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INDUCTION OF MICROSOMAL *N*-HYDROXYLATION OF *N*-2-FLUORENYLACETAMIDE IN RAT LIVER*

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Abstract—Single or multiple injections of *N*-2-fluorenyl-acetamide (2-FAA) to Sprague–Dawley rats increased *N*-hydroxylation of 2-FAA by hepatic microsomes 3- to 12-fold without changing the content of microsomal hemoprotein (cytochrome P-450 or P_1-450) measured either by carbon monoxide difference spectra, by gel electrophoresis of microsomal preparations or by formation of the ethyl isocyanide cytochrome P_1-450 complex. Carbon monoxide inhibited *N*-hydroxylation of 2-FAA by hepatic microsomes of 2-FAA-treated rats. Inhibition by carbon monoxide indicated that either cytochrome P-450 or P_1-450 is the terminal oxidase in *N*-hydroxylation by microsomes of 2-FAA-treated rats. Unlike pretreatment of rats with phenobarbital or 3-methylcholanthrene, pretreatment with 2-FAA did not appear to induce the synthesis of microsomal hemoprotein. The activities of NADPH–cytochrome *c* reductase, NADPH–cytochrome P-450 reductase and of amine oxidase in microsomes of 2-FAA-treated rats were not increased and thus did not account for the stimulation of *N*-hydroxylation. The induction, by 2-FAA, of a heretofore unknown electron carrier associated with the hepatic mixed-function oxidase is postulated and under investigation.

The present study originated from the observation that prolonged feeding of *N*-2-fluorenylacetamide (2-FAA)¶ to rats increased the urinary excretion of the metabolite, *N*-hydroxy-2-FAA, during carcinogenesis [1, 2]. Since *N*-hydroxylation of 2-FAA, an activation reaction essential for carcinogenesis, is performed by hepatic microsomes of the rat [3–7], we investigated the possibility that 2-FAA induces its own microsomal *N*-hydroxylation. Because the microsomal *N*-hydroxylation of 2-FAA appears to be catalyzed by cytochrome P-450 [5] and cytochrome P_1-450 [5, 6], we compared the induction of *N*-hydroxylation by 2-FAA with the induction of the reaction by PB and 3-MC. These compounds are known inducers of cytochrome P-450 and cytochrome P_1-450. In additional experiments the effect of 2-FAA on other components of the hepatic mixed-function oxidase, NADPH–cytochrome P-450 reductase and NADPH-cytochrome *c* reductase, was studied. Finally, the possibility was investigated whether 2-FAA activates monoamine oxidase, an oxidative enzyme located in hepatic microsomes but not associated with the hepatic mixed-function oxidase.

MATERIALS AND METHODS

Labeled and unlabeled compounds. 2-[9-^{14}C]FAA (New England Nuclear Corp., Boston, MA) after recrystallization from ethanol–water (7:3) had a specific activity of 2.65 mCi/m-mole. 2-[1′-^{14}C]FAA was obtained by acetylation of 2-fluorenamine with [^{14}C-carbonyl]acetic anhydride (3.3 mCi/m-mole) (New England Nuclear Corp., Boston, MA) [6]. The product was recrystallized from ethanol–water (7:3) to yield 2-[1′-^{14}C]FAA (1.80 mCi/m-mole), m.p. 196°. *N*-hydroxy-2-[1′-^{14}C] FAA (0.089 mCi/m-mole), m.p. 146°, was prepared as described previously [8]. The purity of the labeled compounds was examined by thin-layer chromatography (t.l.c.) on sheets of Eastman 6061 Silica gel (Distillation Products Industries, Rochester, NY) with chloroform–methanol (95:5) [9]. Scans of the radiochromatograms with a thin-layer scanner (model LB 2721, Berthold Laboratories, Wildbad, West Germany) showed a single radioactive peak coincident with the fluorescence-quenching spot given by the authentic compound.

2-FAA (Aldrich Chemical Co., Milwaukee, WI) was recrystallized from ethanol–water (7:3). The compound, m.p. 196–98°, gave a single fluorescence-quenching spot (R_f 0.43) on t.l.c. *N*-hydroxy-2-FAA, m.p. 150–51°, was prepared by the published procedure [10]. The i.r. and u.v. spectra of the arylhydroxamic acid matched those of the authentic sample. 3-MC, m.p. 181–82°, was purchased from Eastman Kodak Co., Rochester, NY. PB (sodium phenobarbital injection USP) was a product of Winthrop Labs., NY. Ethyl isocyanide, semicarbazide hydrochloride and cytochrome *c* from horse heart, type III, were purchased from Sigma Chemical Co., St. Louis, MO. NADPH was obtained from CalBiochem, LaJolla, CA.

Animals, maintenance and treatment. Male and female Sprague–Dawley rats (Holtzman Co., Madison, WI), 8–10 weeks old, were maintained on regular Purina Chow pellets and water *ad lib.* The

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¶ The following abbreviations are used: 2-FAA, *N*-2-fluorenylacetamide; 3-MC, 3-methylcholanthrene; PB, phenobarbital; DMA, *N*,*N*-dimethylaniline; CO, carbon monoxide; and SDS, sodium dodecyl sulfate.

rats were injected i.p. with 3-MC, PB or 2-FAA according to regimens indicated in the tables and figures. Food was withdrawn 24 hr before preparation of the microsomes. The rats were sacrificed by decapitation. Female rats used for carcinogenicity tests were 6 weeks old at the beginning of treatment and maintained on a semi-synthetic diet containing 20% casein [11]. The animals were kept in individual cages for 10 months after administration of the compounds had been completed. During this period the rats were weighed and examined weekly for the presence of tumors. Routine preparation of tissues for histologic examination was performed as described previously [8].

Preparation of hepatic microsomes and determination of protein. Livers were perfused with cold 0.9% NaCl solution, excised and cut into small pieces. The chopped livers were weighed and suspended in 0.25 M sucrose–0.05 M Tris buffer, pH 7.5, to yield 20% (w/w) suspensions. The suspensions were homogenized with a Polytron homogenizer (type PT 10, Kinematica GMBH, Luzern, Switzerland) for 30–60 sec at the highest speed. Microsomes were obtained by differential centrifugation of the homogenates [12]. The microsomes were washed once with 0.05 M Tris–0.025 M KCl buffer, pH 7.5, and recentrifuged at 105,000 *g* for 30 min. The microsomes from each liver were suspended in the buffer (2 ml) and homogenized gently in a Teflon pestle homogenizer. Aliquots (0.1 ml) of the microsomal homogenates were diluted with the buffer (10 ml) and the protein content was determined by the method of Lowry *et al.* [13] with crystalline bovine serum albumin as a standard.

Determination of N*-hydroxylation of 2-FAA by hepatic microsomes.* Each incubation system consisted of 2-[9-^{14}C]FAA or 2-[1′-^{14}C]FAA (0.4 to 0.5 μmole in 0.1 ml of 95% ethanol), microsomal protein (12 mg) and NADPH (12 μmoles) in a total volume of 6.0 ml of 0.05 M Tris–0.025 M KCl buffer, pH 7.5. The mixtures were incubated in flasks open to air at 37° for 30 min. At the completion of the incubations, methanol (1 ml) containing *N*-hydroxy-2-FAA (1.0 μmole) and cold 1 M acetate buffer (4 ml), pH 6.0, were added. The mixtures were partitioned by solvent extraction [8]. *N*-hydroxy-2-FAA was isolated and purified by t.l.c. and estimated as previously described [8].

Determination of recovery of N*-hydroxy-2-FAA from hepatic microsomes of normal and 2-FAA-treated rats.* *N*-hydroxy-2-[1′-^{14}C]FAA (1.0 μmole in 0.2 ml of 95% ethanol) was added to microsomes (24 mg protein) and NADPH (24 μmoles) in a total volume of 12 ml of 0.05 M Tris buffer, pH 7.5, containing 0.025 M KCl. The incubations and isolation of the hydroxamic acid were carried out as described above.

Determination of cytochrome P-450, binding of 2-FAA and of ethyl isocyanide to hepatic microsomes. Cytochrome P-450 was measured spectroscopically as the reduced CO–hemoprotein complex with the use of an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ [14]. Binding spectra of 2-FAA were obtained by adding 2-FAA (0.3 μmole) in propylene glycol (0.1 ml) to microsomes (6.0 mg protein) suspended in a mixture of 0.05 M Tris–0.025 M KCl buffer (2.1 ml), pH 7.5, and of propylene glycol (0.9 ml) [6]. The reference cuvette contained only the microsomes suspended in the buffer (2.0 ml) and propylene glycol (1.0 ml). Ethyl isocyanide difference spectra were obtained by adding a 5% (w/v) solution of ethyl isocyanide in distilled water (15 μl) to the sample cuvette [15] containing microsomal suspension in 0.05 M Tris–0.025 M KCl buffer, pH 7.5 (1.0 mg protein/ml). The reference cuvette contained the same microsomal suspension to which distilled water (15 μl) was added. The spectra were recorded from 500 to 380 nm with a Beckman Acta III spectrophotometer fitted with a scattered transmission accessory (sensitivity of 0.1 absorbance unit full scale).

SDS–polyacrylamide gel electrophoresis. Gels in glass tubes (9 cm long) or flat-plate separating gels (0.15 cm thick) consisting of 7.5% (w/v) acrylamide, 0.2% (w/v) *N,N′*-methylene-bis-acrylamide and 0.5 M urea were prepared in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% (w/v) SDS. Flat-plate stacking gels (0.15 cm thick) contained 5% (w/v) acrylamide. Microsomes (2 mg protein) were suspended in 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 3% (w/v) SDS, 2.5% (w/v) 2-mercaptoethanol and 0.005% (w/v) bromophenol blue and placed in a boiling water bath for 5 min. The solubilized protein was applied to gels (20–40 μg protein/gel) or to flat-plate gel (12 μg protein/slot). Electrophoresis was carried out at room temperature (22°). A constant current of 4.5 mA/gel was applied until the tracking dye band had migrated a distance of 8.5 cm. A constant current of 10 mA/flat-plate gel was applied during stacking and then changed to 30 mA/flat-plate gel during separation until the tracking dye band migrated a distance of 10 cm. The developed gels were stained in 0.25% Coomassie brilliant blue R-250 in methanol–water–acetic acid (45:45:10, v/v), destained with methanol–water–acetic acid (25:67.5:7.5, v/v) and stored in 7.5% (v/v) acetic acid. Band densities of the destained gels were recorded at 550 nm with a Beckman DR-Monochromator equipped with a Gilford linear transport attachment, model 2410. Molecular weights of the unknown proteins separated on gels were estimated from the migration of standard proteins [16].

Assays of NADPH–cytochrome P-450 reductase activity and of NADPH–cytochrome c *reductase activity.* Microsomes were suspended in 0.05 M Tris–0.025 M KCl buffer, pH 7.5. The suspension (1 mg protein/ml) was placed in an Aminco anaerobic spectrophotometric cell and gassed for 5 min with deoxygenated CO [17]. The activity of cytochrome P-450 reductase was measured after addition of NADPH (10 μl of 0.25 M solution to 3 ml of microsomal suspension) by determining the initial rate of change of A_{450}-A_{490} absorbance with the use of an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for cytochrome P-450 [14]. The activity of cytochrome *c* reductase was determined in a microsomal suspension (0.2 mg protein/ml) containing cytochrome *c* (1.42 mg/ml) and NADPH (0.83 μmole/ml) [18]. The absorption was measured at 550 nm and an extinction coefficient of 18.5 $mM^{-1}\,cm^{-1}$ was used [19]. The rates of the reduction of cytochrome P-450 and of cytochrome *c* were recorded with an Aminco DW-2 UV-Vis spectrophotometer (American Instrument Co., Silver Spring, MD).

Assay of amine oxidase activity. Microsomes for this

assay were prepared from livers homogenized in 0.25 M sucrose. The microsomes were washed with 0.05 M Tris–0.025 M KCl buffer, pH 8.4, and resuspended in the buffer. Amine oxidase activity was measured essentially by the method of Ziegler and Pettit [20]. The incubation system consisted of semicarbazide (6.0 μmoles), NADPH (12 μmoles), microsomal protein (18 mg) and DMA (15 μmoles) in a total volume of 6.0 ml of 0.05 M Tris–0.025 M KCl buffer, pH 8.4. After semicarbazide had been incubated in the buffer for 4 min at 37°, the other components were added in the order listed above. The reaction mixtures were incubated for 5 min at 37°. At the completion of the incubations, the reaction mixtures were deproteinized with perchloric acid. Free DMA was removed with diethyl ether at pH 9.4 and DMA-*N*-oxide was assayed colorimetrically [20].

Radioactivity measurements. The radioactivity of all samples was determined in Scintisol-Complete (10 ml) (Isolab. Inc., Elkhart, IN) by liquid scintillation spectrometry. All samples were counted in duplicate with an error not exceeding 5 per cent. The counts were corrected for quenching by means of an external standard. The counting efficiency was 70–80 per cent.

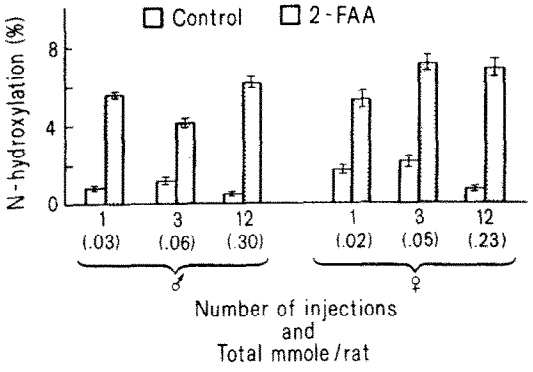

Fig. 1. Induction of microsomal *N*-hydroxylation in rat liver by 2-FAA administered i.p. 2-FAA (0.1 m-mole/kg) was given as a 1 per cent suspension of the compound in 7% gum arabic in 0.9% NaCl. Control rats received i.p. injections of the vehicle (2.2 ml/kg). *N*-hydroxylation of 2-FAA was determined 24 hr after the last injection by inverse isotope dilution as described in Materials and Methods and is expressed as the per cent of substrate converted to the *N*-hydroxy metabolite. Entries are mean values ± S. D. from at least three experiments, each on pooled livers from two rats.

RESULTS

After administration of 2-FAA to male or female Sprague–Dawley rats, *N*-hydroxylation of the arylamide by hepatic microsomes was increased 3- to 12-fold (Fig. 1). The differences in *N*-hydroxylation between normal and 2-FAA-treated rats, although greatest after twelve i.p. injections of 2-FAA in amounts which are tumor-producing, were significant already after a single dose of the compound. In experiments not described here we found that microsomal *C*-hydroxylation to *N*-[5-hydroxy]-2-FAA and, to a lesser degree, to *N*-[7-hydroxy]-2-FAA was also markedly increased in livers of rats treated with 2-FAA.

The possibility was considered that the increase in *N*-hydroxylation of 2-FAA may be due to the retention, within the microsomes, of *N*-hydroxy-2-FAA formed during the pretreatment of rats with the carcinogen. This was tested by incubation of *N*-hydroxy-2-[1′-^{14}C]FAA with microsomes of untreated and 2-FAA-treated rats. The specific radioactivity of *N*-hydroxy-2-[1′-^{14}C]FAA isolated from the incubation mixtures was the same in either case (Table 1). This result led us to conclude that the observed increase in *N*-hydroxylation of 2-FAA was attributable to a stimulation of the activity of microsomal enzyme(s) which *N*-hydroxylate 2-FAA. Determination of the *N*-hydroxylation of 2-FAA by microsomes of untreated and 2-FAA-treated rats prepared at various times after injection of the compound showed that the differences in the formation of *N*-hydroxy-2-FAA by the two sets of microsomes increased in a linear manner up to 12 hr and reached a maximal value at 24 hr (Fig. 2). Accordingly, the microsomes used in further studies on the enzymatic *N*-hydroxylation of 2-FAA were prepared 24 hr after injection of 2-FAA.

Several investigators have shown that microsomal *N*-hydroxylation of 2-FAA in rat liver is catalyzed by cytochrome P-450 and, in the 3-MC-treated rat, by the closely related cytochrome P_1-450 [4–6]. We compared, therefore, the effect of the pretreatment with 2-FAA on the content of microsomal hemoprotein and on *N*-hydroxylation with the effects of pretreatment with 3-MC and with PB, an inducer of cytochrome P-450 [21, 22]. Pretreatment of rats with PB for 3 days increased the content of cytochrome P-450 in hepatic microsomes of male and female rats by 90 and 60 per cent respectively (Fig. 3). However,

Table 1. Recovery of *N*-hydroxy-2-[1′-^{14}C]FAA from incubation mixtures of hepatic microsomes of male rats given a single i.p. dose of 2-FAA

Pretreatment of rat*	*N*-hydroxy-2-[1′-^{14}C]FAA		
	Activity added to microsomes (dis./min/μmole)	Activity isolated† (dis./min/μmole)	Recovery (%)‡
Control	9.8×10^4	8.9×10^4	91
2-FAA	9.8×10^4	8.5×10^4	87

* 2-FAA (0.1 m-mole/kg) was given as a 1 per cent suspension of the compound in 7% gum arabic in 0.9% NaCl 24 hr before preparation of the microsomes. Control rats received the vehicle (2.2 ml/kg). Two rats were used in each group.

† Hydroxamic acid was isolated from the incubation mixtures as described in Materials and Methods.

‡ Values are the means from two experiments.

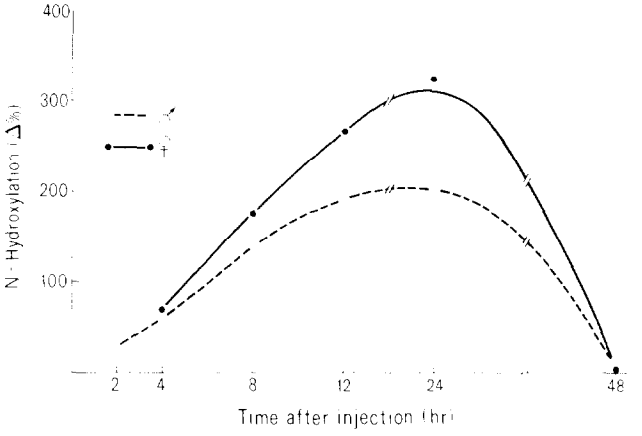

Fig. 2. Time-dependent formation of *N*-hydroxy-2-FAA by rat liver microsomes after a single i.p. injection of 2-FAA. 2-FAA (0.1 m-mole/kg) was given as a 1 per cent suspension of the compound in 7% gum arabic in 0.9% NaCl. Control rats were given the vehicle (2.2 ml/kg). *N*-hydroxylation of 2-FAA was determined by inverse isotope dilution as described in Materials and Methods and is expressed as the per cent of substrate converted to the *N*-hydroxy metabolite. Entries represent differences in *N*-hydroxylation of 2-FAA between control and 2-FAA-treated rats: differences at 4, 8, 12 and 24 hr ($P < 0.001$) and at 2 hr ($P \cong 0.01$) were statistically significant; differences at 48 hr were statistically not significant.

N-hydroxylation of 2-FAA was not increased. This result supported published reports that purified cytochrome P-450 induced by PB is primarily involved in *N*-demethylation reactions and that hydroxylation reactions are stimulated only to a minor extent [23, 24]. 3-MC increased the content of microsomal hemoprotein in livers of male and female rats by 74 and 13 per cent, respectively, and simultaneously increased *N*-hydroxylation of 2-FAA by 535 and 45 per cent respectively (Fig. 3). Female rats appeared to be less responsive to 3-MC induction of cytochrome P_1-450 and of *N*-hydroxylation than male rats. The extent of induction of cytochrome P_1-450 and of *N*-hydroxylation of 2-FAA by 3-MC

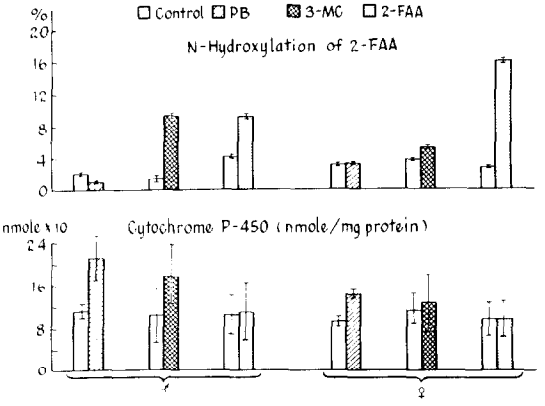

Fig. 3. Effect of pretreatment of rats with PB, 3-MC or 2-FAA on microsomal *N*-hydroxylation of 2-FAA and on content of microsomal hemoprotein. Three injections of PB (0.3 m-mole/kg), a single injection of 3-MC (0.075 m-mole/kg) or 2-FAA (0.1 m-mole/kg) were given i.p. *N*-hydroxylation of 2-FAA was determined by inverse isotope dilution as described in Materials and Methods and is expressed as the per cent of substrate converted to the *N*-hydroxy metabolite. Content of cytochrome P-450 was measured by the method of Omura and Sato [14]. Entries are mean values ± S. D. from three to five experiments, each involving pooled livers of two to three rats.

in male rats observed in this study was similar to that reported earlier [6]. The differences in the average increase of microsomal hemoprotein in the liver of male and female rats treated with 2-FAA (10 and 7 per cent respectively) were not statistically significant ($P > 0.5$) (Fig. 3). Despite the lack of induction of hemoprotein by 2-FAA, there was a marked increase in the N-hydroxylation of 2-FAA (Fig. 3). Thus, in contrast to 3-MC pretreatment, the marked increase in the *N*-hydroxylation of 2-FAA by microsomes of the 2-FAA-treated rats noted above occurred without a concomitant induction of cytochrome P_1-450. This view was further supported by gel electrophoresis of microsomal preparations obtained from untreated, PB-, 3-MC- and 2-FAA-treated rats. Resolution patterns and band densities of microsomal proteins from untreated and 2-FAA-treated rats were virtually indistinguishable (Figs. 4 and 5). Thus, in contrast to pretreatment of rats with PB or 3-MC, pretreatment with 2-FAA did not appear to induce cytochrome P-450 or P_1-450.

In the experiments of Fig. 3, the content of microsomal hemoprotein was estimated from the absorption maxima at 450 and 448 nm of the complex formed by interaction of CO with cytochrome P-450 and P_1-450 respectively [25]. A specific indication of the induction of cytochrome P_1-450 by 3-MC is the complex formation of ethyl isocyanide with this hemoprotein [15]. The ratio A_{455}/A_{430} of the complex of ethyl isocyanide with microsomal hemoprotein from untreated rats is < 1.00. The induction of cytochrome P_1-450 by 3-MC is reflected by an increase in this ratio ($A_{455}/A_{430} > 1.00$) [15, 26]. The ratios of absorbance at 455 and 430 nm of the ethyl isocyanide–hemoprotein complex in hepatic microsomes of 2-FAA-treated rats were essentially the same as those of untreated rats ($A_{455}/A_{430} < 1.00$). These data were in agreement with a recent report [26] and indicated that 2-FAA did not induce a hemoprotein comparable to that induced by 3-MC. Additional evidence for this conclusion came from a study of bind-

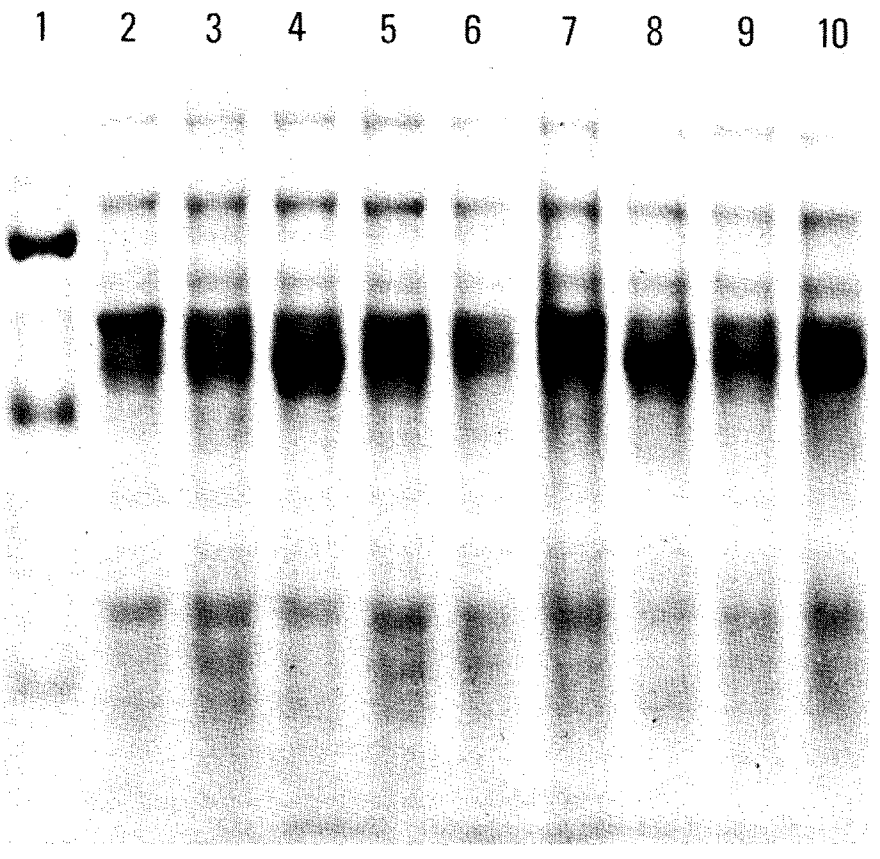

Fig. 4. Electrophoretogram of liver microsomal protein from variously treated rats. Electrophoretic migration is from top to bottom. Microsomal protein (12 μg in 10 μl) in positions 2 to 10 and a mixture of molecular weight standards (160 μg in 10 μl) in position 1 were applied to the flat-plate gel as described in Materials and Methods. Positions 3, 6 and 10 contain microsomes from untreated rats; positions 2 and 7, 4 and 8, and 5 and 9, respectively, contain microsomes from 3-MC-, PB- and 2-FAA-treated rats. Positions 2–6 and 7–10, respectively, contain microsomes from female and male rats. In each experiment, the microsomes were prepared from three pooled livers, and the samples used for electrophoretic analysis were also used for the measurements of enzyme activities and spectral studies shown in Figs. 3 and 6 respectively.

ing spectra. Previous data indicated that 2-FAA added to hepatic microsomes yields a characteristic type I binding spectrum [6]. The magnitude of the spectrum was increased by pretreatment of rats with 3-MC, and the binding of 2-FAA was a function of substrate concentration [6]. In the present study, we confirmed the observation that 2-FAA added to microsomes from 3-MC-treated rats gives a type I binding spectrum (Fig. 6). In contrast, microsomes from untreated, PB, or 2-FAA-treated rats did not yield a binding spectrum of a magnitude comparable to that obtained with 3-MC. All of these data were consistent with the view that 2-FAA given to the rat does not induce cytochrome P_1-450.

It has been shown previously that the *N*-hydroxylation of 2-FAA by hepatic microsomes of untreated or 3-MC-treated male rats was inhibited by CO [6]. Consequently, we examined the effect of CO on *N*-hydroxylation of 2-FAA by hepatic microsomes of 2-FAA-treated rats. In order to obtain data that would permit a direct comparison between 2-FAA-treated and 3-MC-treated rats, the *N*-hydroxylation of 2-FAA by 3-MC-treated male and female rats was reinvestigated (Table 2). The conversion of 2-FAA to *N*-hydroxy-2-FAA by hepatic microsomes was markedly inhibited by CO in each case. These data indicated that cytochrome P-450 or P_1-450 functions as the terminal oxidase in the *N*-hydroxylation of 2-FAA. It should be noted that inhibition of the *N*-hydroxylation of 2-FAA in normal, 3-MC- and 2-FAA-treated female rats was markedly less than in male rats. This finding suggested that microsomal *N*-hydroxylation of 2-FAA by the liver of the female rat involves, in addition to cytochrome P_1-450, an oxidase not related to cytochrome P_1-450 and not inhibited by CO. This suggestion is supported by the observation that female rats are more responsive to the induction of *N*-hydroxylation by 2-FAA and less susceptible to induction of *N*-hydroxylation by 3-MC than are male rats (Figs. 1–3).

Although the role of cytochrome P-450 or P_1-450 in the *N*-hydroxylation of 2-FAA by hepatic microsomes of the male rat has been clearly demonstrated in the present and earlier reports [4, 6], the degree of involvement of these hemoproteins in the *N*-hydroxylation of 2-FAA by hepatic microsomes of female rats is less clear. Because *N*-hydroxylation of 2-FAA is a recognized activation reaction in carcinogenesis [7, 10, 27], we investigated whether a change in the activity of microsomal hemoprotein is reflected

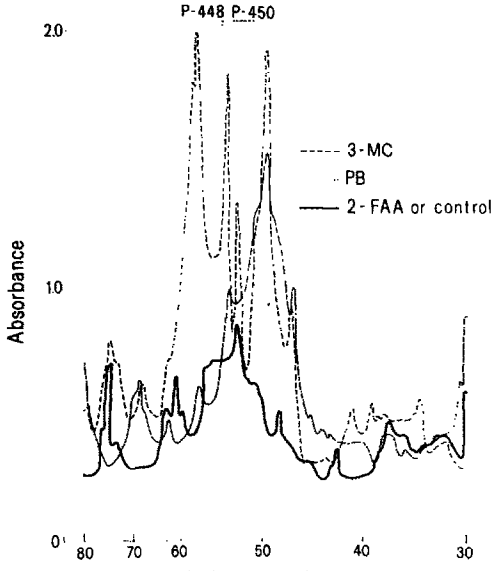

Fig. 5. Coomassie blue protein profiles obtained by SDS-polyacrylamide gel electrophoresis of microsomal preparations from variously treated rats. Microsomes were electrophoresed as described in Materials and Methods. Absorbance at 550 nm is plotted on the ordinate and migration distance corresponding to molecular weight on the abscissa. The figure shows a fragment of the gel between 80 × 10^3 and 30 × 10^3 mol.w.

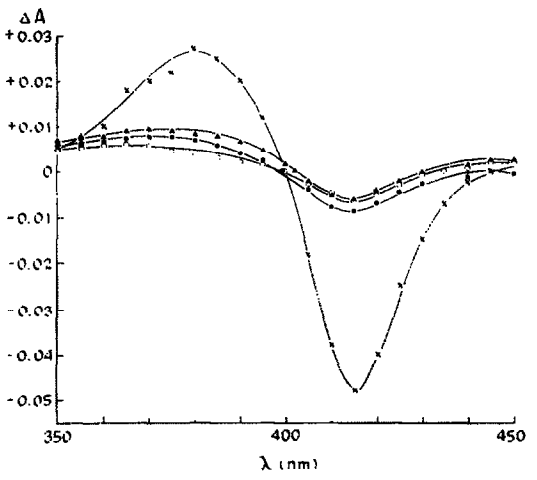

Fig. 6. Binding spectra obtained on addition of 2-FAA to hepatic microsomes of normal (▲ ▲), PB-treated (○ ○), 3-MC-treated (× ×) and 2-FAA-treated (● ●) male rats. 2-FAA (0.3 μmole) in propylene glycol (0.1 ml) was added to microsomes (6.0 mg protein) suspended in a mixture of Tris buffer (2.1 ml, pH 7.5) and propylene glycol (0.9 ml).

in the carcinogenicity of 2-FAA for the female rat. There are data in the literature indicating that 5-allyl-5′-(1-methylbutyl)barbituric acid (secobarbital) degrades cytochrome P-450 *in vivo* [28, 29]. In this study, we determined whether administration of secobarbital to the female rat affects cytochrome P-450 content and *N*-hydroxylation of 2-FAA as well as the carcinogenicity of 2-FAA. These experiments showed that secobarbital alone or together with 2-FAA decreased the content of microsomal hemoprotein, but affected neither *N*-hydroxylation of 2-FAA nor its carcinogenicity (Table 3). These results suggested that the degradation of cytochrome P-450 by secobarbital might not have been sufficiently great and thus the final step in the *N*-hydroxylation of 2-FAA which in-

Table 2. Inhibition by CO of microsomal *N*-hydroxylation of 2-FAA in livers of 3-MC- and 2-FAA-treated rats

Pretreatment of rats*	Sex of rat	*N*-hydroxy-2-FAA formed† (nmoles/12 mg protein/10 min)		Inhibition of *N*-hydroxylation (%)
		In N_2:O_2 (9:1)‡	In CO:O_2 (9:1)‡	
Control	Male	7.66 ± 0.23	1.18 ± 0.26	85
3-MC		29.2 ± 0.74	15.5 ± 0.97	47
Control	Female	2.22 ± 0.01	1.08 ± 0.003	51
3-MC		20.1 ± 1.32	12.5 ± 1.24	38
Control	Male	4.45 ± 0.38	1.22 ± 0.06	73
2-FAA		14.4 ± 1.03	1.60 ± 0.76	89
Control	Female	2.50 ± 0.27	1.92 ± 0.25	23
2-FAA		12.7 ± 0.99	6.73 ± 0.33	47

* A single injection of 3-MC (0.075 m-mole/kg in 2.5 ml corn oil) or 2-FAA (0.1 m-mole/kg in 2.2 ml of 7% gum arabic in 0.9% NaCl solution) was given i.p. 24 hr before preparation of microsomes. Control rats received a single i.p. injection of the appropriate vehicle. Pooled livers of two rats in each group were used.

† *N*-hydroxy-2-FAA was determined by inverse isotope dilution as described in Materials and Methods. Values are means ± S. D. from three incubations.

‡ The gas mixture of the desired volume ratio was provided by a gas-mixing pump, type SA 18-2a (H. Wösthoff, 463 Bochum, West Germany). The Meyer N-EVAP analytical evaporator (model 111, Organomation Assoc. Inc., Shrewsbury, MA) was used to supply the gas mixtures to individual tubes containing reaction mixtures. The incubations were carried out at 37°. The gas mixtures were supplied through needles (19 gauge) at a rate of 15–20 bubbles/min. Buffer (5 ml/tube) was equilibrated with the appropriate gas mixture for 5 min prior to incubations. Microsomes (12 mg protein) and 2-[9-^{14}C]FAA (0.4 to 0.5 μmole in 0.1 ml ethanol) were added and equilibration was continued for 3 min. NADPH (12 μmoles in 0.5 ml of the gas-saturated buffer) was then added and the reaction mixtures were flushed for an additional 10 min. The incubations were terminated and the mixtures were processed as described in the text.

volves cytochrome P-450 was not a rate-limiting step. These data showed that inhibition of the carcinogenicity of an arylamide which requires *N*-hydroxylation for its tumorigenic activity, cannot be accomplished by means of an agent which only partially destroys the biocatalyst responsible for *N*-hydroxylation.

Since the increase in the microsomal *N*-hydroxylation of 2-FAA-treated rats was not accompanied by an increase in microsomal hemoprotein, it seemed possible that the activity of components of the hepatic mixed-function oxidase other than cytochrome P-450 or P_1-450 was stimulated by 2-FAA. Accordingly, we measured the activities of NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase in microsomes of untreated and 2-FAA-treated rats and compared these values to those obtained with microsomes of PB-treated rats (Fig. 7). A single injection of PB to rats increased NADPH–cytochrome P-450 reductase activity by 60 and 80 per cent in male and female rats, respectively, without stimulating the activity of NADPH–cytochrome *c* reductase. Three injections of PB to rats enhanced NADPH–cytochrome P-450 reductase activity 3- and 4-fold in the males and females, respectively, and doubled NADPH–cytochrome *c* reductase activity. In contrast, a single injection of 2-FAA depressed the activities of the reductases. Hence, the marked increase in *N*-hydroxylation of 2-FAA observed after a single dose of 2-FAA was not due to a stimulation of the activities of the reductases. After three injections of 2-FAA, the activity of NADPH–cytochrome *c* reductase was increased by 40 and 30 per cent in males and females, respectively, and the activity of NADPH–cytochrome P-450 reductase was increased by 50 per cent in both males and females. The increase in *N*-hydroxylation of 2-FAA after multiple injections of 2-FAA may therefore, at least in part, be due to stimulation of the electron transport from NADPH to the terminal oxidase, cytochrome P-450.

Since a single injection of 2-FAA did not increase the activities of microsomal NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase,

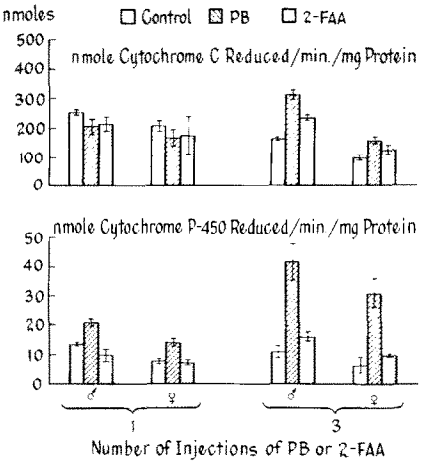

Fig. 7. Effect of PB or 2-FAA on the activities of NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase in microsomes of rat liver. PB (0.3 m-mole/kg) or 2-FAA (0.1 m-mole/kg) was administered i.p. Assays of enzymatic activities are described in Materials and Methods. Entries are mean values ± S. D. from three separate experiments. Differences in microsomal NADPH–cytochrome *c* reductase activity between PB-treated (three doses) male and female rats, 2-FAA-treated (three doses) female rats and the respective controls were statistically significant ($0.05 > P > 0.001$). Differences in microsomal NADPH–cytochrome P-450 activity between PB-treated (one or three doses) male and female rats, 2-FAA-treated (three doses) male rats and the respective controls were statistically significant ($0.05 > P > 0.001$).

we examined the activity of microsomal amine oxidase. This flavoprotein, which is not considered to be a part of the hepatic mixed-function oxidase [30], catalyzes the oxidation of primary, secondary and tertiary amines [31–33]. Although this enzyme did not appear to be involved in *N*-hydroxylation of 2-FAA by hepatic microsomes of 3-MC-treated hamsters [34], its role in the 2-FAA-induced *N*-hydroxylation has not been investigated. The formation of DMA-*N*-oxide by hepatic microsomes in 2-FAA-

Table 3. Lack of inhibition of *N*-hydroxylation and of carcinogenicity of 2-FAA by secobarbital

Compound injected*	Cytochrome P-450† (nmole/mg protein)	*N*-hydroxylation‡ (% of substrate converted)	Rats with tumors/ rats used§
Vehicle	0.74	0.91	0/11
Secobarbital	0.48	0.95	0/11
2-FAA	0.59	5.84	5/13 (16)‖
Secobarbital + 2-FAA	0.47	5.77	5/10 (13)¶

* 2-FAA (0.1 m-mole/kg) as a 1 per cent suspension of the compound in 7% gum arabic in 0.9% NaCl was injected i.p. 3 times weekly for a total of 4 weeks. Control rats received i.p. injections (2.2 ml/kg) of the vehicle. Sodium secobarbital (Eli Lilly Co., Indianapolis, IN) (25 mg/kg) was injected s.c. 1 hr prior to each injection of 2-FAA. Average total dose of sodium secobarbital was 75 mg/rat.

† Determined by the method of Omura and Sato [14].

‡ *N*-hydroxylation of 2-FAA by hepatic microsomes from the pooled livers of two rats was determined 24 hr after the last treatment (see first footnote) as described in Materials and Methods. Each value is the average of three incubations.

§ Tumor incidence assessed 10 months after administration of the compounds had been completed. The numbers in parentheses indicate the total number of tumors.

‖ Fourteen mammary adenocarcinomas, one cellular adenoma and one mixed fibro- and cellular adenoma.

¶ Thirteen mammary adenocarcinomas.

Table 4. Effect of 2-FAA on amine oxidase activity of hepatic microsomes

Pretreatment of rat*	Sex of rat	DMA N-oxide formed† (nmoles/mg protein/min ± S. D.)	Δ Decrease (%)
Control	Male	3.52 ± 0.26	
2-FAA		2.05 ± 0.12	40‡
Control	Female	2.64 ± 0.24	
2-FAA		1.59 ± 0.17	42§

* A single i.p. injection of 2-FAA (0.1 m-mole/kg) as a 1 per cent suspension of the compound in 7% gum arabic in 0.9% NaCl was given to each rat 24 hr before preparation of microsomes. Control rats received a single i.p. injection of the vehicle (2.2 ml/kg). Microsomes were obtained from pooled livers of three to six rats in each group.

† Formation of DMA-N-oxide was measured as described in Materials and Methods. The values are the means ± S. D. from four incubations.

‡ $P < 0.001$, compared to controls.

§ $P \cong 0.001$, compared to controls.

treated rats was decreased significantly in comparison to untreated controls (Table 4. These results led us to conclude that the increased N-hydroxylation of 2-FAA by hepatic microsomes of 2-FAA-treated rats is not attributable to an increased activity of hepatic amine oxidase.

DISCUSSION

The present study suggests that induction of N-hydroxylation by 2-FAA is distinctly different from induction by 3-MC. In contrast to PB- or 3-MC-treated rats, the content of cytochrome P-450 or P_1-450 in hepatic microsomes of 2-FAA-treated rats estimated either by CO difference spectra (see Fig. 3) or by gel electrophoresis (see Figs. 4 and 5) was not increased. While the present studies were in progress, Cameron *et al.* [26] reported that intake of a diet containing 0.02 or 0.05% 2-FAA to male Fischer rats for 2 weeks induced a novel electrophoretic pattern in which the amounts of two polypeptides with a molecular weight of approximately 45,000 were increased. These authors suggested that one of these polypeptides is a hemoprotein. Contrary to their data, we did not observe an increase in the amounts of these proteins in hepatic microsomes of 2-FAA-treated rats (see Figs. 4 and 5). It is possible that the diffferences between our results obtained with Sprague–Dawley rats and those of Cameron *et al.* [26], who used Fischer rats, are due to strain differences. Irrespective of possible differences in the composition of microsomal proteins between the two strains, the data of Cameron *et al.* [26] provide no evidence that the proteins which they reported to be induced by 2-FAA are involved in any way in the increased N-hydroxylation of 2-FAA by hepatic microsomes of the 2-FAA-treated rat. The electrophoretic patterns of these workers are in agreement with our data that 2-FAA does not appear to induce cytochrome P-450 or P_1-450. Cameron *et al.* [26] observed an increase (40 per cent) in the absorbance of the reduced CO–hemoprotein complex in hepatic microsomes of three male Fischer rats pretreated with 2-FAA. We have also observed occasionally similar increases in cytochrome P-450 content in hepatic microsomes 24 hr after treatment of rats with 2-FAA. However, statistical analysis of our data indicated that the average content of cytochrome P-450 in microsomes of 2-FAA-treated rats was not significantly greater than that of untreated rats ($P > 0.5$; see Fig. 3). Occasional differences in the cytochrome P-450 content of hepatic microsomes of untreated and 2-FAA-treated rats may be expected because of individual variations among rats, and these differences are probably not related to a specific induction. Induction of cytochrome P-450 was also not apparent when measurements of absorbance of the reduced CO–hemoprotein complex were taken less than 24 hr after treatment of rats with 2-FAA (see Fig. 2).

Recently, it has been reported that 2-FAA stimulated *in vitro* the formation of 2-hydroxybiphenyl from biphenyl [35]. Although the mechanism of this enhancement remains to be elucidated, no evidence has been presented that the induction of this C-hydroxylation coincided with a major change in the content of cytochrome P-450.

When the increase in N- or C-hydroxylation cannot be correlated with the induction of the terminal oxidase, the possibility remains that the stimulation of the hydroxylation may be attributable to an increase of the activity of the cytochrome P-450 reductases. It has been reported that compounds which give a type I binding spectrum increase the rate of reduction of cytochrome P-450 *in vitro* [19]. It has also been shown that pretreatment of rats with PB, a compound which elicits a type I binding spectrum, increased the activity of NADPH–cytochrome *c* reductase [36]. We have confirmed these findings in that treatment of rats with PB doubled the activity of this enzyme and tripled that of NADPH–cytochrome P-450 reductase. However, administration of a single dose of 2-FAA which also gives a characteristic type I binding spectrum (see Fig. 6) caused a marked increase in N-hydroxylation (see Figs. 1–3) without a measurable increase of the activity of the reductases (see Fig. 7). Hence, compounds exhibiting a type I binding spectrum do not necessarily enhance the activity of the reductases of cytochrome P-450.

Since 2-FAA did not induce any of the known components of the hepatic mixed-function oxidase nor amine oxidase, the possibility remains to be investigated whether 2-FAA induces an electron carrier associated with the hepatic mixed-function oxidase but not identified by the enzyme assays employed in this study. The importance of resolving this problem lies in the fact that the 2-FAA-mediated induction of

N-hydroxylation is more closely tied to arylamide carcinogenesis than the 3-MC-mediated induction. The latter, though of interest for general mechanisms of N-hydroxylation, is clearly not related to carcinogenesis by 2-FAA or arylamides in general. To our knowledge, the findings of this study are the first evidence that a carcinogenic arylamide given to the rat promotes the induction of the enzyme(s) which convert the administered compound to its active form.

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REFERENCES

1. J. W. Cramer, J. A. Miller and E. C. Miller, *J. biol. Chem.* **235,** 885 (1960).
2. J. A. Miller, J. W. Cramer and E. C. Miller, *Cancer Res.* **20,** 950 (1960).
3. C. C. Irving, *J. biol. Chem.* **239,** 1589 (1964).
4. H. R. Gutmann and P. Bell, *Fedn Proc.* **32,** 665 (1973).
5. P. D. Lotlikar and K. Zaleski, *Biochem. J.* **150,** 561 (1975).
6. H. R. Gutmann and P. Bell, *Biochim. biophys. Acta* **498,** 229 (1977).
7. D. Malejka-Giganti, R. E. Rydell and H. R. Gutmann, *Cancer Res.* **37,** 111 (1977).
8. D. Malejka-Giganti, H. R. Gutmann and R. E. Rydell, *Cancer Res.* **33,** 2489 (1973).
9. E. J. Barry, D. Malejka-Giganti and H. R. Gutmann, *Chem. Biol. Interact.* **1,** 139 (1969–70).
10. E. C. Miller, J. A. Miller and H. A. Hartmann, *Cancer Res.* **21,** 815 (1961).
11. C. M. King and H. R. Gutmann, *Cancer Res.* **24,** 770 (1964).
12. U. S. Seal and H. R. Gutmann, *J. biol. Chem.* **234,** 648 (1959).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
14. T. Omura and R. Sato, *J. biol. Chem.* **239,** 2370 (1964).
15. N. E. Sladek and G. J. Mannering, *Biochem. biophys. Res. Commun.* **24,** 668 (1966).
16. K. Weber and M. Osborn, *J. biol. Chem.* **244,** 4406 (1969).
17. G. M. Cohen and G. J. Mannering, *Drug Metab. Dispos.* **2,** 285 (1974).
18. K. W. Renton and G. J. Mannering, *Drug Metab. Dispos.* **4,** 223 (1976).
19. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5,** 109 (1969).
20. D. M. Ziegler and F. H. Pettit, *Biochem. biophys. Res. Commun.* **15,** 188 (1964).
21. A. P. Alvares, G. Schilling, W. Levin and R. Kuntzman, *Biochem. biophys. Res. Commun.* **29,** 521 (1967).
22. A. Hildebrandt, H. Remmer and R. W. Estabrook, *Biochem. biophys. Res. Commun.* **30,** 607 (1968).
23. D. Ryan, A. Y. H. Lu, J. Kawalek, S. B. West and W. Levin, *Biochem. biophys. Res. Commun.* **64,** 1134 (1975).
24. J.-P. Villeneuve, P. Mavier and J.-G. Joly, *Biochem. biophys. Res. Commun.* **70,** 723 (1976).
25. W. Levin, A. Y. H. Lu, D. Ryan, S. West, R. Kuntzman and A. H. Conney, *Archs Biochem. Biophys.* **153,** 543 (1972).
26. R. Cameron, R. N. Sharma, G. D. Sweeney, E. Farber and R. K. Murray, *Biochem. biophys. Res. Commun.* **71,** 1054 (1976).
27. J. A. Miller, *Cancer Res.* **30,** 559 (1970).
28. W. Levin, E. Sernatinger, M. Jacobson and R. Kuntzman, *Science, N.Y.* **176,** 1341 (1972).
29. W. Levin, M. Jacobson, E. Sernatinger and R. Kuntzman, *Drug Metab. Dispos.* **1,** 275 (1973).
30. B. S. S. Masters and D. M. Ziegler, *Archs Biochem. Biophys.* **145,** 358 (1971).
31. D. M. Ziegler, L. L. Poulsen and E. M. McKee, *Xenobiotica* **1,** 523 (1971).
32. D. M. Ziegler and C. H. Mitchell, *Archs Biochem. Biophys.* **150,** 116 (1972).
33. D. M. Ziegler, E. M. McKee and L. L. Poulsen, *Drug Metab. Dispos.* **1,** 314 (1973).
34. P. D. Lotlikar, K. Wertman and L. Luha, *Biochem. J.* **136,** 37 (1973).
35. F. J. McPherson, J. W. Bridges and D. V. Parke, *Biochem. Pharmac.* **25,** 1345 (1976).
36. R. F. Burk and B. S. S. Masters, *Archs Biochem. Biophys.* **170,** 124 (1975).