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SPECIES DIFFERENCES IN THE SUBSTRATE SPECIFICITY OF HEPATIC CYTOCHROME P-448 FROM POLYCYCLIC HYDROCARBON-TREATED ANIMALS*

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Abstract—The *in vitro* effects of α-naphthoflavone on four hepatic mono-oxygenase activities associated with aromatic hydrocarbon responsiveness in the mouse (aryl hydrocarbon hydroxylase, 2-acetylaminofluorene *N*-hydroxylase, biphenyl 2-hydroxylase, and biphenyl 4-hydroxylase) were investigated before and after methylcholanthrene treatment of C57BL/6N and DBA/2N mice, rats, hamsters, guinea pigs and rabbits. The electrophoretic pattern of cytochrome P-450 subunits and reduced CO–hemoprotein difference spectra of the microsomal fractions were also studied. Pretreatment of animals with methylcholanthrene caused: (1) a 1.5 to 2 mm hypsochromic shift in the Soret peak of the reduced hemoprotein–CO complexes in liver microsomes from a C57BL/6N mouse, rat, hamster and rabbit; a 0.5-nm hypsochromic shift in the guinea pig and no shift in the DBA/2N mouse; and (2) an increase in cytochrome P-450 apoproteins of the following molecular weights on sodium dodecyl sulfate–polyacrylamide gel electrophoresis: 54,000 and 55,000 in the C57BL/6N mouse; 48,000, 54,000 and 55,000 in the rat; 49,000 and 54,000 in the hamster; and 54,000 and 57,000 in the rabbit; a small increase in the 54,000 band was seen in the DBA/2N mouse and no increase in the guinea pig. *In vitro* addition of α-naphthoflavone selectively inhibited all four mono-oxygenase activities from the methylcholanthrene-treated C57BL/6N mouse, rat and hamster; 2-acetylaminofluorene *N*-hydroxylase and biphenyl 4-hydroxylase activities in the rabbit; and aryl hydrocarbon hydroxylase, 2-acetylaminofluorene *N*-hydroxylase and biphenyl 4-hydroxylase activities in the guinea pig. The addition of α-naphthoflavone enhanced the activities of aryl hydrocarbon hydroxylase and biphenyl 2-hydroxylase in liver microsomes from both control and methylcholanthrene-treated rabbits, but only biphenyl 2-hydroxylase activity was increased in the guinea pig: the activitity of 2-acetylaminofluorene *N*-hydroxylase was increased in both control and methylcholanthrene-treated DBA/2M mice, but only in the control C57BL/6N mouse. These data indicate that hepatic cytochrome P-448 is composed of multiple cytochromes, which differ among animal species, each catalyzing different mono-oxygenase activities.

In recent years substantial evidence has accumulated indicating that multiple forms of hepatic cytochrome P-450 exist in most experimental animals, and presumably also in man. Most of this evidence stems from studies on CO-difference spectra, ethyl isocyanide difference spectra, substrate specificity, immunochemical differences, and sodium dodecyl sulfate gel electrophoresis patterns in liver microsomes or partially purified cytochrome P-450 fractions from animals pretreated with inducers such as polycyclic hydrocarbons and phenobarbital [1–16]. In mice, the genetic trait of aromatic hydrocarbon responsiveness refers to the capacity of various polycyclic hydrocarbons to induce hepatic cytochrome(s), which is characterized by a 2-nm hypsochromic shift in the Soret maximum of the CO–hemoprotein complex (cytochrome P-448), and an increase in two microsomal polypeptides (having molecular weights of approximately 54,000 and 55,000) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis as well as increases in numerous mono-oxygenase activities [17]. In the mouse these polycyclic hydrocarbon-induced mono-oxygenase activities can be selectively inhibited *in vitro* by α-naphthoflavone [18]. Studies on polycyclic hydrocarbon induction in adult rabbit liver have shown that, despite the occurrence of the 2-nm shift in the Soret maximum and an increase in the 54,000 mol. wt. electrophoretic band, only two of the mono-oxygenases activities that are increased in the responsive mouse are similarly induced [19, 20]. These data suggest that more than one form of cytochrome may be responsible for the mono-oxygenase activities catalyzed by cytochrome P-448 in different species. We sought, therefore, to determine whether the pattern of α-naphthoflavone inhibition of selected mono-oxygenase activities (i.e. aryl hydrocarbon

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hydroxylase, 2-acetylaminofluorene *N*-hydroxylase, biphenyl 4-hydroxylase, and biphenyl 2-hydroxylase) associated with aromatic hydrocarbon responsiveness in the mouse could be used to differentiate among the various forms of cytochrome P-448 in mice, rats, hamsters, guinea pigs and rabbits. In addition, the electrophoretic patterns of liver microsomal proteins from control and methylcholanthrene-treated animals were compared. The results of these studies indicate that cytochrome P-448 is composed of several hemoproteins.

MATERIALS AND METHODS

Chemicals. Materials, and their sources, were as follows: biphenyl, 2-hydroxybiphenyl, 4-hydroxybiphenyl and 2-acetylaminofluorene were obtained from Eastman Kodak Co. (Rochester, NY); benzo[a]pyrene, bovine serum albumin fraction V, bovine liver catalase, bovine liver glutamate dehydrogenase, ovalbumin, and NADH were purchased from Sigma (St. Louis, MO); methylcholanthrene from J. T. Baker Chemical Co. (Phillipsburg, NJ); NADPH from CalBiochem (LaJolla, CA); sodium dodecyl sulfate electrophoresis purity from BDH Chemicals, Ltd. (Poole, England); α-naphthoflavone (7,8-benzoflavone) from Aldrich Chemical Co. (Milwaukee, WI); and acrylamide and *N,N*-methylene-bis-acrylamide from Bio-Rad Laboratories (Rockville Center, NY). All polycyclic hydrocarbon and flavones were recrystallized from benzene–methanol prior to use. [9-^{14}C]-2-acetylaminofluorene (10.5 mCi/m-mole), purchased from New England Nuclear (Boston, MA), was shown to be more than 99.9 per cent pure by thin-layer chromatography (silica gel GF plates using the chromatographic solvent system of chloroform–methanol, 97:3 vol./vol.). *N*-hydroxy-2-acetylaminofluorene was a generous gift from Dr. E. Weisburger, National Cancer Institute, Bethesda, MD. All other reagents and chemicals used were of the highest grade and purity obtainable.

Animals. The inbred strains of male mice, C57BL/6N (B6) and DBA/2N (D2) (15–18 g), as well as male Sprague–Dawley rats (80–110 g), male Syrian hamsters (90–120 g), male guinea pigs (100–130 g), and male 35 day-old New Zealand white rabbits (2.0 to 2.5 kg), where obtained from the National Institutes of Health Veterinary Resources Branch.

Treatment of animals. Animal room conditions and the preparation of liver microsomes have been described previously [21]. Methylcholanthrene-treated animals received an intraperitoneal injection of 80 mg kg^{-1} of the inducer in corn oil 48 hr prior to death and control animals received an equivalent volume of corn oil alone.

Analytical methods. Microsomal *N*-acetylarylamine *N*-hydroxylase [21, 22], aryl hydrocarbon hydroxylase [18], biphenyl 2-hydroxylase [23], and biphenyl 4-hydroxylase [23] activities were determined by methods described in the references cited.

The pH optima for these enzymes in liver microsomes from rats, hamsters and guinea pigs were not significantly different from those in mice and rabbits [19, 22]. In each case, one unit of activity is arbitrarily defined as that amount of enzyme catalyzing, per min at 37°, the formation of 1 pmole *N*-hydroxy-2-acetylaminofluorene, 3-hydroxybenzo[a]pyrene, 2-hydroxybiphenyl, and 4-hydroxybiphenyl respectively. Specific activities are expressed as units per mg of microsomal protein.

Microsomal cytochrome P-450 content was estimated according to Omura and Sato [24] using an extinction coefficient of 91 mM^{-1}. Wavelength measurements were standardized with the use of a holmium oxide crystal (Fisher Scientific Co. Silver Spring, MD).

The slab gel electrophoresis was performed by the method of Laemmli [25]. Gels (1.5 mm thick) containing ten 1.0-cm wide tracks usually received 30 μg of liver microsomal protein/track and those with twenty 0.5 cm-wide tracks received 15 μg of liver microsomal protein/track. The standards, with their subunit molecular weights, included: ovalbumin, 43,000; glutamate dehydrogenase, 53,000; catalase, 58,000; and albumin, 68,000. The gels were stained with Coomassie Blue R solution (0.2% wt./vol. in methanol–glacial acetic acid–water, 5:1:4) for 1 hr and then destained overnight in methanol–glacial acetic acid–water, 10:3:27 [26]. Protein was determined according to the method of Lowry *et al.* [27].

RESULTS

Methylcholanthrene induction of cytochrome P-450 content and mono-oxygenase activities in liver microsomes from mouse, rat, hamster, guinea pig and rabbit. The effects of methylcholanthrene treatment on aryl hydrocarbon hydroxylase, 2-acetylaminofluorene-*N*-hydroxylase and biphenyl hydroxylase activities in the various species under consideration are summarised in Table 1. The aryl hydrocarbon hydroxylase induction was most pronounced (6- to 10-fold) in the B6 mouse and rat, whereas the hydroxylase induction was small (≤2-fold) in hamster, guinea pig and rabbit, and no induction was observed in the D2 mouse. Methylcholanthrene induction of 2-acetylaminofluorene *N*-hydroxylase was 4- to 8-fold in B6 mice, rats, hamsters and rabbits, but no induction was observed in D2 mice and guinea pigs. The activities of biphenyl 2- and 4-hydroxylases were induced to the same degree in B6 mice and rats, but only the 2-hydroxylase was induced in the hamsters and 4-hydroxylase in the guinea pigs. Both the 2- and 4-hydroxylases were induced in the rabbits, but the activity of the 2-hydroxylase was low (< 50 and 126 pmoles mg^{-1} min^{-1} in control and methylcholanthrene microsomes respectively). No induction of the biphenyl hydroxylases was observed in the D2 mice.

Cytochrome P-450 content was increased in B6 mice, rats, hamsters and rabbits after methylcholanthrene administration, but no increase in cytochrome P-450 content was observed in D2 mice and guinea pigs. The Soret maxima of the reduced cytochrome–CO complex in liver microsomes from mice, rats, hamsters, guinea pigs and rabbits 48 hr after treatment with methylcholanthrene are shown in Table 1. Two-nm hypsochromic shifts from the control Soret maxima (450 nm) were observed in B6 mice, rats and rabbits; a 1.5-nm shift to the blue was observed in hamsters, and no change was seen in D2 mice and guinea pig microsomes.

Inhibition or stimulation by α-naphthoflavone of

Table 1. Methylcholanthrene induction of cytochrome P-450 content and mono-oxygenase activities in liver microsomes from mice, rats, hamsters, guinea pigs and rabbits*

	Enzyme units/Cytochrome P-450											
	AHH		AAF-*N*-OH-ase		BØ-4-OH-ase		BØ:2-OH-ase		Content		Soret maximum (nm)	
Species	Control	MC	Control	MC	Control	MC	Control	MC	Control	MC	Control	MC
B6 mouse	240	1330	108	620	2580	4930	1050	4580	690	1280	450.0	448.0
D2 mouse	220	250	81	65	2330	2210	1060	940	670	820	450.0	450.0
Rat	61	605	60	182	900	1930	<50	290	720	1430	450.0	448.0
Hamster	370	453	127	568	2200	2400	922	1990	650	1230	450.0	448.5
Guinea pig	103	151	2.0	2.7	930	1550	127	152	560	640	450.0	449.5
Rabbit	111	250	97	380	1960	5070	<50	126	810	2050	450.0	448.0

* Enzyme activities and cytochrome P-450 content and spectra were measured according to methods described in Materials and Methods. Each value is the mean of two to six experiments, and the coefficient of variation ranged from 5 to 18 per cent Enzyme activities are expressed as units per mg of microsomal protein and cytochrome P-450 content as pmoles/mg of microsomal protein. Abbreviations are as follows: AHH, aryl hydrocarbon hydroxylase; AAF-N-OH-ase, 2-acetylaminofluorene *N*-hydroxylase; BØ-4-OH-ase, biphenyl 4-hydroxylase; BØ-2-OH-ase, biphenyl 2-hydroxylase; and MC, methylcholanthrene.

enzyme activity from control and methylcholanthrene-treated animals. α-Naphthoflavone has been shown to inhibit preferentially the *in vitro* activity of aryl hydrocarbon hydroxylase from methylcholanthrene-treated rats and genetically responsive mice, and at low concentrations to cause some enhancement of control aryl hydrocarbon hydroxylase activity [28, 29]. In mice, biphenyl 4-hydroxylase activity is inhibited *in vitro* by α-naphthoflavone in a fashion similar to aryl hydrocarbon hydroxylase; that is, inhibition is observed in microsomes from methylcholanthrene-treated responsive (B6) mice, but not in microsomes from control or methylcholanthrene-treated nonresponsive (D2) mice [23]. The α-naphthoflavone inhibition of biphenyl 2-hydroxylase activity differs in that liver microsomes from both control and methylcholanthrene-treated mice are inhibited [23].

Figure 1 shows the effect of α-naphthoflavone *in vitro* on 2-acetylaminofluorene *N*-hydroxylase in liver microsomes from control and methylcholanthrene-treated B6 and D2 mice. The activity of the *N*-hydroxy- in liver microsomes from control B6 and D2 mice was enhanced in a dose-dependent fashion to a maximum of about 3-fold upon *in vitro* addition of α-naphthoflavone (1–500 μM). Similar enhancement of *N*-hydroxylase activity was observed with liver microsomes from methylcholanthrene-treated D2 mice

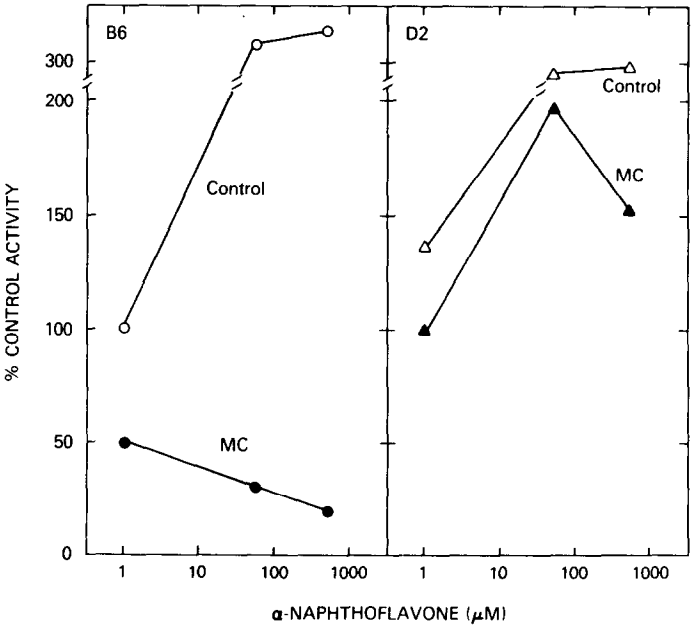

Fig. 1. Effect of α-naphthoflavone *in vitro* on hepatic 2-acetylaminofluorene (2-AAF) *N*-hydroxylase from control (○,△) and methylcholanthrene (MC)-treated (●,▲) C57BL/6N (B6) and DBA(2N (D2) mice. α-Naphthoflavone was added to the incubations in 10 μl demethylsulfoxide, and the 2-AAF *N*-hydroxylase assay was performed as previously described [22]. The absolute values for the enzyme activities in this and subsequent figures are given in Table 1.

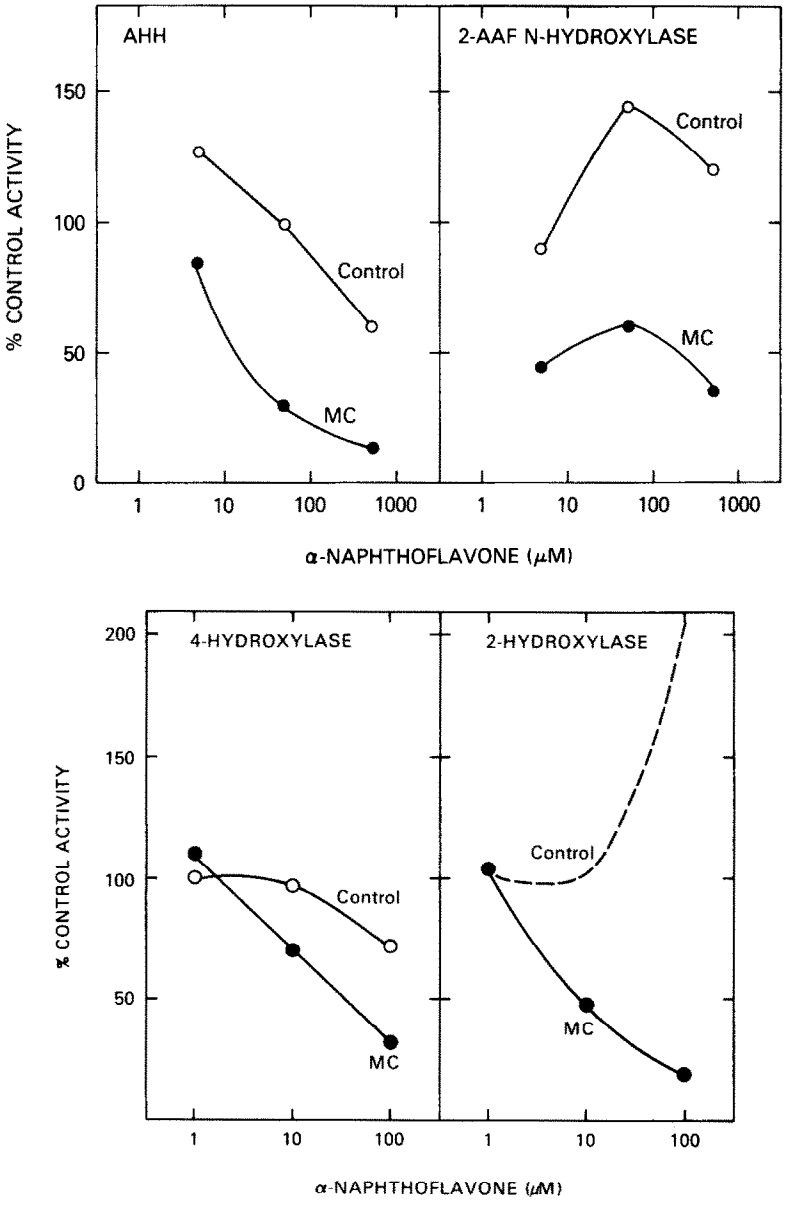

Fig. 2. Effect of α-naphthoflavone *in vitro* on hepatic aryl hydrocarbon hydroxylase (AHH), 2-AAF *N*-hydroxylase (A), and biphenyl 2- and 4-hydroxylases (B) in control (○) and methylcholanthrene (MC)-treated (●) rats. The enzyme assays were performed as described in Materials and Methods.

except at the highest concentration (i.e. 500 μM), where the enhancement was smaller than that seen in control microsomes. In contrast, α-naphthoflavone caused marked inhibition of *N*-hydroxylase activity in liver microsomes from methylcholanthrene-treated B6 mice. Fifty per cent inhibition of *N*-hydroxylase activity was obtained at 1 μM α-naphthoflavone, which is about 5-fold lower than the concentration required for 50 per cent inhibition of aryl hydrocarbon hydroxylase under the same conditions [29].

Figures 2A and 2B shows the effect of α-naphthoflavone on aryl hydrocarbon hydroxylase, 2-acetylaminofluorene *N*-hydroxylase, biphenyl 4-hydroxylase, and biphenyl 2-hydroxylase activities in liver microsomes from control and methylcholanthrene-treated rats. α-Naphthoflavone addition to control microsomes stimulated both aryl hydrocarbon hydroxylase and *N*-hydroxylase activities (Fig. 2A). Aryl hydrocarbon hydroxylase activity was stimulated at a lower concentration of α-naphthoflavone than was the *N*-hydroxylase. The activity of aryl hydrocarbon hydroxylase was inhibited by 30 per cent at the highest concentration of α-naphthoflavone (i.e. 500 μM), whereas the activity of *N*-hydroxylase was still enhanced 20 per cent. However, α-naphthoflavone inhibited both aryl hydrocarbon hydroxylase and *N*-hydroxylase activities in liver microsomes from methylcholanthrene-treated rats. Although the *N*-hydroxy-

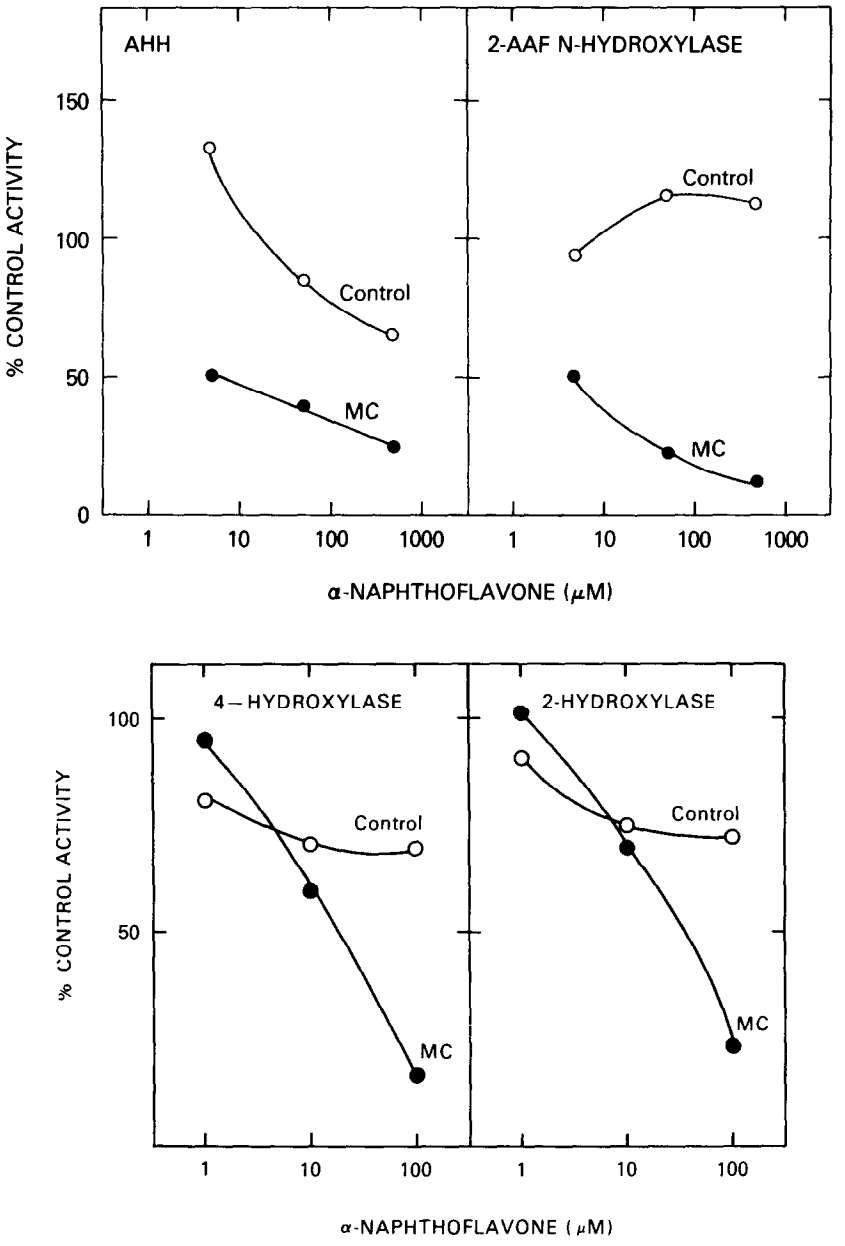

Fig. 3. Effect of α-naphthoflavone *in vitro* on hepatic aryl hydrocarbon hydroxylase (AHH), 2-AAF *N*-hydroxylase (A), and biphenyl 2- and 4-hydroxylases (B) in control (○) and methylcholanthrene (MC)-treated (●) hamsters.

lase was more sensitive than aryl hydrocarbon hydroxylase to α-naphthoflavone inhibition at the lowest concentration, it was somewhat less sensitive at higher concentrations of α-naphthoflavone. Indeed, the profile of *N*-hydroxylase inhibition in microsomes from methylcholanthrene-treated rats was very similar, i.e. enhancement at 50 μM compared with 5 μM α-naphthoflavone, to the inhibition profile (Fig. 2A) observed for *N*-hydroxylase activity in the control microsomes. The effect of α-naphthoflavone on biphenyl 4-hydroxylase (Fig. 2B) was similar to that on aryl hydrocarbon hydroxylase, with differential inhibition of activity in liver microsomes from methylcholanthrene-treated rats. However, unlike aryl hydrocarbon hydroxylase, no enhancement was observed in control microsomes. Similarly, no enhancement of biphenyl 4-hydroxylase activity by α-naphthoflavone was found in liver microsomes from untreated B6 or D2 mice [23]. Biphenyl 2-hydroxylase activity was not measurable in liver microsomes from control rats, but activity was detected in the presence of 100 μM α-naphthoflavone (Fig. 2B), suggesting that control activity was below the limit of sensitivity of the method and was enhanced *in vitro* by α-naphthoflavone. The methylcholanthrene-induced 2-hydroxylase activity was inhibited by α-naphthoflavone simi-

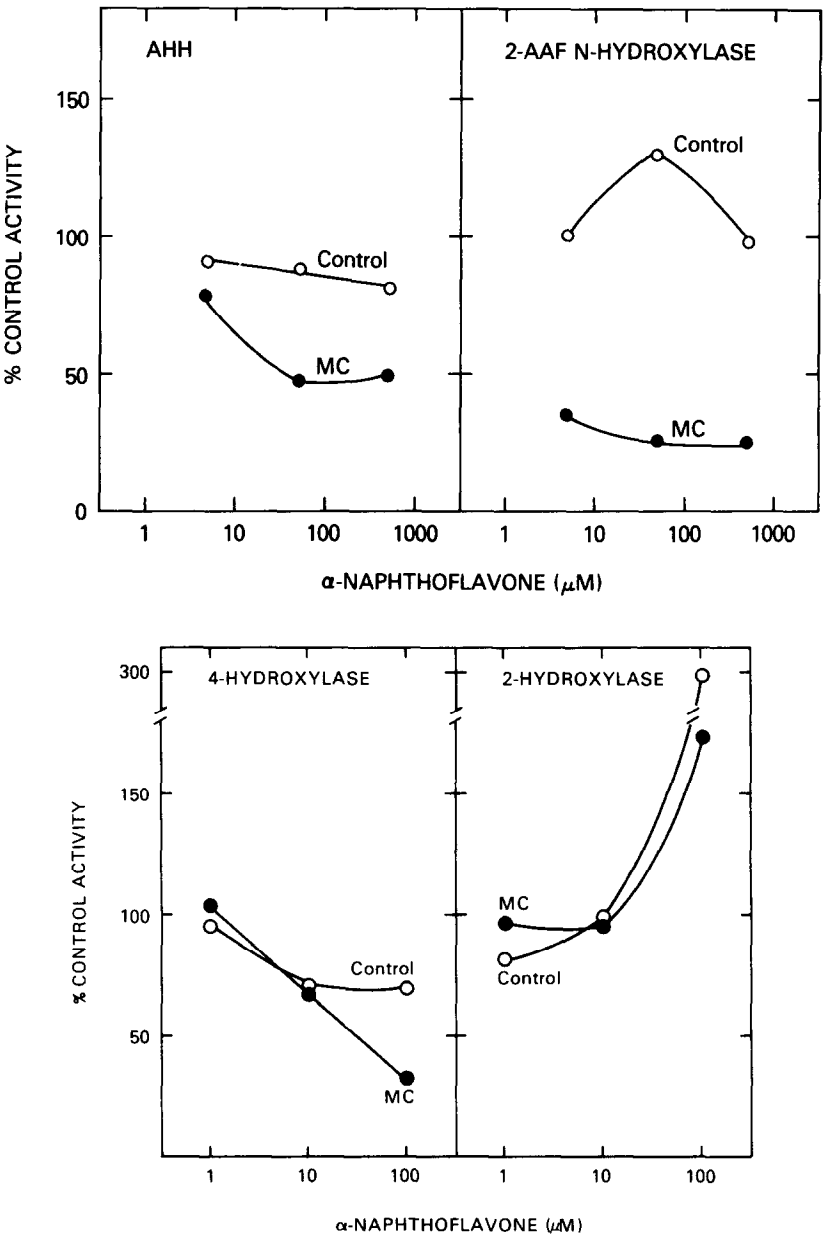

Fig. 4. Effect of α-naphthoflavone *in vitro* on hepatic aryl hydrocarbon hydroxylase (AHH), 2-AAF *N*-hydroxylase (A), and biphenyl 2- and 4-hydroxylases (B) in control (○) and methylcholanthrene (MC)-treated (●) guinea pigs.

larly to the 4-hydroxylase activity; both activities were more sensitive to α-naphthoflavone inhibition than was aryl hydrocarbon hydroxylase activity.

The effects of α-naphthoflavone *in vitro* on the above-mentioned four hepatic mono-oxygenase activities from control and methylcholanthrene-treated hamsters, guinea pigs and rabbits are shown, respectively, in Figs. 3–5. α-Naphthoflavone selectively inhibited the methylcholanthrene-induced mono-oxygenase activities in the hamster liver microsomes (Figs. 3A and 3B). Once again in control microsomes both aryl hydrocarbon hydroxylase and *N*-hydroxylase activities were enhanced upon addition of α-naphthoflavone in a manner similar to that observed for the rat (Fig. 2).

The effect of α-naphthoflavone on the mono-oxygenase activities in liver microsomes from guinea pigs was no more uniform (Fig. 4A and 4B). Although none of the four enzyme activities showed a net increase in the guinea pig after methylcholanthrene treatment, the inhibition of aryl hydrocarbon hydroxylase, *N*-hydroxylase, and biphenyl 4-hydroxylase activities by α-naphthoflavone was much more pronounced in liver microsomes from methylcholanthrene-treated animals. No differential effect of α-naphthoflavone was observed on biphenyl 2-hydroxy-

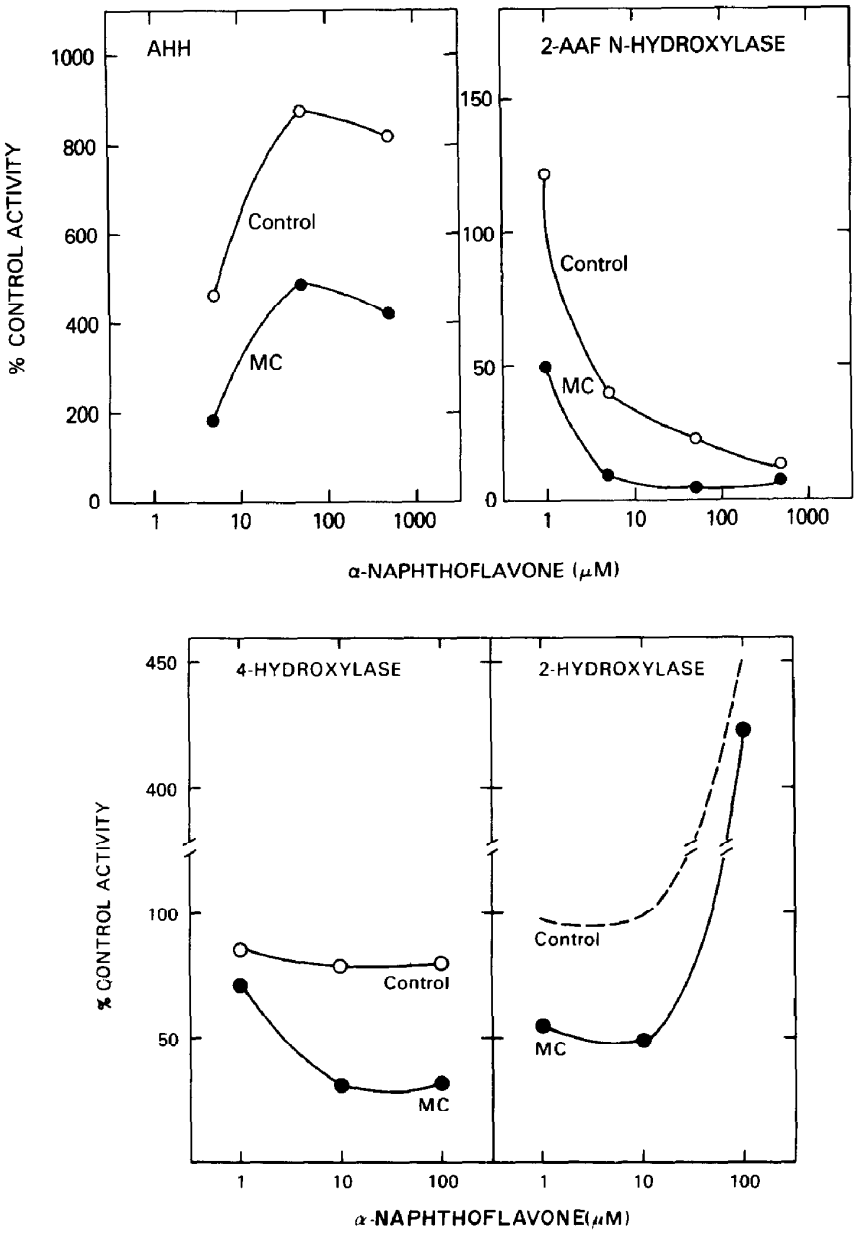

Fig. 5. Effect of α-naphthoflavone *in vitro* on hepatic aryl hydrocarbon hydroxylase (AHH), 2-AAF *N*-hydroxylase (A), and biphenyl 2- and 4-hydroxylases (B) in control (O) and methylcholanthrene (MC)-treated (●) rabbits.

lase activity from control and methylcholanthrene-treated guinea pigs; in both cases about 3-fold enhancement of the 2-hydroxylase activity was found (Fig. 4B).

Addition of α-naphthoflavone to liver microsomes from control and methylcholanthrene-treated rabbits caused different effects on all four mono-oxygenase activities (Figs. 5A and 5B). Aryl hydrocarbon hydroxylase activity was increased several-fold by α-naphthoflavone in both control and methylcholanthrene microsomes; the dose-dependent increase was more pronounced in control microsomes even though methylcholanthrene produced only very small net induction (Table 1). In marked contrast, α-naphthoflavone caused inhibition of *N*-hydroxylase activity in liver microsomes from both control and methylcholanthrene-treated rabbits. Although the *N*-hydroxylase activity in the methylcholanthrene-treated rabbits was more sensitive to α-naphthoflavone inhibition, the profile of α-naphthoflavone inhibition in the control microsomes was the same. The activity of biphenyl 4-hydroxylase was selectivity inhibited by α-naphthoflavone in liver microsomes from methylcholanthrene-treated rabbits, whereas insignificant effects were observed in control microsomes (Fig. 5B). The activity of biphenyl 2-hydroxylase was not measurable in control liver microsomes from rabbits but activity was found in the presence of 100 μM

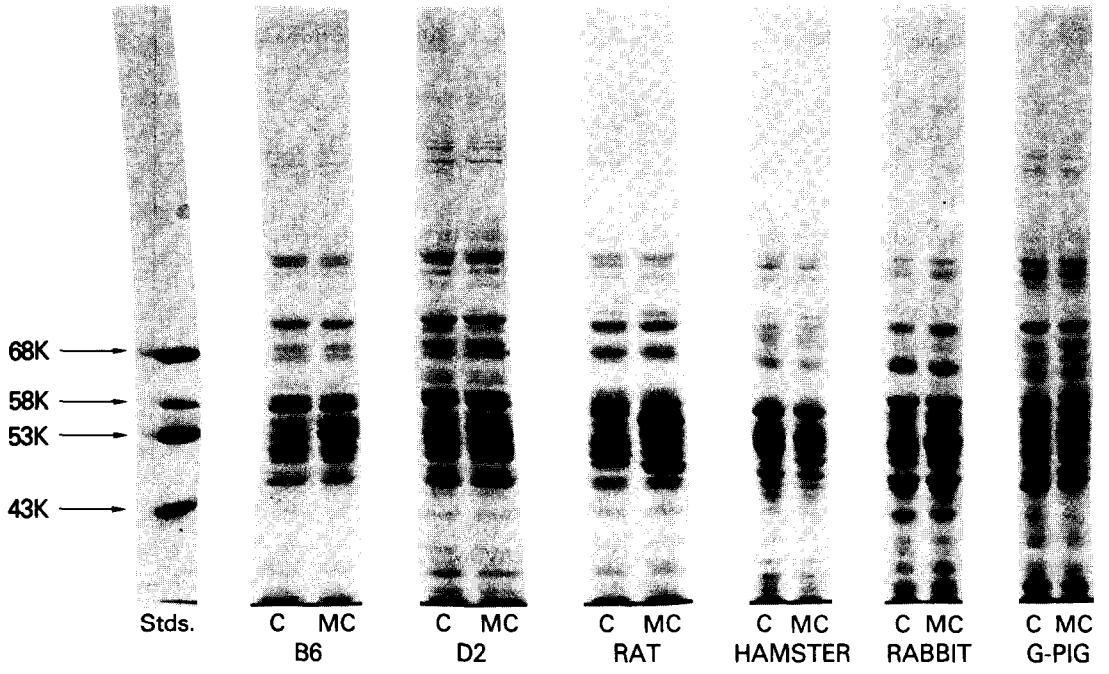

Fig. 6. Electrophoretogram of liver microsomes from control (C) and methylcholanthrene-treated (MC) B6 and D2 mice, rats, hamsters, guinea pigs and rabbits. Electrophoretic migration is from top to bottom. A mixture of molecular weight standards was applied to the outermost well (Stds.), and the microsomal protein (5 μg in 5 μl) to the other wells of the 0.75-mm analytical gels described in Materials and Methods.

α-naphthoflavone, similar to the effects in the rats. Pretreatment of rabbits with methylcholanthrene produced detectable biphenyl 2-hydroxylase activity, which was inhibited by a low concentration (1 and 10 μM) of α-naphthoflavone but markedly enhanced by a high concentration of α-naphthoflavone (100 μM).

Electrophoretic changes in liver microsomes after methylcholanthrene treatment. Figure 6 shows increases in several different cytochrome P-450 subunits, detectable by electrophoresis, in B6 mice, rats, hamsters and rabbits after treatment with methylcholanthrene, whereas no increases were observed in the cytochrome P-450 region in guinea pigs and only a slight increase in the 54,000 mol. wt. protein was seen in the D2 mice after methylcholanthrene treatment. The approximate molecular weights of the polypeptides which showed increase after methylcholanthrene treatment were: 54,000 and 55,000 in B6 mice; 54,000, 55,000 and 48,000 in the rats; 49,000 and 54,000 in the hamsters; and 54,000 and 57,000 in the rabbits.

DISCUSSION

In this study we have shown that the selective inhibition by α-naphthoflavone of four hepatic mono-oxygenase activities, which are associated with aromatic responsiveness in mice, is similar in methylcholanthrene-treated responsive mice, rats and hamsters. Only 2-acetylaminofluorene *N*-hydroxylase and biphenyl 4-hydroxylase show pure inhibition by α-naphthoflavone in the methylcholanthrene-treated rabbits; the activities of aryl hydrocarbon hydroxylase and biphenyl 2-hydroxylase are enhanced but there is still a differential effect on activities from methylcholanthrene microsomes. In the methylcholanthrene-treated guinea pigs, which show very small (35 per cent) increases in the four mono-oxygenase activities and only a 0.5-nm shift to the blue in the reduced CO–hemoprotein complex, selective inhibition of aryl hydrocarbon hydroxylase, 2-acetylaminofluorene *N*-hydroxylase and biphenyl 4-hydroxylase activities is nonetheless observed, whereas the activity of biphenyl 2-hydroxylase is enhanced in both control and methylcholanthrene-treated animals. Polyacrylamide gel electrophoresis of liver microsomes from the methylcholanthrene-treated animals shows increases in the following polypeptide bands: 54,000 and 55,000 in responsive B6 mice; 48,000, 54,000 and 55,000 in rats; 49,000 and 54,000 in hamsters; 54,000, and a small increase in 57,000 in rabbits; and a small increase in the 54,000 band in nonresponsive D2 mice. No increase is seen in the intensity of the electrophoretic bands in liver microsomes from guinea pigs after methylcholanthrene treatment.

The differential effect of α-naphthoflavone on aryl hydrocarbon activity in liver microsomes from control and polycyclic hydrocarbon-treated animals is well established [1, 18, 28]. All the mono-oxygenase activities in the mouse that are associated with aromatic hydrocarbon responsiveness are selectively inhibited by α-naphthoflavone after polycyclic hydrocarbon treatment [17, 29]. Evidence obtained from immunochemical studies with purified hepatic cytochrome P-448 from rats indicates that more than one cytochrome forms the "complex" collectively referred

to as cytochrome P-448 [14, 15]. Our earlier studies have also shown that the substrate specificity of rabbit cytochrome P-448 differs from that in the mouse [20] and more recent studies have confirmed the existence of at least two forms of cytochrome P-448 in rabbits with differing substrate specificities [30]. The present study further supports the hypothesis that polycyclic hydrocarbon-induced cytochrome P-448 is composed of several different forms of hemoprotein, which may differ among animal species.

α-Naphthoflavone selectively inhibits the four mono-oxygenase activities in three (i.e. B6 mouse, rat and hamster) of the four species which showed the most pronounced hypsochromic shift in the Soret maximum of the reduced CO–hemoprotein complex after methylcholanthrene treatment (Figs. 1–5, Table 1). Electrophoresis of liver microsomes from methylcholanthrene-treated B6 mice, rats or hamsters shows an increase of staining density in several polypeptide bands, but the increase in the band around molecular weight of 54,000 is common to all three. A similar increase is also observed in the 54,000 polypeptide band in the rabbit, which shows the same hypsochromic shift in the Soret maximum after methylcholanthrene treatment as that observed with rat, mouse and hamster. However, α-naphthoflavone inhibition in the rabbit differs from that in the other species. It is therefore concluded, as previously suggested [20], that, in all the species investigated, the form of cytochrome(s) whose reduced complex with CO has a Soret maximum of approximately 448 nm and which has a subunit molecular weight of 54,000 is associated with polycyclic hydrocabon-inducible 2-acetylaminofluorene-*N*-hydroxylase activity. This most likely corresponds to a recently described form of cytochrome P-448 in rabbits which has a high degree of specificity for acetanilide [30].

The inhibition of the four mono-oxygenase activities in liver microsomes from control and methylcholanthrene-treated rabbits by α-naphthoflavone shows several interesting features (Figs. 5A and 5B). Inhibition of 2-acetylaminofluorene *N*-hydroxylase is observed in both control and methylcholanthrene microsomes, with more pronounced effects after methylcholanthrene treatment. This suggests that the constitutive *N*-hydroxylase is very similar to the polycyclic hydrocarbon-induced *N*-hydroxylase, and that both activities might be associated with the 54,000-mol. wt. electrophoretic band. This suggestion is further supported by the presence of a substantial polypeptide band of about 54,000 mol. wt. in liver microsomes from the control rabbits (Fig. 6), which appears developmentally at about the same time as does inducible *N*-hydroxylase [20]. α-Naphthoflavone does not, to any significant degree, inhibit the constitutive level of rabbit biphenyl 4-hydroxylase, whereas selective inhibition is observed in liver microsomes from methylcholanthrene-treated rabbits, indicating a clear difference in these two forms of biphenyl 4-hydroxylase. The activity of aryl hydrocarbon hydroxylase is enhanced substantially by α-naphthoflavone in liver microsomes from methylcholanthrene-treated rabbits and to an even greater extent in control rabbits (Fig. 5A). These data and our previous finding on the differential effects of α-naphthoflavone on control and methylcholanthrene-induced aryl hydrocarbon hydroxylase activity in neonatal and adult rabbits [20], as well as recent evidence [30], suggest strongly that more than one form of P-450 is associated with the control aryl hydrocarbon hydroxylase activity in adult rabbit liver microsomes and that these forms are differentially affected (e.g. one is slightly increased and one slightly decreased) by methylcholanthrene treatment *in vivo*. Biphenyl 2-hydroxylase activity is not detectable in liver microsomes from control adult rabbits (Table 1), but measurable activity is found in neonatal rabbits [20]. The biphenyl 2-hydroxylase activity is induced by methylcholanthrene treatment in both the neonatal and young adult rabbits (Ref. 20, Table 1). *In vitro* addition of α-naphthoflavone to liver microsomes from methylcholanthrene-treated adult rabbits activates a presumably inactive form of the 2-hydroxylase, to a level equal to or greater than that observed in the methylcholanthrene-treated neonatal rabbit (Fig. 5B, Table 1, Ref. 19). These results demonstrate similarities between aryl hydrocarbon hydroxylase and biphenyl 2-hydroxylase which differentiate them from the other mono-oxygenases, and imply that a separate and unique hemoprotein may catalyze the 2-hydroxylation of biphenyl and 3-hydroxylation of benzo[a]pyrene. Biphenyl 4-hydroxylase activity is not induced by methylcholanthrene treatment in adult rabbits (i.e. older than 50 days), but induction is observed in neonatal and young rabbits [20]. In spite of this lack of biphenyl 4-hydroxylase induction in adult rabbits, *in vitro* addition of α-naphthoflavone causes selective inhibition in both adult and younger rabbits (Ref. 20, Table 1) and differentiates the biphenyl 4-hydroxylase from aryl hydrocarbon hydroxylase and biphenyl 2-hydroxylase as well as 2-acetylaminofluorene *N*-hydroxylase.

Methylcholanthrene treatment of hamsters causes only a very small induction of all the enzymes except 2-acetylaminofluorene *N*-hydroxylase, which is increased 4- to 5-fold (Fig. 3, Table 1) although there is a near maximum hypsochromic shift in the Soret maximum of the reduced CO–hemoprotein complex. However, as in rats and mice, *in vitro* α-naphthoflavone selectively inhibits all four mono-oxygenase activities after methylcholanthrene treatment. Polyacrylamide gel electrophoretograms show increases in polypeptide bands at 49,000 and 54,000 mol. wt. in liver microsomes from methylcholanthrene-treated hamsters. There are indications that polycyclic hydrocarbon induction of aryl hydrocarbon hydroxylase activity in hamsters is much more pronounced in the fetus and neonate than in the adult animal [31]. In this respect, hamsters may be similar to rabbits in that polycyclic hydrocarbon induction of hepatic aryl hydrocarbon hydroxylase activity during the perinatal period may represent a residual fetal phenomenon [20]. However, the hemoproteins resulting from methylcholanthrene treatment of hamsters differ from those in rabbits with respect to α-naphthoflavone inhibition and more closely resemble those in rats and mice.

In methylcholanthrene-treated guinea pigs, which show a very small (20–40 per cent) induction of the four mono-oxygenase activities and only a 0.5-nm hypsochromic shift in the Soret maximum of the reduced CO–hemoprotein complex, α-naphthoflavone selecti-

vely inhibits aryl hydrocarbon hydroxylase, 2-acetylaminofluorene *N*-hydroxylase and biphenyl 4-hydroxylase, whereas the activity of biphenyl 2-hydroxylase is enhanced. These findings are rather surprising since no electrophoretic band from guinea pig liver microsomes is significantly altered by *in vivo* methylcholanthrene treatment (Fig. 6). The enhancement of biphenyl 2-hydroxylase activity in liver microsomes from both control and methylcholanthrene-treated guinea pigs is similar to the enhancement observed in methylcholanthrene-treated rabbits (Figs. 4B and 5B).

The mechanism(s) by which α-naphthoflavone inhibits or enhances the mono-oxygenase activities are not clear at this point. It seems likely, however, that more than one mode of action is operant (i.e. inhibition could be competitive, noncompetitive or mixed) and evidence to this effect has been obtained from both animals and man [32–34]. The enhancement of mono-oxygenase activities by the *in vitro* addition of α-naphthoflavone may be caused by an interaction with the allostric binding site(s) on the hemoproteins, in a fashion similar to the one that has been observed for the mixed-function amine oxidase [35].

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REFERENCES

1. S. S. Thorgeirsson, S. A. Atlas, A. R. Boobis, J. S. Felton and D. W. Nebert, *Pharmacologist* **17**, 217 (1975).
2. A. P. Alvares, G. Schilling, W. Levin and R. Kuntzman, *Biochem. biophys. Res. Commun.* **29**, 521 (1967).
3. R. Kuntzman, W. Levin, M. Jacobson and A. H. Conney, *Life Sci.* **7**, 215 (1968).
4. N. E. Sladek and G. J. Mannering, *Biochem. biophys. Res. Commun.* **24**, 668 (1966).
5. A. H. Conney, W. Levin, M. Jacobson, R. Kuntzman, D. Y. Cooper and O. Rosenthal, in *Microsomes and Drug Oxidation* (Eds. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering), pp. 279–95. Academic Press, New York (1969).
6. A. Y. H. Lu, R. Kuntzman, S. B. West, M. Jacobson and A. H. Conney, *J. biol. Chem.* **247**, 1727 (1972).
7. A. Y. H. Lu. W. Levin, S. B. West, M. Jacobson, D. Ryan, R. Kuntzman and A. H. Conney, *J. biol. Chem.* **248**, 456 (1973).
8. N. Zampaglione, D. J. Jollow, J. R. Mitchell, B. Stripp, M. Hamrick and J. R. Gillette, *J. Pharmac. exp. Ther.* **187**, 218 (1973).
9. R. E. Rasmussen and I. Y. Wang, *Cancer Res.* **34**, 2290 (1974).
10. T. Matsushima, P. H. Grantham, E. K. Weisburger and J. H. Weisburger, *Biochem. Pharmac.* **21**, 2043 (1972).
11. G. Holder, H. Yagi, P. Dansette, D. M. Jerina, W. Levin, A. Y. H. Lu and A. H. Conney, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4356 (1974).
12. A. F. Welton and S. D. Aust. *Biochem. biophys. Res. Commun.* **54**, 923 (1974).
13. D. A. Haugen, T. A. van der Hoeven and M. J. Coon, *J. biol. Chem.* **250**, 3567 (1975).
14. J. C. Kawalek, W. Levin, D. Ryan, P. E. Thomas and A. Y. H. Lu, *Molec. Pharmac.* **11**, 874 (1975).
15. P. E. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek and W. Levin, *Molec. Pharmac.* **12**, 746 (1976).
16. D. A. Haugen, M. J. Coon and D. W. Nebert, *J. biol. Chem.* **251**, 1817 (1976).
17. S. S. Thorgeirsson and D. W. Nebert, *Adv. Cancer Res.* **24**, 149 (1977).
18. F. M. Goujon, D. W. Nebert and J. E. Gielen, *Molec. Pharmac.* **8**, 667 (1972).
19. S. A. Atlas, S. S. Thorgeirsson, A. R. Boobis, K. Kumaki and D. W. Nebert, *Biochem. Pharmac.* **24**, 2111 (1975).
20. S. A. Atlas, A. R. Boobis, J. S. Felton, S. S. Thorgeirsson and D. W. Nebert, *J. biol. Chem.* **242**, 4712 (1977).
21. S. S. Thorgeirsson, J. S. Felton and D. W. Nebert, *Molec. Pharmac.* **11**, 159 (1975).
22. S. S. Thorgeirsson and W. L. Nelson, *Analyt. Biochem.* 122 (1976).
23 S. A. Atlas and D. W. Nebert, *Archs Biochem. Biophys.* **175**, 495 (1976).
24. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
25. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
26. J. V. Maizel, Jr., in *Methods in Virology* (Eds. K. Muramaro and H. Koprowski), Vol. 5, pp. 179–200. Academic Press, New York (1971).
27. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
28. F. J. Wiebel, J. C. Leutz, L. Diamond and H. V. Gelboin, *Archs Biochem. Biophys.* **144**, 78 (1971).
29. D. W. Nebert, J. R. Robinson, A. Niwa, K. Kumaki and A. P. Poland, *J. Cell. Physiol.* **85**, 393 (1975).
30. E. F. Johnson and U. Muller-Eberhard, *J. biol. Chem.* **252**, 2839 (1977).
31. D. W. Nebert and H. V. Gelboin, *Archs Biochem. Biophys.* **134**, 76 (1969).
32. F. J. Wiebel and H. V. Gelboin, *Biochem. Pharmac.* **24**, 1511 (1975).
33. O. Pelkonen, in *Carcinogenesis Polynuclear Aromatic Hydrocarbons: Chemistry, Metabolism, and Carcinogenesis* (Eds. R. I. Freudenthal and P. W. Jones), Vol. 1, pp. 9–21. Raven Press, New York (1976).
34. J. Kapitulnik, P. J. Poppers, M. K. Buening, J. G. Fortner and A. H. Conney, *Clin. Pharmac. Ther.* **22**, 475 (1977).
35. D. M. Ziegler and C. A. Mitchell, *Archs Biochem. Biophys.* **150**, 116 (1972).