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MICROSOMAL METABOLISM OF ARYLAMIDES BY THE RAT AND GUINEA PIG—I.

OXIDATION OF *N*-3-FLUORENYLACETAMIDE AT CARBON-ATOM-9—A MAJOR METABOLIC REACTION*

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Abstract—The *N*-hydroxylation of *N*-3-fluorenylacetamide (3-FAA), an isomer of the carcinogen, *N*-2-fluorenylacetamide (2-FAA), by hepatic microsomes of untreated and 3-methylcholanthrene (3-MC)-treated guinea pigs was found to be of a similar low order as that previously observed in the rat. Hepatic microsomes of the guinea pig and of the rat converted 3-FAA to *N*-(9-hydroxy)-3-FAA and to *N*-(9-oxo)-3-FAA. These new metabolites were separated and identified by high-pressure liquid chromatography (h.p.l.c.). *N*-(9-hydroxy)-3-FAA was the major product of the hydroxylation of 3-FAA by hepatic microsomes of the rat or guinea pig exceeding the formation of *N*-(7-hydroxy)-3-FAA, the principal phenolic metabolite of 3-FAA. The amounts of *N*-(9-oxo)-3-FAA formed were about one-third of the amounts of *N*-(9-hydroxy)-3-FAA produced. In contrast to the formation of phenolic metabolities, the hydroxylation of 3-FAA to *N*-(9-hydroxy)-3-FAA was not increased by pretreatment of guinea pigs or rats with 3-MC. Similarly, pretreatment of rats with PB did not enhance the yield of *N*-(9-hydroxy)-3-FAA. CO inhibited the formation of *N*-(9-hydroxy)-3-FAA by 80 per cent. These data lead us to conclude that the formation of *N*-(9-hydroxy)-3-FAA is catalyzed by a microsomal hemoprotein not identical with cytochrome P_1-450 or P-450. In contrast to 3-FAA, 2-FAA appeared to be a poor substrate for hydroxylation to the *N*-(9-hydroxy)-2-FAA. The susceptibility of 3-FAA to hydroxylation at carbon-atom 9 of the fluorene moiety may be rationalized by resonance structures in which carbon-atom 9 is positively charged and acts as a electrophilic center. Similar resonance structures cannot be written for 2-FAA.

Previous investigations have shown that the carcinogenic arylamide, 2-FAA,* is bound to cytochrome P_1-450 of rat liver microsomes, as indicated by a type I binding spectrum [1], and that the compound is metabolized by hepatic microsomes of 3-MC-treated rats to the proximate carcinogen, *N*-hydroxy-2-FAA [1–3]. In contrast, the non-carcinogenic isomer, 3-FAA, produces an identical type I binding spectrum with hepatic microsomes of 3-MC-treated rats or guinea pigs, but yields only trace amounts of *N*-hydroxy-3-FAA on incubation with hepatic microsomes of the 3-MC-stimulated rat [1]. In the current study, we have explored the *N*-hydroxylation and *C*-hydroxylation of 3-FAA by hepatic microsomes of the untreated and 3-MC-treated guinea pig. On the basis of structural considerations, we hypothesized that 3-FAA would be hydroxylated at C-9 of the fluorene moiety. Accordingly, we investigated the conversion of 3-FAA to *N*-(9-hydroxy)-3-FAA by hepatic microsomes of the guinea pig and of the rat and compared the formation of the alcohol, *N*-(9-hydroxy)-3-FAA, with the microsomal formation of phenolic metabolites under various conditions. Some preliminary observations were made concerning the microsomal enzyme(s) which catalyze the hydroxylation of 3-FAA to *N*-(9-hydroxy)-3-FAA. The data obtained in these studies form the basis of this report.

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* The following abbreviations are used in the text: 2-FAA, *N*-2-Fluorenylacetamide; 3-MC, 3-methylcholanthrene; PB, phenobarbital; DMSO, dimethyl sulfoxide; 3-FAA, *N*-3-fluorenylacetamide; t.l.c., thin-layer chromatography; h.p.l.c., high-pressure liquid chromatography; and C-9, carbon-atom 9.

MATERIALS AND METHODS

Labeled and unlabeled compounds. *N*[^{14}C-acetyl]3-FAA was prepared by treating 3-fluorenamine (73 mg, 0.40 m-mole) in benzene (4 ml) and triethylamine (0.01 ml) with [^{14}C-carbonyl]acetic anhydride (2.5 mCi, 0.50 m-mole) (New England Nuclear Corp., Boston, MA). The material was collected, washed with *n*-hexane and recrystallized from ethanol–water. The specific radioactivity of the recrystallized compound (65 mg), m.p. 192–194°, was 2.2 mCi/m-mole. Thin-layer chromatography of the compound on Silica gel GF_{254} with chloroform–methanol (95:5) and a radioscan of the chromatogram with a thin-layer scanner (model LB 2721, Berthold Laboratories, Wildbad, West Germany) showed a single radioactive peak, $R_f = 0.53$. *N*[^{14}C-acetyl]2-FAA, m.p. 196° (specific radioactivity = 3.55 mCi/m-mole) was obtained as described above for *N*[^{14}C-acetyl]3-FAA. High-pressure liquid chromatography of the compound on Corasil II with diethyl ether–*n*-hexane (9:1) gave a

single peak (retention time = 23 min). *N*-hydroxy-3-FAA, m.p. 132–134°[4], 3-FAA, m.p. 191–193°[5], *N*-(9-hydroxy)-3-FAA, m.p. 210–211°[6], *N*-(9-oxo)-3-FAA, m.p. 219–221°[6], *N*-(2-hydroxy)-3-FAA, m.p. 230–231°[7], *N*-(9-hydroxy)-2-FAA, m.p. 249–250°[6, 8], and *N*-(9-oxo)-2-FAA, m.p. 233–236°[6, 8], were synthesized by published methods. *N*-(7-hydroxy)-3-FAA, m.p. 269–272°, m/e = 239 (M^+), was prepared by a modification of the literature procedure[9]. Anal. Calc. for $C_{15}H_{13}NO_2$: C, 75.30; H, 5.48; N, 5.60. Found: C, 75.19; H, 5.57; N, 5.79. All compounds gave single fluorescence-quenching spots on t.l.c. (Silica gel GF_{254}) with chloroform–methanol (95:5) as a solvent or single peaks by h.p.l.c. on Corasil II with diethyl ether–*n*–hexane (9:1) as a solvent[6]. 3-MC, m.p. 181–182°, and PB were obtained from Distillation Products Industries (Division of Eastman Kodak Co.) Rochester, NY, and from Winthrop Laboratories, New York, NY respectively. NADPH and NADH were obtained from CalBiochem, La Jolla, CA. Crystalline serum albumin was purchased from Sigma Chemical Co., St. Louis, MO.

Preparations of microsomes. Microsomes were prepared from the pooled livers of male Sprague–Dawley rats (175–200 g) or male guinea pigs (175–200 g) of the Duncan–Hartley strain. The animals were purchased from Bio-Lab, White Bear Lake, MN, and maintained on Purina Chow pellets and water *ad lib*. The animals were injected with 3-MC or PB according to the schedule indicated in Table 1 (footnote ‡) or Table 4 (footnote †). Food was withdrawn 24 hr prior to sacrifice by decapitation. The livers were perfused with cold 0.9% NaCl, excised, weighed and frozen to a thin sheet between two blocks of dry ice. The frozen tissue was powdered under liquid N_2 and the powder was homogenized in 0.25 M sucrose–0.05 M KH_2PO_4, pH 7.4, with a Polytron homogenizer (type PT 10, Kinematica GMBH, Luzern, Switzerland), for 30–60 sec at the highest speed. Microsomes were obtained from the 20% homogenate (w/v) by differential centrifugation[10]. The microsomal pellet was washed once with the 0.05 M KH_2PO_4–0.025 M KCl buffer, pH 7.4, and was then homogenized manually in a homogenizer equipped with a Teflon pestle. Aliquots (0.05 ml) of this suspension were diluted 100-fold with deionized water and the protein content of this solution was determined colorimetrically with the use of crystalline bovine serum albumin as the standard [11].

Assay for cytochrome P-450, conditions of incubation and determination of metabolites. Cytochrome P-450 in hepatic microsomes was measured spectrophotometrically as the reduced CO:hemoprotein complex in a Beckman Acta VI spectrophotometer with the use of an extinction coefficient of 91 $mM^{-1}\ cm^{-1}$[12]. Each incubation mixture contained *N*[^{14}C-acetyl]3-FAA or *N*[^{14}C-acetyl]2-FAA (0.5 μmole in 0.05 ml DMSO), NADPH (12 μmoles) unless stated otherwise, and microsomes equivalent to 10–12 mg protein[1]. The relationship between protein concentration and oxidation at C-9 of 3-FAA was determined within a range of 0.5 to 2.0 mg protein/ml. The extent of the reaction was a non-linear function of protein concentration within that range and tended to reach a plateau near 2.0 mg protein/ml, the concentration used throughout this study. The total volume was adjusted to 6.0 ml with 0.05 M KH_2PO_4–0.025 M KCl buffer, pH 7.4. Incubations were carried out for 0.5 hr in a rotary shaker at 37° and in an atmosphere of air. The incubations were terminated by addition of 1 N HCl (0.5 ml). The metabolites were extracted from the medium with diethyl ether and partitioned into neutral and phenolic fractions[13]. The ether was evaporated and the residues were dissolved in 1.0 ml MeOH. The methanol solutions were assayed for radioactivity. Aliquots containing 10,000–20,000 dis./min were dried under N_2 and the residue was dissolved in 0.05 ml ethyl acetate. Appropriate markers (0.2 to 0.5 μg) were added and aliquots of the ethyl acetate solution containing 2000–4000 dis./min were subjected to h.p.l.c. with Corasil II as the adsorbent and with diethyl ether–*n*–hexane (9:1) as the solvent[6]. The effluent corresponding to the markers as well as to several as yet unidentified peaks in the elution profile (see Figs. 1 and 2) was collected and assayed for radioactivity. The solvent was evaporated and the residue was dissolved in Scintisol Complete (10 ml) (Isolab, Inc., Akron, OH). Radioactivity was measured in a Packard liquid scintillation spectrometer, model 3375. Counts were corrected for quenching by means of a calibration curve established with external standards. The counting efficiency was 83–90 per cent. All samples were counted with an error not exceeding 5 per cent. Samples containing less radioactivity than twice background were disregarded. The radioactivity of the metabolites in the neutral or phenolic fraction was calculated from the radioactivity associated with the respective peaks in the elution profile and the ratio of the volume of the fraction chromatographed to the total volume of the fraction. These values were used to determine the extent of conversion of the substrate to a particular metabolite. As shown in Figs. 1 and 2A, the radioactivity associated with the various peaks was clearly separated provided that the total radioactivity applied to the column did not exceed 4000 cpm.

RESULTS AND DISCUSSION

We have reported previously that 3-FAA and 2-FAA bind to cytochrome P_1-450 of hepatic microsomes of the guinea pig and rat[1, 14]. Judged by the appearance of the binding spectra[1] and by determinations of the binding constants[14], the binding of 3-FAA to cytochrome P_1-450 appeared to be of a magnitude similar to that of 2-FAA. Binding of an arylamide to cytochrome P_1-450 presumably precedes its microsomal oxidation [15]. Consequently, we determined the *N*-hydroxylation of 3-FAA by hepatic microsomes of untreated and of 3-MC-treated guinea pigs (Table 1). Although hepatic microsomes from the guinea pig contain an active deacetylase[17], a deacetylase inhibitor, such as NaF, was not added to the incubation mixtures because NaF inhibited the microsomal enzymatic system involved in the *N*-hydroxylation of

Table 1. *N*-hydroxylation of *N*[^{14}C-acetyl]3-FAA by hepatic microsomes of untreated and 3-MC-treated guinea pigs*

Expt. No.	3-MC treatment†	Cytochrome P-450‡ (nmoles/mg protein)	*N*-hydroxy-[^{14}C-acetyl]-3-FAA found§ (nmoles)	*N*-hydroxylation (%)
1a	–	1.82	ND‖	ND
1b	+	2.58	0.46 ± 0.20	0.09
2a	–	1.42	0.86 ± 0.48	0.17
2b	+	2.45	1.56 ± 0.81¶	0.31

* Microsomes were prepared by Ca^{2+}-aggregation [1, 16] from the pooled livers of two animals in Expts. 1a and 1b and from the pooled livers of three animals in Expts. 2a and 2b. The incubation system contained microsomes (12 mg protein), NADPH (12 μmoles) and *N*[^{14}C-acetyl]3-FAA (0.5 μmole in 0.05 ml DMSO, sp. act. = 2.18 mCi/m-mole) in Tris buffer (0.03 M, pH 7.5). The final volume of the incubation system was 6.0 ml. Incubations were carried out for 0.5 hr in air at 37° in a rotary shaker. At the end of the incubation period, carrier compound (*N*-hydroxy-3-FAA, 2 μmoles) was added. The *N*-hydroxy metabolite was isolated by solvent extraction, purified by t.l.c. and its radioactivity was assayed as described previously [1].

† 3-MC (20 mg/kg of body weight) in corn oil (8 mg/ml) was injected intraperitoneally at 48 and 24 hr prior to preparation of the microsomes. The untreated animals were injected with corn oil only.

‡ Cytochrome P-450 was determined by the standard procedure [12].

§ The values are the means ± S.E. of three to four separate incubation mixtures.

‖ Not detected.

¶ This value is not significantly different from that found in Expt. 2a ($P > 0.05$).

2-FAA [1]. Since the *N*-hydroxylation of 2-FAA by guinea pig-liver microsomes was lower, by several orders of magnitude, than the *N*-hydroxylation of the carcinogen by rat-liver microsomes, we felt that the low capacity of guinea pig liver for microsomal *N*-hydroxylation would be even further depressed by addition of NaF. Quantitatively, microsomal *N*-hydroxylation of 3-FAA was of the same low order as that previously reported for 2-FAA [1]. The low capacity of the guinea pig for *N*-hydroxylation reported previously for 2-FAA [1–3] and shown in this study for 3-FAA explains the resistance of this species to the carcinogenicity of 2-FAA and related arylamides [18].

The data on the spectral binding of 3-FAA to microsomal cytochrome P_1-450 of the guinea pig, along with the absence of *N*-hydroxylation of 3-FAA, suggested that the compound might be metabolized to other oxidation products. In the case of 2-FAA, hepatic microsomes of untreated and 3-MC-treated guinea pigs produced large amounts of the phenolamides, *N*-(7-hydroxy)-2-FAA and *N*-(5-hydroxy)-2-FAA [2]. Accordingly, we investigated the formation of *C*-hydroxylated metabolites of 3-FAA.

High pressure liquid chromatography of the phenolic fraction obtained by extraction of incubation mixtures of hepatic microsomes with *N*[^{14}C-acetyl]3-FAA in the presence of NADPH indicated that the effluent radioactivity was largely associated with *N*-(7-hydroxy)-3-FAA (peak IV) and two other unidentified metabolites (peaks VI and VII, Fig. 1A). *N*-(7-hydroxy)-3-FAA was a major phenolic metabolite produced by microsomal oxidation in untreated as well as in 3-MC-treated guinea pig (Table 2). The unidentified compound, designated peak VII, was a major microsomal metabolite only when animals had been treated with 3-MC. *N*-(2-hydroxy)-3-FAA, which in addition to *N*-(7-hydroxy)-3-FAA is a major urinary metabolite of 3-FAA in the rat [7], was not formed by hepatic microsomes of the untreated guinea pig. Only trace amounts of this compound were detectable in microsomal suspensions obtained from 3-MC-treated guinea pigs. The addition of NADPH was a requirement for the formation of any phenolic metabolite by hepatic microsomes of the guinea pig. This is in agreement with findings that microsomal *C*-hydroxylation required NADPH [19, 20], is stimulated by 3-MC [21] and is carried out by one of the multiple forms of cytochrome P-450 [21, 22].

High pressure liquid chromatography of the neutral fraction obtained by extraction of the incubation mixtures of hepatic microsomes from guinea pigs disclosed, in addition to the labeled substrate, *N*[^{14}C-acetyl]3-FAA, radioactivity associated with *N*-(9-hydroxy)-3-FAA and *N*-(9-oxo)-3-FAA (Fig. 1B). The figure shows that the labeled metabolites were clearly separated from each other as well as from remaining substrate. The major metabolite of *N*[^{14}C-acetyl]3-FAA produced by microsomes of untreated guinea pigs was *N*-(9-hydroxy)[^{14}C-acetyl]3-FAA. Conversion of the arylamide to this metabolite accounted for 19 per cent of the substrate (Table 3). This value may represent a lower limit, since no attempt was made to inhibit the deacetylase present in guinea pig-liver microsomes [17]. Nevertheless, the magnitude of the value clearly indicates

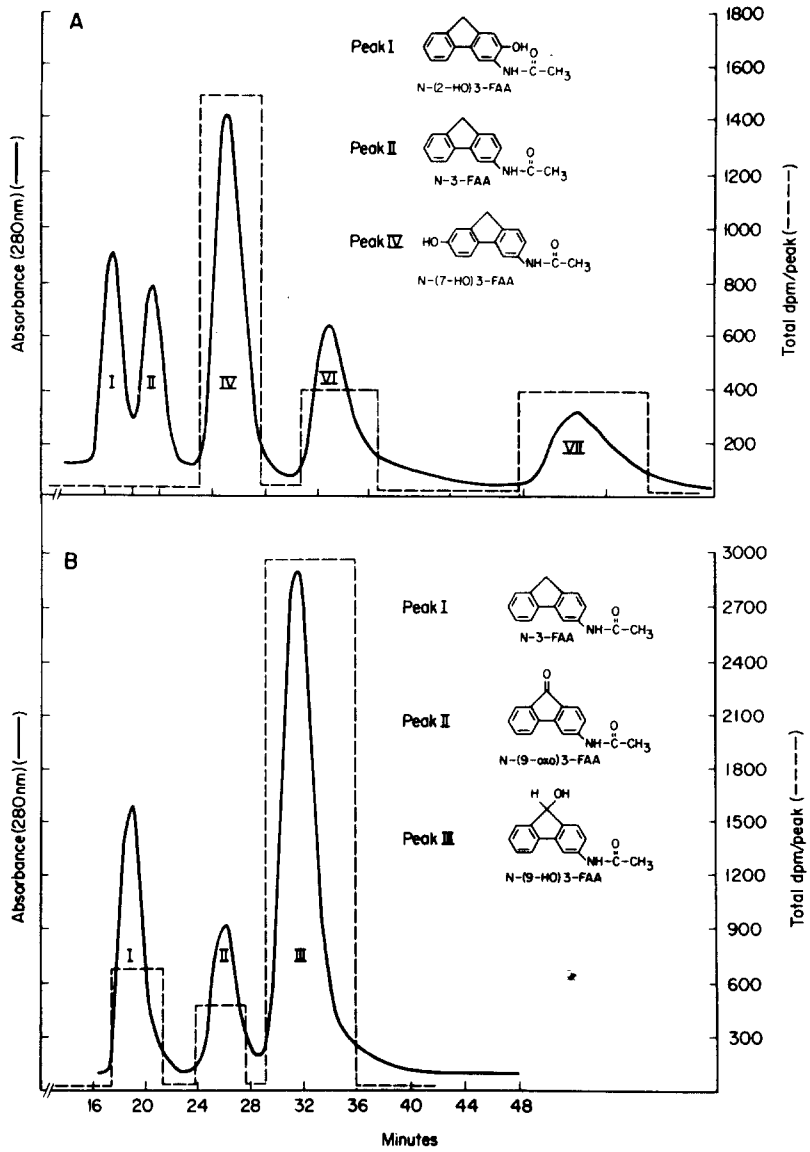

Fig. 1. Identification by h.p.l.c. of the phenolic and neutral metabolites of *N*[^{14}C-acetyl]3-FAA formed by hepatic microsomes of the untreated and 3-MC-treated guinea pig. The microsomes were prepared from the pooled livers of two to three animals by differential centrifugation in 0.25 M sucrose–0.05 M KH_2PO_4 buffer, pH 7.4, containing 0.025 M KCl [10]. Each incubation system contained microsomes (10–12 mg protein), *N*[^{14}C-acetyl]3-FAA (0.5 μmole in 0.05 ml DMSO, sp. act. = 1.0 mCi/m-mole), in 0.05 M KH_2PO_4–0.025 M KCl buffer, pH 7.4, and NADPH (12 μmoles). The total volume of the incubation system was 6.0 ml and the incubations were carried out in air for 0.5 hr at 37° in a rotary shaker. The incubations were terminated by addition of 1 N HCl (0.5 ml). The metabolites were isolated from the microsomal incubation system by solvent extraction [13] and the compounds (0.2 to 0.5 μg) corresponding to peaks I, II and IV of A were added to the phenolic fractions as markers. The compounds (0.2 to 0.5 μg) corresponding to peaks I, II and III of B were added to the neutral fraction as markers. The solid line represents the absorbance of the compounds in the effluent at 280 nm. The columns indicated by the dotted line represent the radioactivity eluted simultaneously with the respective markers and with unidentified metabolites. (A) elution of the radioactivity associated with the phenolic metabolites produced by the hepatic microsomes of the 3-MC-treated guinea pig; (B) elution of the radioactivity associated with the neutral metabolites produced by hepatic microsomes of the untreated or 3-MC-treated guinea pig.

that hydroxylation of 3-FAA at C-9 is a major reaction by hepatic microsomes of this species. This hydroxylation was dependent on the presence of NADPH and appeared to be inhibited by pretreatment of guinea pigs with 3-MC. NADPH could not be replaced by NADH. The yield of *N*-(9-oxo)-3-FAA was one-third of that of *N*-(9-hydroxy)-3-FAA. Oxidation of 3-FAA to the ketone also appeared to require NADPH and was inhibited by pretreatment of the animals with 3-MC. Since neither hydroxylation nor ketone formation was inducible by 3-MC, we concluded tentatively that the

Table 2. Formation of phenolic metabolites of *N*[^{14}C-acetyl]3-FAA by hepatic microsomes of untreated and 3-MC-treated guinea pigs*

Pretreatment of guinea pigs†	NADPH present in incubation system	Cytochrome P-450‡ (nmoles/mg protein)	Amount of metabolite found§ (nmoles)			
			N-(2-hydroxy)-3-FAA	*N*-(7-hydroxy)-3-FAA	Peak VI	Peak VII
	−	0.98	ND‖	ND	ND	ND
	+	0.98	ND	7.7 ± 3.7	2.9 ± 1.4	ND
3-MC	+	1.38	0.5¶	19.9 ± 2.2	8.3 ± 1.4	31.2 ± 14.5

* The preparation of the microsomes and the composition of the incubation system were those described in Fig. 1. The metabolites were isolated and identified by h.p.l.c. [6].

† Pretreatment of animals with 3-MC was that described in Table 1 (footnote †).

‡ Cytochrome P-450 was determined by the standard procedure [12].

§ The values are the means ± S.E. of two to three separate experiments each consisting of one to two incubation mixtures.

‖ Not detected.

¶ This value is derived from one experiment consisting of one incubation mixture. In another experiment this metabolite was not detected.

Table 3. Oxidation of *N*[^{14}C-acetyl]3-FAA at C-atom 9 by hepatic microsomes of untreated and 3-MC-treated guinea pigs*

Pretreatment of guinea pigs†	NADPH present in incubation system	Cytochrome P-450‡ (nmoles/mg protein)	Amount of metabolite found§ (nmoles)	
			N-(9-hydroxy)-3-FAA	*N*-(9-oxo)-3-FAA
	−	0.98	1.9 ± 0.9	1.7 ± 0.5
	+	0.98	93.3 ± 15.2	32.6 ± 4.7
3-MC	+	1.38	20.9 ± 3.3	8.8 ± 2.6

* The preparation of the microsomes and the composition of the incubation systems are those described in Fig. 1. The metabolites were isolated and identified by h.p.l.c. [6].

† Pretreatment of animal with 3-MC was that described in Table 1 (footnote †).

‡ Cytochrome P-450 was determined by the standard procedure [12].

§ The values are the means ± S.E. of two to three separate experiments each consisting of one to two incubation mixtures.

microsomal enzyme(s) catalyzing these reactions were distinct from cytochrome P_1-450. Preliminary experiments using *N*-(9-hydroxy)[^{14}C-acetyl]3-FAA as substrate suggested that the alcohol is the immediate precursor of the ketone.

To our knowledge, this is the first instance that microsomal oxidation of a fluorenylacetamide at C-9 has been observed to produce major amounts of the respective alcohol and ketone. Because of the novelty of the reactions with regard to 3-FAA, the question arose as to whether microsomes of other species known to metabolize fluorenylamides also

Table 4. Oxidation of *N*[^{14}C-acetyl]3-FAA at C-atom 9 by hepatic microsomes of untreated rats and of rats treated with PB or 3-MC*

Pretreatment of rats†	NADPH present in incubation system	Cytochrome P-450‡ (nmoles/mg protein)	Amount of metabolite found§ (nmoles)	
			N-(9-hydroxy)-3-FAA	*N*-(9-oxo)-3-FAA
	−	1.16	2.5	1.2
	+	1.16	109.6 ± 25.6	21.0 ± 5.3
PB	+	2.99	97.3 ± 16.7	36.5 ± 3.7
3-MC	+	1.47	96.1 ± 2.7	24.4 ± 2.4

* The preparation of the microsomes and the composition of the incubation system were those described in Fig. 1. The metabolites were isolated and identified by h.p.l.c. [6].

† PB (80 mg/kg of body weight) in saline (40 mg/ml) was injected intraperitoneally 72, 48 and 24 hr prior to the preparation of the microsomes. The untreated animals were injected with saline only. 3-MC was administered as described in Table 1 (footnote †).

‡ Cytochrome P-450 was determined by the standard procedure [12].

§ The values are the means ± S.E. of one to two separate experiments each consisting of two incubation mixtures.

hydroxylate 3-FAA at C-9. Accordingly, we examined the 9-hydroxylation of 3-FAA and the formation of *N*-(9-oxo)-3-FAA by hepatic microsomes of untreated, PB-treated and 3-MC-treated rats. As in the guinea pig, 3-FAA was metabolized to major amounts of *N*-(9-hydroxy)-3-FAA and to *N*-(9-oxo)-3-FAA by hepatic microsomes of the rat (Table 4). The reaction also required the presence of NADPH. Twenty-two and 4.2% of the substrate were converted to *N*-(9-hydroxy)-3-FAA and to *N*-(9-oxo)-3-FAA respectively (Table 4). The hydroxylation and oxidation of 3-FAA at C-9 were not affected by pretreatment of the rats with 3-MC (Table 4). The lack of stimulation by 3-MC confirmed the view that cytochrome P_1-450 was not involved in the formation of *N*-(9-hydroxy)-3-FAA or of *N*-(9-oxo)-3-FAA from 3-FAA.

To determine whether cytochrome P-450 rather than cytochrome P_1-450 catalyzed these reactions, the formation of *N*-(9-hydroxy)-3-FAA and of *N*-(9-oxo)-3-FAA by hepatic microsomes of PB-treated rats was measured (Table 4). The data showed that PB, a known inducer of cytochrome P-450 [23], did not increase the yield of *N*-(9-hydroxy)-3-FAA over

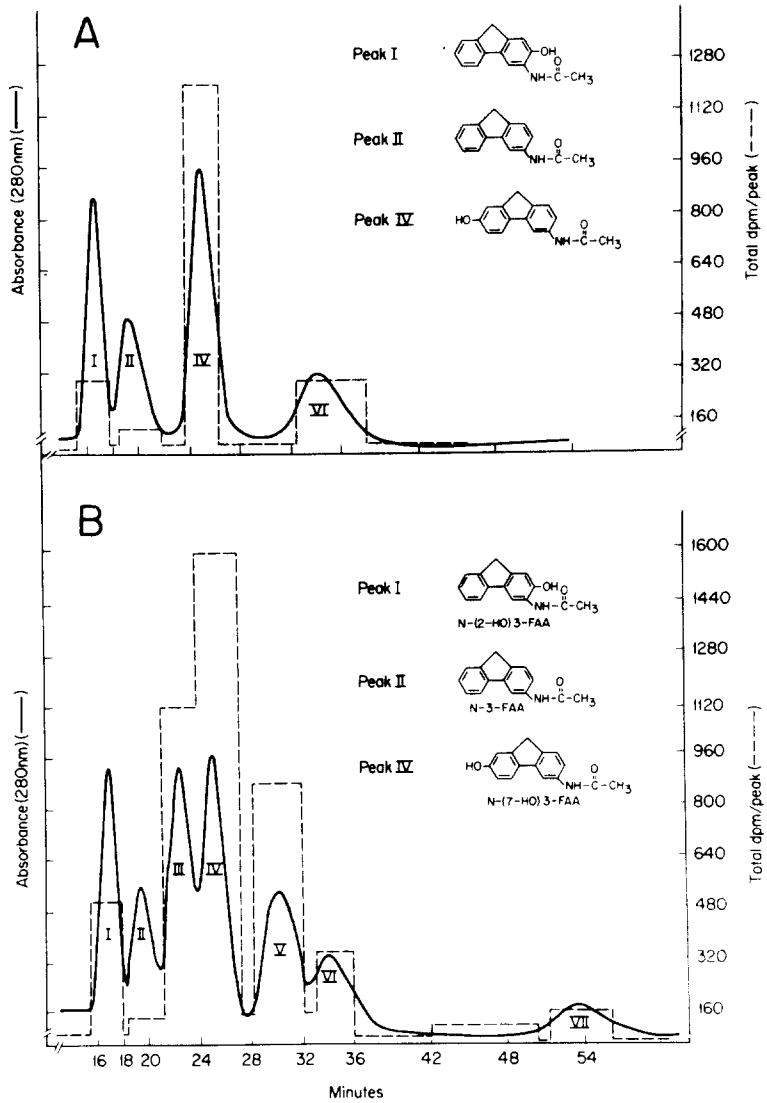

Fig. 2. Identification by h.p.l.c. of phenolic metabolites of *N*[^{14}C-acetyl]-3-FAA by hepatic microsomes of untreated and 3-MC-treated rats. The preparation of the microsomes and the composition of the incubation systems were those described in Fig. 1. The phenolic metabolites were isolated from the incubation mixtures as previously described [13] and the compounds (0.2 to 0.4 μg) corresponding to peaks I, II and IV were added to the phenolic fraction as markers. One-fifth of the total radioactivity of the phenolic fraction was subjected to h.p.l.c. The radioactivity of the phenolic fraction obtained from the microsomal incubation systems of untreated rats (6000 dis./min) was 38 per cent of the radioactivity of the phenolic fraction extracted from the incubation systems of 3-MC-treated rats (16,000 dis./min). The solid line represents the absorbance of the compounds in the effluent at 280 nm. The columns shown by the dotted line represent the radioactivity eluted simultaneously with the respective markers (peaks, I, II and IV) or unknown metabolites (peaks III, V, VI and VII). (A) identification of phenolic metabolites from hepatic microsomes of the untreated rat; (B) identification of phenolic metabolites from the hepatic microsomes of the 3-MC-treated rat.

that of the microsomes of untreated rats. These data suggested that cytochrome P-450 is not involved in the hydroxylation of 3-FAA at C-9.

In order to demonstrate that 3-MC which did not increase the microsomal formation of the alcohol, *N*-(9-hydroxy)-3-FAA, was effective in stimulating the production of phenolamides by rat-liver microsomes, we examined the effect of 3-MC on the conversion of *N*[^{14}C-acetyl]3-FAA to phenolic metabolites. Microsomal *C*-hydroxylation of arylamides to phenolamides has been shown to be stimulated by pretreatment of the rat with 3-MC [1, 2, 20]. The results of these experiments are represented by the elution profiles of Fig. 2. The upper part of the figure shows that the major portion of the eluted radioactivity was associated with *N*-(7-hydroxy)-3-FAA (peak IV). This phenolamide has been identified as a major urinary metabolite of 3-FAA in the rat [9]. *N*-(2-hydroxy)-3-FAA, another urinary metabolite of 3-FAA of the rat [7], was also detected in the eluate (peak I). In addition, there was an unknown metabolite designated peak VI. The elution profile depicted in Fig. 2B shows the stimulatory effect of 3-MC by an increase in the radioactivity associated with *N*-(7-hydroxy)-3-FAA and *N*-(2-hydroxy)-3-FAA. Furthermore, there were three new metabolites (peaks III, V and VII) which, though unidentified, were presumably phenolamides. The amounts of the phenolic metabolites were not assessed quantitatively because there was incomplete separation of the two major peaks, *N*-(7-hydroxy)-3-FAA and peak III. The fact that a stimulatory effect of 3-MC on phenol formation, a cytochrome P_1-450-catalyzed reaction [21, 22], was clearly demonstrated suggested that hydroxylation at C-9 should also have been increased if the reaction were catalyzed by cytochrome P_1-450. The data strengthened the conclusion that cytochrome P_1-450 is involved in the formation of the phenolic metabolites of 3-FAA, but not in the formation of *N*-(9-hydroxy)-3-FAA or *N*-(9-oxo)-3-FAA.

The formation of *N*-(9-hydroxy)-3-FAA from 3-FAA by hepatic microsomes is an oxidative reaction. Microsomal oxidations are carried out by the hepatic mixed-function oxidase which contains multiple forms of the hemoprotein, cytochrome P-450 [24, 25]. Since hydroxylation reactions catalyzed by microsomal hemoprotein(s) are inhibited

Table 5. Effect of CO on hydroxylation at C-atoms 9 and 7 of *N*[^{14}C-acetyl]3-FAA by hepatic microsomes of the untreated rat*

Exposure of incubation system to gas mixture†	Amount of *N*-(9-hydroxy)-3-FAA found (nmoles)	Inhibition of hydroxylation (%)	Amount of *N*-(7-hydroxy)-3-FAA found (nmoles)	Inhibition of hydroxylation (%)
A	101.6 ± 5.0		16.0 ± 7.9	
B	22.7 ± 1.5	77.7	3.9 ± 2.2	76.7

* The preparation of microsomes and the composition of the incubation system were those described in Fig. 1. The values listed in columns 2 and 4 are the means ± S.E. of two separate experiments each consisting of two incubation mixtures. The content of cytochrome P-450 was 1.06 nmoles/mg of protein.

† A = 90% N_2: 10% O_2; B = 90% CO: 10% O_2. Gas mixture A and B were made with a gas-mixing pump, type SA18-2a (Wösthoff, 463 Bochum, West Germany). The buffer was equilibrated for 20 min. After addition of *N*[^{14}C-acetyl]3-FAA, NADPH and microsomes, the incubation flasks were closed with a stopper through which a needle (gauge 18) was inserted in such a way that the tip of the needle was above the level of the liquid. A positive gas pressure was maintained throughout the incubation (0.5 hr) in a rotary shaker at 37°.

Table 6. *C*-hydroxylation and oxidation at C-atom 9 of *N*[^{14}C-acetyl]2-FAA by hepatic microsomes of untreated and 3-MC-treated rats*

3-MC treatment†	Amount of metabolite found‡ (nmoles)				
	N-(9-hydroxy)-2-FAA	*N*-(9-oxo)-2-FAA	*N*-(7-hydroxy)-2-FAA	*N*-(5-hydroxy)-2-FAA	*N*-(3-hydroxy)-2-FAA
−	10.0	ND§	7.5	2.9	1.1
+	6.0	4.1	67.5	26.8	41.6

* Microsomes were prepared as described in Fig. 1. The incubation system (6.0 ml) contained microsomes (12 mg protein), *N*[^{14}C-acetyl]2-FAA (0.5 μmole in 0.05 ml DMSO, sp. act. = 0.1 mCi/m-mole) in a 0.05 M KH_2PO_4–0.025 M KCl buffer, pH 7.4. NADPH (12 μmoles) was added to initiate the reaction. The conditions of incubation and the extraction of the metabolites were those described in Fig. 1. The metabolites were separated and identified by h.p.l.c. [6].

† Pretreatment of animals with 3-MC was that described in Table 1 (footnote †).

‡ Each value represents single determinations from one incubation system containing hepatic microsomes of untreated rats and from one incubation system containing hepatic microsomes of 3-MC-treated rats.

§ Not detected.

by CO[26, 27], the effect of CO on the formation of *N*-(9-hydroxy)-3-FAA was tested. Exposure of microsomes to a mixture of 90% CO: 10% O_2 inhibited the formation of *N*-(9-hydroxy)-3-FAA as well as that of *N*-(7-hydroxy)-3-FAA by about 75 per cent (Table 5). These data, in conjunction with the non-inducibility of the 9-hydroxylation of 3-FAA by 3-MC or PB, led us to conclude that the enzyme catalyzing the 9-hydroxylation of 3-FAA is a microsomal hemoprotein not identical with cytochrome P_1-450 or P-450.

The 9-hydroxylation of a fluorenylacetamide on a major scale has not been reported heretofore. However, data have recently been published indicating that the carcinogenic isomer, 2-FAA, undergoes microsomal hydroxylation at C-9[28, 29]. However, the metabolites, *N*-(9-hydroxy)-2-FAA and *N*-(9-oxo)-2-FAA, were produced only in trace amounts[28]. Since the method of h.p.l.c. developed in this laboratory permits the ready identification of *N*-(9-hydroxy)-2-FAA [6], we determined the formation of *N*-(9-hydroxy)-2-FAA from 2-FAA by hepatic microsomes of the rat (Table 6). The results confirmed those of Benkert *et al.* [28] in that only trace amounts (1–2 per cent) of the substrate, 2-FAA, were converted to *N*-(9-hydroxy)-3-FAA (Table 6). Feller *et al.* [29] reported no quantitative data on the amounts of *N*-(9-hydroxy)-2-FAA formed from 2-FAA. In contrast to the formation of phenolamides, 3-MC had no stimulatory effect on the formation of *N*-(9-hydroxy)-2-FAA.

The question may be raised as to why 3-FAA, but not 2-FAA, is hydroxylated in major amounts at C-9. The ionic mechanism depicted in Fig. 3 rationalizes the 9-hydroxylation of 3-FAA. Structure A is in equilibrium with structure B, in which the electron shift leads to displacement of a hydride ion from C-9. Structure B is in resonance with C, which creates an electrophilic center at C-9 susceptible to nucleophilic attack by hydroxyl ions. The oxygen may come from the microsomal hemoprotein shown to catalyze the 9-hydroxylation of 3-FAA. In the case of 2-FAA, a resonance form involving C-9 cannot be written. Instead, a similar electron shift would lead to extended conjugation involving both phenyl rings and would create an electrophilic center at carbon-atom 7.

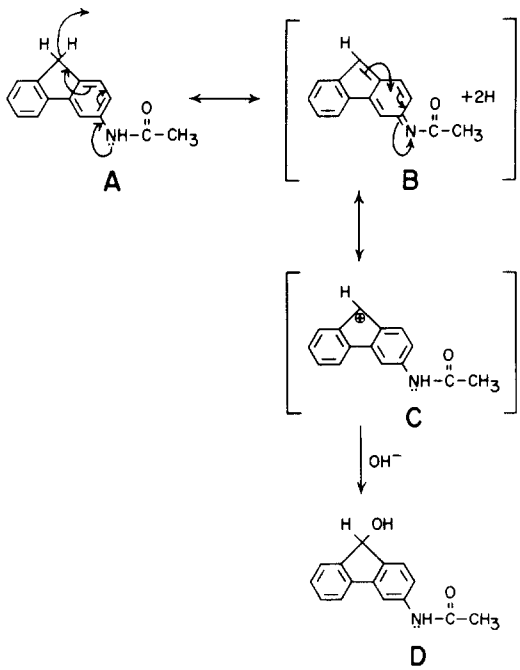

Fig. 3. Hypothetical mechanism for microsomal hydroxylation of 3-FAA at carbon-atom 9.

In summary, the data presented in this study indicate that there are microsomal hydroxylations of arylamides, exemplified by the 9-hydroxylation of 3-FAA by hepatic microsomes of the rat and guinea pig, which appear to be catalyzed by a CO-sensitive hemoprotein(s) not identical with cytochrome P-450 or P_1-450. Further studies concerned with the isolation and purification of this enzyme(s) are required to ascertain whether or not it is a variant of cytochrome P-450.

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