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INDUCTION OF BENZPYRENE HYDROXYLASE BY FLAVONE AND ITS DERIVATIVES IN FETAL RAT LIVER EXPLANTS

K. R. CUTRONEO, R. A. SEIBERT and EDWARD BRESNICK

Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Ga. 30902, U.S.A.

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Abstract—Fetal rat liver organ culture affords a system for the comparison of the mechanism(s) of induction* of benzpyrene (BP) hydroxylase by flavones and 3-methylcholanthrene (3-MC). Flavone and β-naphthoflavone (BNF) were equally potent inducers at 10^{-5} M. 4′-halogenated flavone derivatives proved even more effective in this regard; flavanone and the naturally occurring flavone, tangeretin, were inactive. Although BP hydroxylase activity was inhibited by BNF, 3-MC or 4′-bromoflavone when added to homogenates of fetal rat liver explants, no interference by the inducer was observed under the experimental conditions employed in this study. When equal amounts of the induced and control enzyme were mixed, no less than additive enzyme activity was observed. Flavone derivatives and 3-MC appear to act by a similar mechanism in elevating BP hydroxylase, since combinations of inducers at unsaturating levels resulted in an additive effect, while at saturating levels, enzyme induction was no greater than that of the most potent agent alone.

THE ACTIVITY and amount of the microsomal mixed function oxidases which effect the metabolism of many polycyclic hydrocarbons and other foreign compounds[1–3] determine at least in part the response of an organism to both pharmacologically and toxicologically active substances. One of the characteristics of this enzyme system is its response to the administration of various substrates of the biotransformation reaction, i.e. the enzyme activity is elevated.[4] Thus, the inducibility* of this enzyme system in mammalian tissues may play an important role as a protective mechanism against ingested and inhaled environmental pollutants.

Benzpyrene[2] (BP) hydroxylase catalyzes the conversion of BP to a number of hydroxy and quinone derivatives.[5] This microsomal mixed function oxidase is inducible by polycyclic hydrocarbons *in vivo*,[4,6–8] in rat lung organ culture,[9] in hamster cell culture,[10] and in fetal rat liver explants.† Furthermore, BP hydroxylase activity is elevated in the placentas of women who have a history of cigarette smoking.[6,11,12]

The flavones, a class of polycyclic structures many of which occur naturally in a wide variety of plants, possess the same spectrum of inducing activity as 3-methylcholanthrene (3-MC),[9,13] yet lack the carcinogenic potential.[13] Consequently, a more intensive study was undertaken of the effect of flavone and of its derivatives upon BP hydroxylase in fetal rat liver explants. These studies are reported in this manuscript.

* The term "induction" is used in a general sense to describe an elevation in enzyme activity, without having any genetic connotations.

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MATERIALS AND METHODS

Materials. Pregnant Sprague–Dawley rats (18–20 days of gestation) obtained from the Holtzman Rat Co., Wis., were used throughout this investigation. β-Naphthoflavone (BNF) and 3-MC were obtained from the Aldrich Chemical Co., Wis., and Eastman Organic Chemicals, N.Y. respectively. *O*-hydroxyacetophenone, *p*-iodobenzoylchloride, *p*-bromobenzoylchloride and *p*-chlorobenzoylchloride were purchased from Eastman Organic Chemicals and were used in the preparation of the 4′-halogenated flavone derivatives.[14] The purity of BNF was assessed by thin-layer chromatography on Silica gel-coated plastic foils (with fluorescent indicator) that had been purchased from Eastman Organic Chemicals using toluene–ethyl formate–formic acid, 50:40:10 (by vol.) as a developing solvent. The purity of 4′-bromoflavone (BrF), m.p. 177–179°, was determined by mass spectroscopy and by melting point. The purity of the 4′-chloroflavone (CLF), m.p. 187–188°, and 4′-iodoflavone (IF), m.p. 168–169°, was partly assessed by the narrow range of melting points. We thank Mr. M. K. Veldhuis of the U.S. Fruit and Vegetable Products Laboratory, Winter Haven, Fl., for supplying (tangeretin) 4′, 5, 6, 7, 8 pentamethoxy-flavone (PMF).

Fetal liver organ culture. A modification of the explant technique of Wicks[15] was employed. This technique was learned by one of us (E.B.) in the laboratory of Dr. W. D. Wicks and the authors are grateful for this opportunity. One or two pregnant rats were sacrificed by decapitation, and the two uterine horns with the intact fetuses were removed. All other operations were carried out under sterile conditions. The fetuses were removed from the amniotic sacs and the livers were extirpated. The fetal livers were cut into 2-mm cubes with a scalpel blade and placed immediately into sterile plastic petri dishes containing sterile Eagle's minimal essential medium (MEM) with Hanks' balanced salt solution (Grand Island Biological Co.), pH 7·4,2 × glucose and 2 × $NaHCO_3$, 0·05 M tricine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cubes were washed twice with medium and randomized. Seven cubes (approximately 2 mg protein) were placed on stainless steel grids (prepared from mesh of No. 16 wire diameter) in plastic culture dishes. The dishes were then vapor-sealed, and the cultures were incubated at 37° in an atmosphere of 95% air–5% CO_2 in a humidified Napco incubator. The pH of the medium was maintained at 7·4 with 0·5 N NaOH. The fetal liver explants were preincubated for 24 hr prior to the addition of drugs. The drugs, dissolved in dimethylsulfoxide (DMSO), were added to the medium in a final DMSO concentration of 0·1 or 0·2% (v/v). Control cultures were treated with the same volume of DMSO. These concentrations of DMSO produced no demonstrable effects on BP hydroxylase activity. Following incubation, the liver tissue was rinsed in 0·9% NaCl (w/v) and homogenized in 1·1 ml of cold 0·25 M sucrose by use of a plastic plexiglass homogenizer with a teflon coated pestle.

Enzyme assay. BP hydroxylase activity was assayed by a modification of the method of Nebert and Gelboin.[10] The reaction mixture in a total volume of 1 ml contained 50 μmoles of Tris–HCl buffer (pH 7·5), 0·54 μmole of NADPH, 3 μmoles of $MgCl_2$, 0·32 μmole of glucose 6-phosphate, 0·6 unit of glucose 6-phosphate dehydrogenase, 80 mμmoles of BP in 50 μl of DMSO and 0·4 ml of fetal liver homogenate (0·7–1·2 mg protein). Control and induced enzyme activity was shown to be linear to 3·0 mg protein. Although DMSO has been reported to effect BP hydroxylase activity when added *in vitro*,[16] the addition of DMSO up to 100 μl to our incubation mixture exerted no demonstrable effect on enzyme activity. The samples were incubated in the dark for

10 min at 37°. After incubation, the tubes were placed in an ice bath and the reaction was stopped by the addition of 1 ml of acetone and 3·4 ml of hexane. The samples were shaken for 20 min at room temperature and centrifuged for 10 min. The hexane phase (2 ml) was placed in 3·0 ml of 1 N NaOH, shaken for 1 min and centrifuged for 5 min. The alkaline phase was read in an Aminco spectrophotofluorometer at activation and fluorescence wavelengths of 400 and 522 nm respectively. Quinine sulfate in 0·1 N H_2SO_4 was used as a reference standard and read at activation and fluorescence wavelengths of 352 and 452 nm respectively. One unit of enzyme activity was arbitrarily defined as the amount of product with a fluorescence equivalent of 1 ng of 3-hydroxy-BP formed during the 10-min incubation at 37°. Enzyme activity was proportional to both amount of enzyme and time of incubation. Authentic 3-hydroxy-BP for calibration of our assay was kindly supplied by Dr. H. V. Gelboin of the National Cancer Institute.

Determination of "soluble" BNF and BrF. Either BNF or BrF in DMSO was added to 4 ml of media. The mixture was then filtered through a 0·45 μ Millipore filter. The filter was washed with two equal volumes of deionized water, air-dried, dissolved in 4 ml of ethyl acetate, centrifuged at room temperature and read at 320 nm. A blank consisting of 4 ml of medium was treated similarly; the absorbance at 320 nm (A_{320}) of the blank filter was 0·246. The reproducibility of this method at a particular concentration was within 10 per cent over a broad range of concentrations of BNF and BrF. The amount of "soluble" flavone derivative was determined by subtracting the amount of the compound on the filter from the amount originally added to the medium (as determined from standard curves of the flavone derivatives in DMSO).

Protein determination. The amount of protein of the homogenates was determined by the method of Lowry *et al.*[17] using bovine serum albumin as the reference standard.

RESULTS

The ability of flavones to induce BP hydroxylase in preincubated fetal liver explants was determined (Fig. 1). Addition of flavone (F) and BNF to preincubated cultures resulted in a 5 to 6-fold increase in enzyme activity in 24 hr. The halogenated derivatives were the most potent inducers tested. Treatment of explants with either the 4′-bromo, iodo, or chloro derivative elicited a 10 to 11-fold increase in enzyme activity. The introduction of a bromo moiety in the 4′ position of flavone more than doubled the inducing activity in liver and lung tissue after intubation of the drug to female rats.[13] Treatment of fetal liver explants with BrF was accompanied by more than twice the induction of BP hydroxylase as was observed with BNF. PMF, a naturally occurring methoxy derivative, and flavanone (FLAV), the unsubstituted compound, had no inducing activity in fetal liver explants.

Addition of α-naphthoflavone, i.e. 7,8-benzoflavone, and BNF to homogenates of mouse skin was reported to inhibit BP hydroxylase activity.[18] In Table 1, the inhibition is shown of the control and induced enzyme systems of fetal rat liver explants by BNF, BrF and 3-MC. BNF proved the most potent inhibitor of enzyme activity. The possibility existed that the flavones may inhibit enzyme activity when assayed *in vitro* which would make comparison of their relative inducing capacities difficult to quantitate. However, no interference in the enzyme assay *in vitro* by the inducer was observed

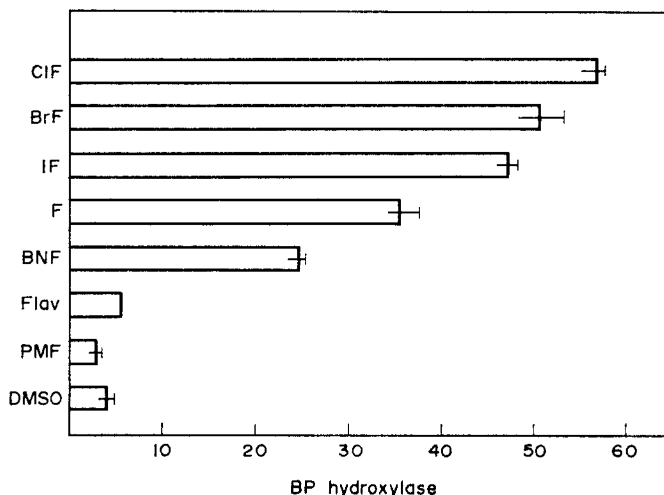

FIG. 1. BP hydroxylase. Cultures were preincubated for 24 hr and treated with either DMSO at a final concentration of 0·1% (v/v) or drugs (10^{-5} M) for 24 hr. Bars represent the mean ± S.E. of enzyme activities of three cultures (expressed as units per milligram of protein).

(Table 2). This was determined by the mixing of equal amounts of control and induced enzyme preparations; no less than additive enzyme activity was observed.

F and its derivatives were added to 24-hr preincubated cultures at doses ranging from 10^{-9} to 10^{-5} (Figs. 2 and 3). PMF at all doses tested was inactive (Fig. 2). Although BNF and F produced approximately the same fold induction of enzyme activity at 10^{-5} M, significant induction was observed at 10^{-7} M with only the former.

TABLE 1. EFFECT OF 3-METHYLCHOLANTHRENE, β-NAPHTHOFLAVONE AND 4'-BROMOFLAVONE ON BP HYDROXYLASE ACTIVITY *in vitro**

Addition	Per cent inhibition	
	Control	Induced
BNF	44	58
3-MC	29	34
BrF	29	39

* All agents were added to the assay system at a final molar concentration of $5{\cdot}5 \times 10^{-5}$ M just prior to incubation. The values represent the mean of three determinations. The induced enzyme was prepared by preincubating cultures for 24 hr followed by incubation for 24 hr with 4'-bromoflavone at 10^{-5} M. The control enzyme refers to BP hydroxylase in 48-hr incubated cultures. At 48 hr after explantation, liver homogenates, 3% (w/v), were prepared with 0·25 M sucrose.

TABLE 2. EFFECT OF MIXING CONTROL AND INDUCED ENZYME PREPARATIONS ON BP HYDROXYLASE ACTIVITY *in vitro**

Enzyme source	BP hydroxylase (units/mg protein)
DMSO	2·8
BrF-induced	18·0
DMSO + BrF-induced	20·7

* All cultures were preincubated for 24 hr and incubated with either DMSO at 0·1% or BrF (4'-bromoflavone) at 10^{-5} M final concentration for the next 24 hr. Cultures were then homogenized in 0·25 M sucrose, and BP hydroxylase activity of a 0·4-ml aliquot (0·85–0·9 mg protein) of control and induced samples was determined. Aliquots (0·4 ml) of the control and induced enzyme systems were mixed, and BP hydroxylase activity of the mixture was determined.

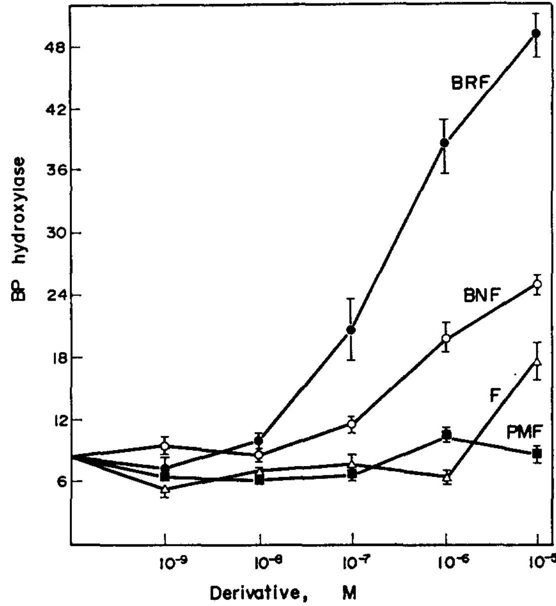

FIG. 2. Dose–response of induction of BP hydroxylase by flavone and derivatives. Explants of fetal liver were preincubated for 24 hr and then either DMSO at a final concentration of 0·1% (v/v) or the flavone derivatives were added at the specified molar concentration. Each point represents the mean ± S.E. of enzyme activities of three cultures. Enzyme activity is expressed as units per milligram of protein.

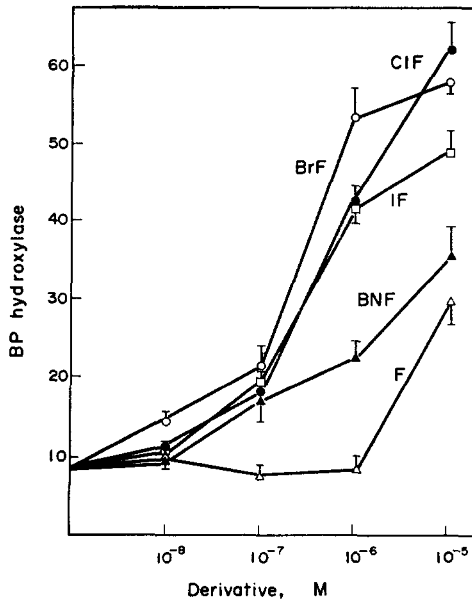

FIG. 3. Dose–response of induction of BP hydroxylase by flavone and derivatives. See legend to Fig. 2. Enzyme activity is expressed as units per milligram of protein.

A very dramatic elevation of enzyme activity was observed between 1×10^{-6} M and 1×10^{-5} M of F (Fig. 3).

Since the inducing capacity of a compound is related to the amount of inducer in the medium of organ culture, the relative solubilities of BrF and BNF were determined (Table 3). The amount of inducer initially added to the medium was directly related to the absolute amount of "soluble" inducer and was inversely related to the per cent of inducer which was "soluble". At final molar concentrations above $1{\cdot}38 \times 10^{-5}$ M, BNF and BrF were equally "soluble" in the medium. Although both compounds are equally soluble in the medium, BrF is a much more potent inducer than BNF at 10^{-6} and 10^{-5} M (see Figs. 2 and 3).

TABLE 3. SOLUBILITY OF β-NAPHTHOFLAVONE AND 4′-BROMOFLAVONE IN THE MEDIUM*

Molarity ($\times 10^5$)	Per cent "soluble"	
	BNF	BrF
0·55	88	96
1·38	51	61
2·75	53	55
5·5	44	41
11·0	36	40

* Either BNF or BrF dissolved in DMSO was added to 4 ml of media to give the greatest final molar concentrations. After filtering through a 0·45 μ Millipore, the amount of BNF or BrF remaining on the filter was determined as described in Materials and Methods. The per cent "soluble" was calculated by dividing the amount of BNF or BrF passing through the filter by the amount added to the culture and multiplying this value by 100. Under identical conditions, 3-MC when added at 10^{-5} M was "soluble" to the extent of 10^{-7} M.

Because of the similar spectrum of induction of BNF and 3-MC,[9,13]* we determined the effect of simultaneous administration of BNF, 3-MC and BrF on the induction of BP hydroxylase. If 3-MC and the flavones act through the same mechanism, then the net effect of a combination of the two substances would be a function of their affinities for the target of induction and their intrinsic stimulatory activities. At saturating levels of inducers, i.e. 1×10^{-5} M, the total elevation should be equal to that of the most potent agent as indicted in Fig. 4. The level of induction expressed as BP hydroxylase activity in treated cultures minus BP hydroxylase activity in control cultures was no greater than the level obtained with the most potent agent alone. On the other hand, at unsaturating doses, the level of induction should be equal to the additive effects of the individual agents alone. These results are seen in Fig. 4. The data suggest that 3-MC, BNF and BrF act through a similar mechanism upon the same cellular target for induction of BP hydroxylase in fetal rat liver explants.

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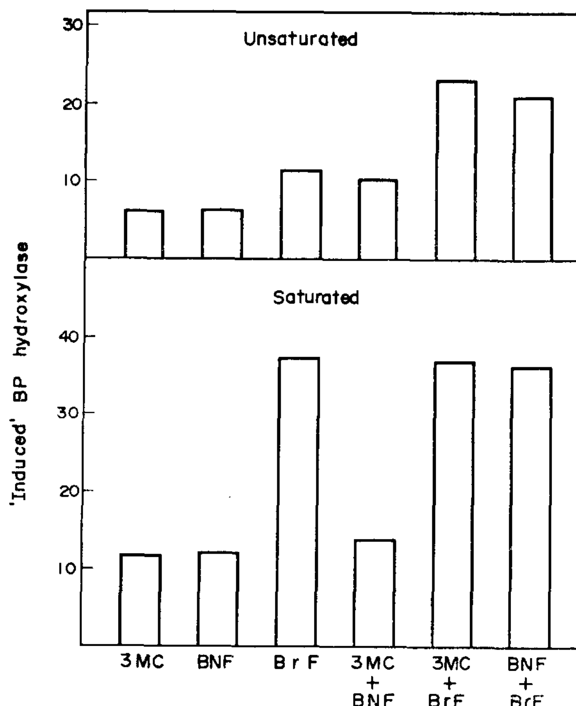

FIG. 4. Induction of BP hydroxylase by simultaneous administration of either 3-MC and BNF, 3-MC and BrF or BNF and BrF. Explants were preincubated for 24 hr and incubated with either DMSO (0·1 %, v/v) or the drug combination specified. Drugs were either given at unsaturating level, i.e. 3×10^{-7} M (top) or at saturating level, i.e. 1×10^{-5} M (bottom). Each bar represents the induced enzyme (units per milligram of protein) which was calculated by subtracting the mean enzyme activity of control cultures from the mean activity of treated cultures. Each group consisted of three cultures.

DISCUSSION

The reliance on studies *in vivo* to estimate the inducing capacity of a series of compounds could be complicated by absorption, distribution and metabolism of the inducer, nutritional and hormonal variables and systemic toxicity in the whole animal. Thus, these factors may lead to ambiguities in determining the relative inducing potential of a series of compounds.

The ability of flavone derivatives to inhibit chemical-induced carcinogenesis has been reported.[18–20] Wattenberg and Leong[19] have reported that BNF inhibits lung and mammary gland tumor formation induced by 9,10-dimethylbenzanthracene (DMBA), pulmonary adenoma formation[19] and epidermal neoplasia induced by BP.[20] These workers felt that the mixed function oxidase, BP hydroxylase, converted the polycyclic hydrocarbon to weakly carcinogenic or noncarcinogenic hydroxy derivatives.[5,21,22] Consequently, BP hydroxylase would function in detoxification. On the other hand, Gelboin *et al.*[23] have correlated the inhibition of BP hydroxylase activity *in vitro* by the non-inducer, 7,8-benzoflavone, after topical application with inhibition of DMBA-induced mouse skin tumorigenesis. Gelboin *et al.*[23] and Alfred and Gelboin[24] feel that 7,8-benzoflavone inhibited the high level of the polycyclic hydrocarbon-induced enzyme which then prevented the activation of DMBA to potent

carcinogenic metabolites. Determination of the capacity of flavone derivatives to induce BP hydroxylase activity becomes extremely important in regard to the tumorigenic responsiveness of the target tissue to various carcinogenic polycyclic hydrocarbons. Consequently, it is of prime importance to ascertain whether the action of BP hydroxylase is required for the formation of a proximal carcinogen or in detoxification.

The inducing spectrum of flavone and its derivatives of BP hydroxylase is quite different *in vitro* in fetal liver organ culture (Fig. 1) as compared to the administration *in vivo* to rats.[13] The presence of a halogen at the para position of the phenyl ring of the flavone markedly enhanced induction in both fetal liver organ culture and in lung and in liver tissue after administration to rats. BrF elicited as much or a little lower induction of BP hydroxylase as compared to that observed after BNF treatment.[13] After testing *in vivo*, BNF was the most potent inducer of the flavones.[13] However, when added to liver organ culture, BrF as well as the other 4′-halogenated derivatives proved more potent in this regard. These differences may be due to absorption or metabolism differences which would result in different efficacies of induction of these two inducers *in vivo* and in culture.

Another flavone derivative which showed a marked dissimilarity of induction of BP hydroxylase *in vivo* and in liver organ culture was tangeretin. This naturally occurring flavone was shown to be a moderate inducer of BP hydroxylase in liver and lung tissue after administration to rats.[13] Since quercetin pentamethyl ether which has an inducing capacity and structural similarities to the naturally occurring flavones inhibited pulmonary adenoma formation, the possibility existed that dietary factors might alter an organism's responsiveness to environmental carcinogenic pollutants. However, no data have been presented concerning the amount of tangeretin in the diets of man and animals.[20] In liver organ culture, tangeretin was not an effective inducer of BP hydroxylase activity over a wide dose range (Fig. 2). Thus, the ability of this naturally occurring flavone to induce BP hydroxylase activity in tissues immediately exposed to ingested carcinogenic polycyclic hydrocarbons, i.e. lung and intestine, and thereby to inhibit carcinogenesis, would appear unlikely without possible systemic metabolic alteration to an active inducer.

In conclusion, the ability of a number of flavone derivatives to induce BP hydroxylase activity in fetal rat liver organ culture has been reported. Marked differences exist between the inductive capacities of various flavone derivatives after administration to rats[13] and after addition to fetal liver explants. The possibility exists that differences observed in liver tissue *in vivo* and in culture were due to the fetal stage of development of the tissue in organ culture. Likewise, the induction of BP hydroxylase in lung and liver tissue after administration of the flavones to rats may be complicated by such factors as systemic toxicity, hormonal interactions, absorption, and distribution of inducer and metabolism of the inducer by tissues other than the one under study. Such complications may be eliminated by a systematic study of the inducing capacities of the flavones in fetal liver organ culture as has been presented in this report.

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REFERENCES

1. A. H. Conney and J. J. Burns, *Adv. Pharmac.* **1,** 31 (1962).
2. A. H. Conney, R. G. Davidson and J. J. Burns, *J. Pharmac. exp. Ther.* **130,** 1 (1960).
3. H. Remmer, in *First International Pharmacological Meeting*, p. 235. Macmillan, New York (1962).
4. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
5. A. H. Conney, E. C. Miller and J. A. Miller, *J. biol. Chem.* **228,** 753 (1957).
6. R. M. Welch, Y. E. Harrison, B. W. Gommi, P. J. Poppers, M. Finster and A. H. Conney, *Clin. Pharmac. Ther.* **10,** 100 (1969).
7. H. V. Gelboin and N. R. Blackburn, *Cancer Res.* **24,** 356 (1964).
8. D. W. Nebert and H. V. Gelboin, *Archs Biochem. Biophys.* **134,** 76 (1969).
9. L. W. Wattenberg, J. L. Leong and A. R. Galbraith, *Proc. Soc. exp. Biol. Med.* **127,** 467 (1968).
10. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **245,** 160 (1970)
11. R. M. Welch, Y. E. Harrison, A. H. Conney, P. J. Poppers and M. Finster, *Science, N.Y.* **160,** 541 (1968).
12. D. W. Nebert, J. Winker and H. V. Gelboin, *Cancer Res.* **29,** 1763 (1969).
13. L. W. Wattenberg, M. A. Page and J. L. Leong, *Cancer Res.* **28,** 934 (1968).
14. T. S. Wheeler, in *Organic Synthesis*, Vol. 32, p. 72. John Wiley, New York (1952).
15. W. D. Wicks, *J. biol. Chem.* **243,** 900 (1968).
16. F. Wiebel, L. Diamond and H. V. Gelboin, *Proc. Am. Ass. Cancer Res.* **11,** 84 (1970).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
18. H. V. Gelboin, F. Wiebel and L. Diamond, *Science, N.Y.* **170,** 169 (1970).
19. L. W. Wattenberg and J. L. Leong, *Proc Soc. exp. Biol. Med.* **128,** 940 (1968).
20. L. W. Wattenberg and J. L. Leong, *Cancer Res.* **30,** 1922 (1970).
21. I. Berenblum and R. Schoental, *Cancer Res.* **3,** 145 (1943).
22. J. W. Cook and R. Schoental, *Br. J. Cancer* **6,** 400 (1952).
23. H. V. Gelboin, E. Huberman and L. Sachs, *Proc. natn Acad. Sci. U.S.A.* **64,** 1188 (1969).
24. L. J. Alfred and H. V. Gelboin, *Science, N.Y.* **157,** 75 (1967).