[CANCER RESEARCH 38, 4640-4644, December 1978]
0008-5472/78/0038-0000$02.00

Modulation of the Binding of the Carcinogen Benzo(*a*)pyrene to Rat Liver DNA *in Vivo* by Selective Induction of Microsomal and Nuclear Aryl Hydrocarbon Hydroxylase Activity[1]

Andreas Viviani and Werner K. Lutz

Institute of Toxicology, Federal Institute of Technology, and University of Zurich, CH-8603 Schwerzenbach, Zurich, Switzerland

ABSTRACT

The influence of microsomal and nuclear aryl hydrocarbon hydroxylase (AHH) activity on the covalent binding of [G-^{3}H]benzo(*a*)pyrene to rat liver DNA was evaluated *in vivo*. Induction of microsomal AHH was obtained after phenobarbital treatment (160% of control), which also increased DNA binding to 190%, but left the nuclear activity unchanged. Nuclear AHH was induced with dieldrin (150%), and the binding was decreased to 75%, whereas the microsomal AHH was at control level. The increasing effect of microsomal AHH induction as well as the decreasing effect of nuclear AHH induction on the binding was shown clearly when the data of the individual rats were used to solve the equation

$$\text{Binding} = a^*(\text{microsomal AHH}) + b^*(\text{nuclear AHH}) + c$$

Multiple linear regression analysis with the data from 10 animals resulted in positive values for *a* and *c*, a negative value for *b*, and a good multiple correlation coefficient of $r = 0.974$. Pretreatment with 3-methylcholanthrene induced microsomal AHH to 380% of control and nuclear AHH to 590% and increased the binding to 175%. The binding was higher than predicted by the formula found, probably because the increasing influence of induced microsomal AHH overshadowed the decreasing effect of the nuclear AHH. The study shows clearly that the binding of a foreign compound to DNA *in vivo* is dependent not only on microsomal enzyme activities but also on nuclear activities even if the latter are considerably lower than those of microsomes.

INTRODUCTION

The mixed-function oxygenase AHH[2] (EC 1.14.14.2), an inducible enzyme system found in most mammalian tissues, catalyzes the metabolism of BP and other carcinogenic PAH to products that can bind covalently to DNA (6, 7, 13). This binding to DNA is accepted as an early and critical event in chemical carcinogenesis, and it has been shown that the carcinogenic potency of different PAH is correlated to the extent of their binding to DNA (2, 3).

The tumor incidence from exposure to PAH is affected by other compounds, and there are indications that this is due to an alteration of the PAH metabolism, particularly to changes in AHH activity (1, 10, 15). The first aim of this study was therefore to determine whether an induction of AHH has any influence on the binding of BP to DNA *in vivo*.

In the liver cell, AHH is located in the endoplasmic reticulum and, with much less activity, in the outer nuclear membrane (15, 17). Both microsomal and nuclear AHH's are capable of activating BP to DNA-binding metabolites, and an induction in general increased the binding *in vitro* (1, 8, 15, 16). The extent of induction of AHH was found to be different in the 2 compartments and to be dependent on the inducer used (8, 15, 18).

A study of the time dependence of the AHH induction revealed different induction levels in microsomes and nuclei in the first days of the treament, with an equilibration of the induction levels after a longer time period (18). This finding enabled us to evaluate *in vivo* the role of microsomal or nuclear AHH in the activation of BP to DNA-binding metabolites.

MATERIALS AND METHODS

Chemicals. BP, MC, and DI were obtained from Fluka AG, Buchs, Switzerland, and Mazola corn oil was from Maizena GmbH, Hamburg, Germany. [G-^{3}H]BP with a specific activity of 24 Ci/mmol was from the Radiochemical Centre, Amersham, England. BP was purified further by column chromatography in hexane on silica gel, and DI was purified by recrystallization. Sodium phenobarbital was obtained from the Kantonsapotheke, Zurich, Switzerland. Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories, Richmond, Calif. 94804.

Animals. Adult male rats (Sprague-Dawley derived SIV50, Kantonales Tierspital, Zurich, Switzerland), weighing 280 to 330 g at the day of sacrifice, were housed 2 per cage on sawdust in clear plastic cages and held untreated for at least 1 week after receipt. Each pair of rats was given the same treatment. Nafag laboratory chow (Nafag AG, Gossau, Switzerland) and tap water were provided ad libitum.

Enzyme Induction. Inducers were administered as described (18). PB was given in the drinking water at 1 g per liter of sodium phenobarbital. MC and DI (20 mg/kg) in corn oil were injected i.p. daily.

Administration of Tritiated BP. In Experiment A, [G-^{3}H]BP was diluted with inactive BP to a specific activity of 1.44 Ci/mmol. Twenty hr before sacrifice, a solution of 250 μg BP per ml corn oil (2 ml/kg) was injected i.p. (500 μg BP/kg; 6.3^*10^9 dpm/kg). This dose does not induce the AHH (11). In Experiment B, [G-^{3}H]BP was diluted to a specific activity of 4.9 Ci/mmol and 700 μg were dissolved in hexane and

---

[1] Part of this work has been presented at the 10th Annual Meeting of the Union of the Swiss Societies for Experimental Biology, Davos, Switzerland, May 19 to 20, 1978, and at the 20th Congress of the European Society of Toxicology, Berlin, GFR, June 25 to 28, 1978.

[2] The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; BP, benzo(*a*)pyrene; PAH, polycyclic aromatic hydrocarbons; MC, 3-methylcholanthrene; DI, dieldrin; NaP, sodium phosphate buffer, pH 6.8; EH, epoxide hydratase.

Received July 13, 1978; accepted August 18, 1978.

dispersed as a fine film in a 50-ml round-bottomed flask by rotary evaporation of the hexane. Ten ml of serum from the same strain of rats were added, and the flask was filled with nitrogen and vigorously shaken in the dark at room temperature for 48 hr. Complete dissolution of the BP was checked by spectrophotometry and liquid scintillation counting of the supernatant of a diluted serum sample centrifuged at 105,000 × *g* for 20 min. Sixteen hr before sacrifice, the rats were constrained in a plastic bottle, and 2 ml/kg (140 μg BP/kg; $6.0*10^9$ dpm/kg) were injected into the penis vein without anesthesia.

Determination of AHH Activity. Animals were killed by stunning and cervical dislocation at 0800 hr. The liver was excised and washed twice in ice-cold 0.25 M sucrose, 50 mM Tris-HCl, pH 7.5. Liver microsomes and nuclei were prepared, as described (18), by a calcium precipitation method and sucrose density centrifugation, respectively. AHH activity was determined with an assay based upon the fluorescence of hydroxylated BP products as described before (18).

Isolation and Purification of DNA. The big lobe from the washed liver was frozen in liquid nitrogen and stored 5 days until further use. The DNA was isolated according to Markov and Ivanov (12) with several modifications.

Four g of tissue were blended with 25 ml of 8 M urea, 0.24 M sodium phosphate (hereafter known as urea buffer) containing 1% sodium dodecyl sulfate and 10 mM EDTA in a custom-made, air-tight aluminum headpiece on a Waring Blendor at high speed for 4 times 30 sec with intervals of 30 sec to cool the headpiece in an ethanol bath (−30°). The foamy lysate was centrifuged for 3 min at 3000 × *g*, and 10 ml of chloroform/isopentyl alcohol/phenol 24/1/25 were added. The mixture was vigorously shaken for 10 min at room temperature and centrifuged for 15 min at 20,000 × *g*. The aqueous supernatant was pipetted off, and the extraction step was repeated. To remove the phenol, the supernatant was extracted twice with 25 ml of diethyl ether. For the DNA adsorption by hydroxyapatite chromatography, 3 g of dry hydroxyapatite were suspended in urea buffer, stored overnight at room temperature, poured into a column of 2.5 cm diameter, and equilibrated with three bed volumes of urea buffer. The column was loaded with the aqueous sample and washed with urea buffer with a flow rate of 1 ml/min by gravity until no absorption at 260 nm was recorded (70 ml). The same flow rate was maintained for the removal of the urea with 25 ml of 14 mM NaP. DNA was eluted with 0.48 M NaP, monitored by spectrophotometry at 260 nm. The DNA fraction was dialyzed against 500 volumes of deionized water for 16 hr at 4° and made 0.2 M with respect to NaCl by adding 2 M NaCl. Two volumes of ethanol were added, and the DNA was precipitated at −20° for 24 hr. After centrifugation for 20 min at 1000 × *g*, the supernatant was decanted, and the DNA was dried in a vacuum desiccator and redissolved in 14 mM NaP.

The amount and purity of the DNA was determined by spectrophotometry. An absorbance of 6.18 at 260 nm was established for 1 μmol DNA-phosphate per ml by phosphate determination following Chen *et al.* (4). The purity of the final preparation was at least equal to the one specified by Markov and Ivanov (12) as judged by the ratio A 260 nm/A 230 nm, which was better than 2.30 (0.1% protein).

One to 2 mg dried DNA were redissolved in 4 ml of 14 mM NaP, and the radioactivity was counted in a BF 5000 scintillation counter (Laboratorium Berthold, Wildbad, Germany) after the addition of 10 ml Insta-Gel (Packard Instruments Co., Downers Grove, Ill. 60515). The net counts ranged from 17 to 180 cpm.

RESULTS

The binding of BP to rat liver DNA after AHH induction by different inducers is given in Table 1, together with the corresponding AHH activities in microsomes and nuclei.

Experiment A: i.p. Administration of Tritiated BP. Fourteen-day pretreatment with PB markedly enhanced the microsomal AHH activity and doubled the amount of BP

Table 1

Binding of tritiated BP to rat liver DNA after different induction of microsomal and nuclear AHH

Adult male rats were treated with an inducer for the time period indicated until sacrifice. In Experiment A, 500 μg BP per kg in corn oil were administered p.o. 20 hr before sacrifice. In Experiment B, 140 μg BP per kg in serum were injected i.v. 16 hr before sacrifice. AHH activity is expressed as pmol 3-OH-BP formed in 1 min per mg of protein, measured by a fluorimetric assay; binding to DNA as μmol BP bound per mol DNA-phosphate/mmol BP administered per kg body weight.

Inducer dosages: phenobarbital, given continuously in the drinking water at 1 g/liter; DI, 20 mg/kg in corn oil for 3 consecutive days; MC, 20 mg/kg/day in corn oil; control, no pretreatment.

	Microsomal AHH	Nuclear AHH	Binding to DNA
Experiment A inducer			
None (control)	2040 ± 190[a]	39 ± 6	6.8 ± 0.8
Phenobarbital, 14 days	3620 ± 600[b]	44 ± 26	14.2 ± 8.4
DI, 12 days	2630 ± 250[b]	48 ± 15	7.2 ± 1.9
Experiment B inducer			
None (control)	930 ± 340[a]	24 ± 0	8.7 ± 3.5
Phenobarbital, 8 days	1520 ± 140[b]	26 ± 3	16.9 ± 3.0[b]
DI, 3 days	1070 ± 110	37 ± 4[b]	6.6 ± 3.1
MC, 4 days	3590 ± 300[b]	140 ± 22[b]	15.2 ± 2.3[b]

[a] Mean ± S.D.

[b] Significantly different from control ($p < 0.05$; Student's *t* test).

bound to DNA. DI did not alter the binding, although the microsomal activity was also elevated. Neither of the inducers affected the nuclear AHH activity, but there was a tendency to higher activity seen after DI treatment. One might tentatively conclude from these data that the increase in binding observed after the induction of microsomal AHH is counteracted by increases in nuclear activity.

This hypothesis is corroborated when the data of individual animals are considered. Chart 1 shows, in a 3-dimensional plot, the dependence of the binding on the microsomal and nuclear AHH activities. One block represents the values from 1 animal.

With such a representation, the reducing effect of nuclear AHH on the binding of BP to DNA comes out much more clearly, and the data fit into a linear equation of the form

Binding = +0.0071*(microsomal AHH)

$$- 0.36*(\text{nuclear AHH}) + 6.1$$

with a surprisingly good multiple correlation coefficient r = 0.82 and a standard error of the estimation of the binding of 3.8 units.

This set of experiments prompted us to continue the investigation with some minor modifications. A different administration schedule was used for BP and DI. MC, a potent inducer of both the microsomal and nuclear AHH, was included.

The dose of tritiated BP was lowered to 140 μg/kg. This was done to exclude any inducing effect from BP itself. Previous experiments had shown that an induction of AHH is seen only above a single i.p. dose of 1 mg BP/kg (11).

BP was injected i.v. in order to accelerate distribution and metabolism of BP so that the time between administration and sacrifice could be reduced to 16 hr.

Experiment B: I.v. Administration of Tritiated BP. Phenobarbitol pretreatment had the same enhancing effect on microsomal AHH and binding as found in Experiment A (Table 1). With DI, the microsomal AHH was not changed, but the induction of nuclear AHH was significant this time. We found the expected decrease in binding, which is on a nonsignificant level on the basis of the average data but

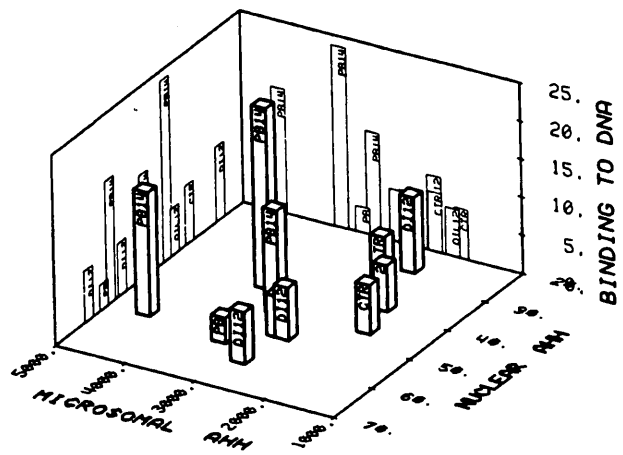

Chart 1. Binding of BP to rat liver DNA as a function of the microsomal and nuclear AHH activity as obtained in Experiment A. Each *bar* represents the 3 values from one individual rat treated as indicated (inducer and days of pretreatment). See legend to Table 1 for further explanations.

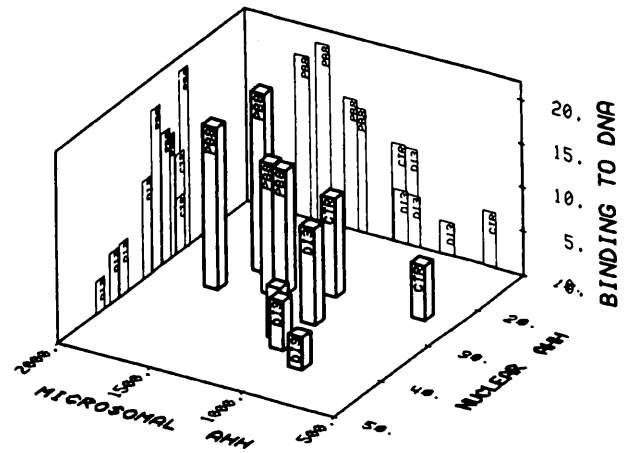

Chart 2. Binding of BP to rat liver DNA as a function of the microsomal and nuclear AHH activity as obtained in Experiment B. Each *bar* represents the 3 values from one individual rat treated as indicated (inducer and days of pretreatment). See legend to Table 1 for further explanations.

very obvious with the individual animal data as seen in Chart 2. Linear regression analysis of the values used in Chart 2 resulted in the equation

Binding = +0.014*(microsomal AHH)

$$- 0.39*(\text{nuclear AHH}) + 5.7$$

with a multiple correlation coefficient r = 0.974 and a standard error of the estimation of 1.5 units. The plain fitting was therefore even better than that from the i.p. Experiment A.

Pretreatment of the animals with MC gave rise to highly significant inductions of microsomal and nuclear AHH activities. The high binding values obtained after this experiment make it clear that the increasing effect of microsomal induction on the binding overshadows the decreasing influence of nuclear induction.

One might calculate theoretical binding values for the MC-treated animals from their AHH activities on the basis of the formula found by linear regression. They come out much lower than the ones actually measured, and even come out negative. This indicates that the theoretical plane formula for the binding as a function of the 2 AHH activities is not linear for the expanded scale of Chart 3, which combines all data of Experiment B.

It is to be noted that MC does not induce the same AHH species as phenobarbitol or DI does. In particular, other types of cytochrome P450 are formed (15, 16).

DISCUSSION

The data presented show that the microsomal AHH increases the amount of binding of BP to DNA *in vivo*, whereas the nuclear AHH decreases it. These findings were somewhat unexpected, since our working hypothesis was that the nuclear AHH, which is closer to the DNA than the microsomal AHH, should have an increasing effect on DNA binding. Rather, it could now be concluded that the activation of BP to reactive metabolites that can bind covalently to DNA takes place in the microsomal fraction, and that the

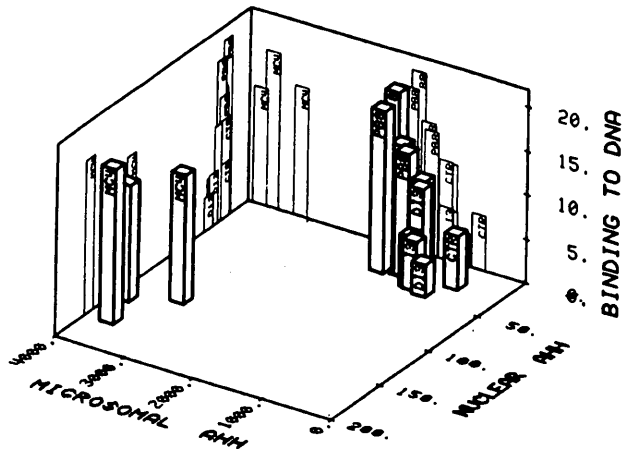

Chart 3. Binding of BP to rat liver DNA as a function of the microsomal and nuclear AHH activity as obtained in Experiment B. Data from rats pretreated with MC are included. Each *bar* represents the 3 values from one individual rat treated as indicated (inducer and days of pretreatment). See legend to Table 1 for further explanations.

comparatively low activity of nuclear AHH inactivates these metabolites through further oxidation.

It is to be noted, however, that the AHH is not the only enzyme involved in the process leading to reactive metabolites that can covalently bind to DNA. In particular, EH plays an important role in the activation of BP by forming BP-dihydrodiols from BP-epoxides, which are then further oxidized by the AHH to the very reactive BP-diolepoxides (14, 16, 21). EH is inducible by various compounds in microsomes and nuclei (14). An induction of EH by the chemicals used in this study could accelerate the intermediate step in the formation of the BP-diolepoxides and could therefore increase the binding of BP to DNA. On the other hand, reactive epoxides are, on their own right, substrates for EH, and are inactivated to BP-dihydrodiols and tetrahydrotetrols. An induction of EH might therefore lower the binding. There are no data available to date to decide whether this enzyme would produce a positive or negative change in our *in vivo* study.

Additional detoxifying enzyme activities which are also inducible are present in microsomes and nuclei (5, 9, 20). Glutathione-S-epoxide transferase, 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase, and glucuronyl transferase are known to inactivate BP metabolites, and they may also contribute to alterations in the binding of BP to DNA when changed in their activity by an inducer.

Long-term treatment with inducing agents results in an equal level of AHH induction in microsomes and nuclei (18). The binding of BP to DNA would then be enhanced because the increasing effect of the microsomal AHH outweighs the decreasing influence of the nuclear AHH. Such a situation is typical for an exposure of humans to environmental agents, and our animal model predicts increased binding to DNA and tumor formation under these circumstances.

Wattenberg and Leong (19) performed such an experiment with mice, where a 16-day pretreatment with 5,6-benzoflavone induced lung AHH and resulted in a decrease in lung tumor formation from orally administered BP. This finding cannot be explained on the basis of our experiments with rats.

Kouri (10) showed that there is a correlation between levels of AHH activity and susceptibility to BP-induced lung cancer in various strains of mice. Moreover, after pretreatment of the mice with 2,3,7,8-tetrachloro-dibenzo-p-dioxin, an AHH inducer similar to MC, he observed a doubling of the lung cancer occurrence.

These findings with experimental animals show that we still lack a scientific basis for a prediction of human cancer incidence after exposure to inducing agents. Many more *in vivo* experiments are needed before any further conclusions can be drawn.

REFERENCES

1. Alexandrov, K. Effects of Inducers and Inhibitors of the Benzo(*a*)pyrene Hydroxylase of Isolated Rat Liver Nuclei and Nuclear Envelopes on the Binding of Benzo(*a*)pyrene to DNA. European J. Cancer, *13:* 847–853, 1977.
2. 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.
3. Buty, S. G., Thompson, S., and Slaga, T. J. The Role of Epidermal Aryl Hydrocarbon Hydroxylase in the Covalent Binding of Polycyclic Hydrocarbon to DNA and Its Relationship to Tumor Initiation. Biochem. Biophys. Res. Commun., *70:* 1102–1108, 1976.
4. Chen, P. S., Toribara, T. Y., and Warner, H. Microdetermination of Phosphorus. Anal. Chem., *28:* 1756–1758, 1956.
5. Cohen, G. M., Haws, S. M., Moore, B. P., and Bridges, J. W. Benzo(*a*)-pyren-3-yl Hydrogen Sulphate, a Major Ethyl Acetate-Extractable Metabolite of Benzo(*a*)pyrene in Human, Hamster and Rat Lung Cultures. Biochem. Pharmacol., *25:* 2561–2570, 1976.
6. Conney, A. H. Pharmacological Implications of Microsomal Enzyme Induction. Pharmacol. Rev., *19:* 317–360, 1967.
7. Heidelberger, C. Chemical Carcinogenesis. Ann. Rev. Biochem., *44:* 79–121, 1975.
8. Jernström, B., Vadi, H., and Orrenius, S. Formation in Isolated Rat Liver Microsomes and Nuclei of Benzo(*a*)pyrene Metabolites that Bind to DNA. Cancer Res., *36:* 4107–4113, 1976.
9. Kaplowitz, N., Kuhlenkamp, J., and Clifton, G. Drug Induction of Hepatic Glutathione *S*-Transferases in Male and Female Rats. Biochem. J., *146:* 351–356, 1975.
10. Kouri, R. E. Relationship Between Levels of Aryl Hydrocarbon Hydroxylase Activity and Susceptibility to 3-Methylcholanthrene and Benzo(*a*)pyrene-Induced Cancers in Inbred Strains of Mice. *In:* R. Freudenthal and P. W. Jones (eds.), Carcinogenesis—A Comprehensive Survey, Vol. 1, pp. 139–151. New York: Raven Press, 1976.
11. Lutz, W. K., Viviani, A., and Schlatter, C. Nonlinear Dose-Response Relationship for the Binding of the Carcinogen Benzo(*a*)pyrene to Rat Liver DNA *in Vivo*. Cancer Res., *38:* 575–578, 1978.
12. Markov, G. G., and Ivanov, I. G. Hydroxyapatite Column Chromatography in Procedures for Isolation of Purified DNA. Anal. Biochem., *59:* 555–563, 1974.
13. Nebert, D. W., and Gelboin, H. V. Substrate-Inducible Microsomal Aryl Hydroxylase in Mammalian Cell Culture. I. Assay and Properties of Induced Enzyme. J. Biol. Chem., *243:* 6242–6249, 1968.
14. Oesch, F. Differential Control of Rat Microsomal "Aryl Hydrocarbon" Monooxygenase and Epoxide Hydratase. J. Biol. Chem., *251:* 79–87, 1976.
15. Pezzuto, J. M., Lea, M. A., and Yang, C. S. The Role of Microsomes and Nuclear Envelope in the Metabolic Activation of Benzo(*a*)pyrene Leading to Binding with Nuclear Macromolecules. Cancer Res., *37:* 3427–3433, 1977.
16. Pezzuto, J. M., Yang, C. S., Yang, S. K., McCourt, D. W., and Gelboin, H. V. Metabolism of Benzo(*a*)pyrene and (−)-*trans*-7,8-Dihydroxy-7,8-dihydrobenzo(*a*)pyrene by Rat Liver Nuclei and Microsomes. Cancer Res., *38:* 1241–1245, 1978.
17. Rogan, E. G, and Cavalieri, E. 3-Methylcholanthrene-Inducible Binding of Aromatic Hydrocarbons to DNA in Purified Rat Liver Nuclei. Biochem. Biophys. Res. Commun., *58:* 1119–1126, 1974.
18. Viviani, A., and Lutz, W. K. Time Course of the Induction of Aryl Hydrocarbon Hydroxylase in Rat Liver Nuclei and Microsomes by Phenobarbital, 3-Methylcholanthrene, 2,3,7,8-Tetrachloro-Dibenzo-p-Dioxin, Dieldrin and Other Inducers. Biochem. Pharmacol., in press, 1978.
19. Wattenberg, L. W., and Leong, J. L. Inhibition of the Carcinogenic

Action of Benzo(a)pyrene by Flavones. Cancer Res., *30:* 1922-1925, 1970.
20. Wishart, G. J., and Fry, D. J. Uridine Diphosphate Glucuronyl-transferase Activity in Nuclei and Nuclear Envelopes of Rat Liver and Its Apparent Induction by Phenobarbital. Biochem. Soc. Trans., *5:* 705-706, 1977.
21. Yang, S. K., McCourt, D. W., Roller, P. P., and Gelboin, H. V. Enzymatic Conversion of Benzo(a)pyrene Leading Predominantly to the Diol-epoxide *r*-7,*t*-8-Dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydro-benzo(a)pyrene through a Single Enantiomer of *r*-7,*t*-8-Dihydroxy-7,8-dihydrobenzo-(a)pyrene. Proc. Natl. Acad. Sci. U. S., *73:* 2594-2598, 1976.