heidelberger, C., and Moldenhauer, M. G. The Interaction of Carcinogenic Hydrocarbons with Tissue Constituents. IV. A Quantitative Study of the Binding to Skin Proteins of Several C^{14}-Labeled Hydrocarbons. Cancer Res., *16:* 442–449, 1956.
10. Liquori, A. M., De Lerma, B., Ascoli, F., Botre, C., and Trasciatti, M. Interaction Between DNA and Polycyclic Aromatic Hydrocarbons. J. Mol. Biol., *5:* 521, 1962.
11. Miller, E. C. Studies on the Formation of Protein-Bound Derivatives of 3,4-Benzpyrene in the Epidermal Fraction of Mouse Skin. Cancer Res., *11:* 100–108, 1951.
12. Miller, J. A., and Miller, E. D. The Carcinogenic Aminoazo Dyes. Advan. Cancer Res., *1:* 339–396, 1953.
13. Nebert, D. W., and Gelboin, H. V. Substrate-inducible Microsomal Aryl Hydroxylase in Mammalian Cell Culture. II. Cellular Responses During Enzyme Induction. J. Biol. Chem., *243:* 6250–6261, 1968.
14. O'Sullivan, M. A., and Kirby, K. S. Studies on Nucleic Acids During Carcinogenesis. Brit. J. of Cancer, *18:* 792, 1964.
15. Potter, V. R. Enzyme Studies on the Deletion Hypothesis of Carcinogenesis. *In:* The Molecular Basis of Neoplasia, pp. 367–399. Austin: The University of Texas Press, 1962.
16. Salser, J. S., and Balis, M. E. Amino Acids Associated with DNA of Tumors. Cancer Res., *28:* 595–600, 1968.
17. Sims, P. The Metabolism of Benzo[a]pyrene by Rat-Liver Homogenates. Biochem. Pharmacol., *16:* 613–618, 1967.
18. Ts'o, P. O. P., and Lu, P. Interaction of Nucleic Acids. I. Physical Binding of Thymine, Adenine, Steroids, and Aromatic Hydrocarbons to Nucleic Acids. Proc. Natl. Acad. Sci. U. S., *51:* 17, 1964.
19. Ts'o, P. O. P., and Lu, P. Interaction of Nucleic Acids. II. Chemical Linkage of the Carcinogen 3,4-Benzpyrene to DNA Induced by Photoradiation. Proc. Natl. Acad. Sci. U. S., *51:* 272–280, 1964.
20. Wattenberg, L. W., and Leong, J. L. Histochemical Demonstration of Reduced Pyridine Nucleotide Dependent Polycyclic Hydrocarbon Metabolizing Systems. J. Histochem. Cytochem., *10:* 412–420, 1962.

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