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MICROSOMAL METABOLISM OF ARYLAMIDES BY THE RAT AND GUINEA PIG—II

OXIDATION OF 3-FLUORENYLACETAMIDE AT CARBON ATOM 9 FORMATION OF 3-ACETAMIDO-9-FLUORENONE*,†

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Abstract—Previous work on the oxidation of *N*-3-fluorenyl acetamide at carbon atom 9 yielded two products, 9-hydroxy-3-fluorenylacetamide and 3-acetamido-9-fluorenone. The formation of the alcohol, 9-hydroxy-3-fluorenylacetamide, was catalyzed by a microsomal heme protein. The present study deals with the formation of the ketone, 3-acetamido-9-fluorenone, and with the question of whether the alcohol-forming and the ketone-forming enzymes are identical. 3-Acetamido-9-fluorenone was formed from 9-hydroxy-3-fluorenylacetamide, suggesting that 9-hydroxy-3-fluorenylacetamide is an intermediate in the formation of 3-acetamido-9-fluorenone. Cofactor requirements, pH optimum, effect of carbon monoxide and intracellular distribution indicated that the alcohol-forming and the ketone-forming activities are not identical. Unlike the alcohol-forming enzyme, the ketone-forming utilized $NADP^+$ and NAD^+ and does not appear to be a heme protein.

Previous work from this laboratory has shown that *N*-3-fluorenylacetamide (3-FAA)‖ is hydroxylated, by hepatic microsomes of untreated or 3-MC-treated rats at C-9, to major amounts of 9-hydroxy-3-FAA [1]. The hydroxylation reaction required oxygen and NADPH, and was inhibited by CO. The data led us to conclude that the formation of 9-hydroxy-3-FAA was catalyzed by a hemoprotein not identical with cytochrome P_1-450 or P-450 [1]. Analysis of the incubation mixtures of hepatic microsomes and 3-FAA by h.p.l.c. indicated the presence of a second compound identified as the ketone, 9-oxo-3-FAA [1]. In the present study, the conditions leading to the formation of this metabolite were explored in some detail. Specifically, the substrate and the cofactor requirements, the pH optimum, and the effect of CO were determined. The formation of 9-hydroxy-3-FAA from 3-FAA was examined in parallel experiments in order to ascertain whether the formation of 9-oxo-3-FAA and 9-hydroxy-3-FAA is catalyzed by the same enzyme(s). To clarify this point further, the intracellular distribution of the microsomal enzyme(s) involved in the formation of 9-oxo-3-FAA and of 9-hydroxy-3-FAA was also investigated.

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‖ The following abbreviations are used: 3-FAA, *N*-3-fluorenylacetamide; 9-oxo-3-FAA, 3-acetamido-9-fluorenone; C-9, carbon atom 9; CO, carbon monoxide; h.p.l.c., high-pressure liquid chromatography; 3-MC, 3-methylcholanthrene; and t.l.c., thin-layer chromatography.

MATERIALS AND METHODS

Labeled and unlabeled compounds. 9-Hydroxy-3-[^{14}C-acetyl]FAA was prepared by treating a solution of 3-amino-9-fluorenol (m.p. 157–159°, 35 mg, 0.18 m-mole) [2] in benzene–toluene (2:1, 6 ml) containing triethylamine (0.005 ml) with [^{14}C-carbonyl]acetic anhydride (0.25 m-mole. 4.2 mCi/m-mole) (New England Corp., Boston, MA). The precipitate was recrystallized from ethanol:water and the recrystallized product was purified further by preparative t.l.c. on silica gel GF_{254} with $CHCl_3$:MeOH. The purified 9-hydroxy-3-[^{14}C-acetyl]FAA (28 mg, m.p. 208–210°, specific radioactivity = 1.5 mCi/m-mole) gave a single radioactive peak, R_f = 0.78, on scanning of a radiochromatogram (t.l.c.) with a thin-layer scanner (model LB2721, Berthold Laboratories, Wildbad, West Germany). 3-[^{14}C-acetyl]FAA (m.p. 192–194°, specific radioactivity = 2.2 mCi/m-mole) was prepared as described previously [1]. 3-FAA [3], 9-hydroxy-3-FAA [4], and 9-oxo-3-FAA [4] were synthesized by the published methods and were pure as judged by h.p.l.c. [4]. 3-Amino-9-fluorenone (9-oxo-3-FA) was prepared by reduction of 3-nitro-9-fluorenone (Aldrich Chemical Co., Milwaukee, WI) with ammonium sulfide or with hydrazine hydrate and Raney Nickel [2]. The crude product, m.p. 140–146°, was purified by column chromatography on silica gel (80–200 mesh, J. T. Baker Chemical Co., Fisher, Pittsburgh, PA) with chloroform as the solvent. The second orange band eluted from the column was collected and the solvent was removed at reduced pressure. The residue was recrystallized from ethanol:water or from toluene to give the desired product, m.p. 161–163° (reported m.p. 142–146° [2, 5]); m/e = 195 (M^+); ν_{max}^{KBr} 3500, 3340 (NH), 1690 (C=O), 1590 (NH) cm^{-1}.

Calculated for $C_{13}H_9NO$: C, 79.98; H, 4.65; N, 7.17. Found: C, 79.69; H, 4.69; N, 6.84. NAD^+, $NADP^+$, NADH, NADPH and crystalline serum albumin were purchased from the Sigma Chemical Co., St. Louis, MO.

Preparation of microsomes. Microsomes were prepared as described previously [1, 6]. The protein content of the microsomal suspension, diluted 100-fold with deionized water, was determined colorimetrically with the use of crystalline bovine serum albumin as the standard [7]. Microsomal cytochrome P-450 was measured spectrophotometrically as the reduced CO:hemoprotein complex ($\varepsilon = 91\ mM^{-1}\ cm^{-1}$) in a Beckman Acta VI spectrophotometer [8]. Conditions of the incubation of the microsomes with the ^{14}C-labeled substrates were those described previously [1]. The radioactive metabolites were extracted from the incubation system with diethyl ether [1, 9], and partitioned into acidic/phenolic and neutral fractions [9]. After addition of the appropriate markers, the neutral metabolites were separated by h.p.l.c. [4] and the radioactivity associated with the peaks in the elution profile was measured [1, 4]. The quantities of metabolites in the neutral fraction were calculated from these measurements as described previously [1]. Radioactivity was measured in a Packard liquid scintillation spectrometer, model 3375. Counts were corrected for quenching by means of a calibration curve prepared with the use of 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 that of the background were disregarded.

RESULTS AND DISCUSSION

The enzymatic requirements for the formation of 9-hydroxy-3-FAA from 3-FAA had been studied previously in some detail [1]. However, no information was available regarding the mechanism of formation of the second metabolite of the microsomal oxidation of 3-FAA, 9-oxo-3-FAA. Theoretically, 9-oxo-3-FAA could arise from 3-FAA by oxidation of the intermediate, 9-hydroxy-3-FAA (Fig. 1, pathway 2). Alternatively, the arylamide could yield the ketone by an oxidative mechanism at C-9 not involving the intermediate formation of the alcohol (Fig. 1, pathway 3). Since 9-fluorenone can evidently arise non-enzymatically from fluorene hydroperoxide [10], 3-FAA might conceivably be oxidized to the ketone via the corresponding hydroperoxide. Accordingly, we investigated whether 9-hydroxy-3-FAA could serve as a substrate for the microsomal formation of 9-oxo-3-FAA. Qualitative evidence for the formation of 9-oxo-3-[^{14}C-acetyl]FAA from 9-hydroxy-3-[^{14}C-acetyl]FAA was provided by the elution profiles shown in Fig. 2. Virtually no 9-hydroxy-3-[^{14}C-acetyl]FAA was oxidized by a preparation of boiled microsomes (Fig. 2A), and only minor quantities of the ketone were detected on incubation of 9-hydroxy-3-[^{14}C-acetyl]FAA with microsomes in the absence of any pyridine nucleotide cofactor (Fig. 2B). The trace amounts of ketone formed in the absence of microsomes and cofactors were due probably to the non-enzymatic oxidation of the alcohol by air. Appreciable conversion of 9-hydroxy-3-[^{14}C-acetyl]FAA to 9-oxo-3-[^{14}C-acetyl]FAA was observed upon addition of $NADP^+$ (Fig. 2C). These results indicated that the oxidation of 9-hydroxy-3-[^{14}C-acetyl]FAA to 9-oxo-3[^{14}C-acetyl]FAA is an enzymatic reaction requiring a pyridine nucleotide cofactor. It also supported the view that 9-hydroxy-3-FAA is the precursor of 9-oxo-3-FAA observed in the microsomal oxidation of 3-FAA at C-9 [1]. Peak I, which appeared in the elution profile under all conditions, was due to an unlabeled compound. Since its retention time was the same as that of authentic 9-oxo-3-FA, peak I was identified tentatively as 9-oxo-3-FA. However, the amounts eluted were insufficient for definitive identification.

The yields of 9-oxo-3-[^{14}C-acetyl]FAA from 9-hydroxy-3-[^{14}C-acetyl]FAA under various conditions and with the use of oxidized and reduced pyridine nucleotide cofactors are summarized in Table 1. Maximal yields of ketone (60 per cent) were obtained with $NADP^+$ or NAD^+, whereas microsomal oxidation to

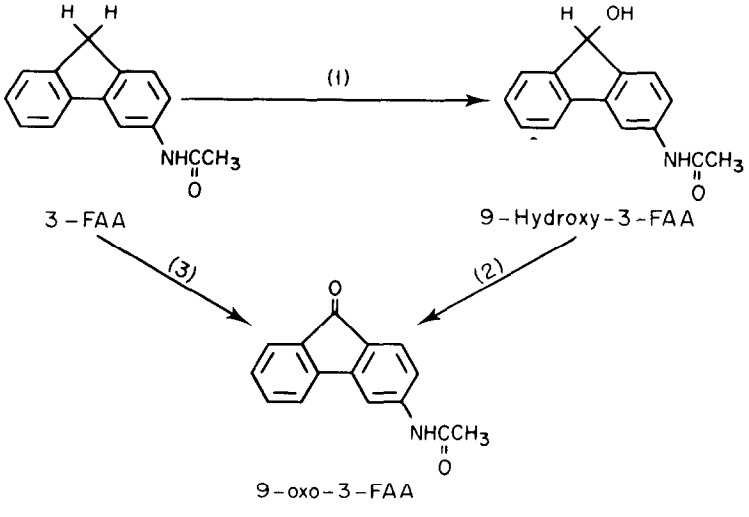

Fig. 1. Possible pathways for the formation of 9-oxo-3-FAA from 3-FAA. The theoretical considerations on which the pathways are based are given in the text.

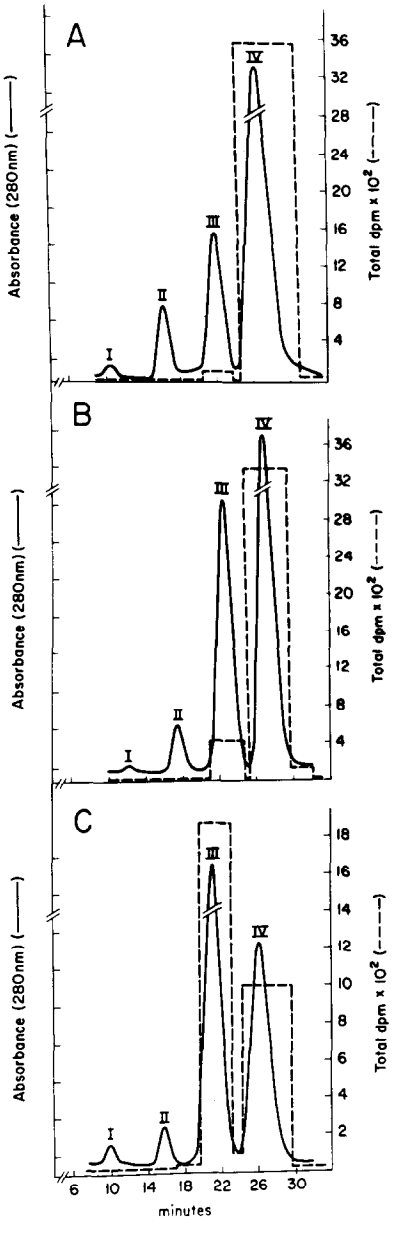

Fig. 2. Identification of 9-oxo-3-[^{14}C-acetyl]FAA as a metabolite of 9-hydroxy-3-[^{14}C-acetyl]FAA by hepatic microsomes of the rat. Microsomes were prepared from two to three pooled livers of untreated rats by differential centrifugation [6]. The incubation conditions were those described in Table 1. The incubation mixture was partitioned by solvent extraction [1, 9]. 9-Hydroxy-3-FAA, 9-oxo-3-FAA and 3-FAA were added as markers to the neutral fraction which was then subjected to h.p.l.c. [4]. 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 associated with the respective markers. Panel A: elution profile after incubation of 9-hydroxy-3-[^{14}C-acetyl]FAA with boiled microsomes. Peaks IV and III correspond to 9-hydroxy-3-[^{14}C-acetyl]FAA and 9-oxo-3-[^{14}C-acetyl]FAA respectively. Peak II is due to marker 3-FAA. Peak I had the same retention time as authentic 9-oxo-3-FA. Panel B: elution profile after incubation of 9-hydroxy-3-[^{14}C-acetyl]FAA and microsomes without NADP$^+$. The assignment of the peaks in the elution profile was the same as in panel A. Panel C: elution profile after incubation of 9-hydroxy-3-[^{14}C-acetyl]FAA in the presence of NADP$^+$. The assignment of the peaks was that indicated in panel A.

the ketone proceeded only to the extent of 25 per cent in the presence of NADPH. It remains to be determined whether or not NAD$^+$ or NADP$^+$ function without oxygen in the oxidation of 9-hydroxy-3-FAA. The greater efficiency of NADP$^+$ or NAD$^+$ compared to NADPH may be due to the fact that the oxidized cofactors accept hydrogen directly from 9-hydroxy-3-FAA. NADPH, on the other hand, may have to be converted by microsomal enzymes to NADP$^+$ before it functions in the reaction. Furthermore, NADPH at the concentration employed in cytochrome P-450-catalyzed reactions [1, 11] exerted an inhibitory effect on the oxidation of 9-hydroxy-3-FAA to 9-oxo-3-FAA (see Fig. 3). The efficiency of NADH in the formation of the ketone from the alcohol is not readily explicable; it is possible that NADH is oxidized more rapidly to NAD$^+$ than NADPH to NADP$^+$.

The first evidence that the oxidation of 3-FAA to 9-hydroxy-3-FAA (and the subsequent oxidation of the alcohol to the ketone) involves two different enzymes came from the investigation of the cofactor requirements for the formation of 9-hydroxy-3-FAA. As shown in Table 2, maximal yields of 9-hydroxy-3-FAA were obtained by the addition of NADPH to microsomes and 3-FAA. This confirmed previous observations that hydroxylation of 3-FAA at C-9 is an NADPH-dependent reaction which resembles cytochrome P-450-catalyzed reactions [1]. In contrast, none of the oxidized cofactors nor NADH supported the 9-hydroxylation of 3-FAA to an extent comparable to that seen with NADPH. As shown by the data of Table 2, the low levels of 9-hydroxy-3-FAA observed with NADP$^+$, NAD$^+$ or NADH could not be attributed to the rapid conversion of 9-hydroxy-3-FAA to 9-oxo-3-FAA. Thus, although both reactions are oxidative, the marked differences in the cofactor requirements for hydroxylation of 3-FAA and for oxidation of 9-hydroxy-3-FAA indicated that the two reactions are catalyzed by different enzymes.

The effect of increasing concentrations of NADPH on the two reactions is shown in Fig. 3. The amount of 9-hydroxy-3-FAA formed from 3-FAA was a linear function of the concentration of NADPH (curve I). In contrast, the formation of 9-oxo-3-FAA either from 3-FAA (curve II) or from 9-hydroxy-3-FAA (curve III) reached a maximum between 2 and 4 μmoles NADPH/incubation system. When 9-hydroxy-3-FAA was the substrate, there was a marked decline in the formation of ketone after the maximum had been attained (curve III). The decline in ketone formation, although less pronounced, was also evident when 3-FAA was the substrate (curve II). The similar shape of curves II and III supported the view that 9-hydroxy-3-FAA is an intermediate on the pathway from 3-FAA to 9-oxo-3-FAA. The different effects of NADPH on the formation of 9-hydroxy-3-FAA and on 9-oxo-3-FAA provided further evidence that formation of the alcohol and of the ketone are catalyzed by different enzymes. The decline in the formation of 9-oxo-3-FAA with increasing concentrations of NADPH may be due to an inhibitory effect of NADPH on the enzyme which catalyzes the oxidation of the alcohol to the ketone. A similar effect of NADPH was reported for the microsomal oxidation of indanol to indanone; specifically, there was an optimal yield of ketone followed by a decline as the concentration of NADPH was increased [12].

Table 1. Oxidation of 9-hydroxy-3-[^{14}C-acetyl]FAA to 9-oxo-3-[^{14}C-acetyl]FAA by hepatic microsomes of the rat in the presence of reduced and oxidized nicotinamide adenine dinucleotides *

Conditions of incubation	Cofactor in incubation system	Amount of cofactor added (μmoles)	9-Oxo-3-[^{14}C-acetyl]FAA found† (nmoles)
No microsomes	None		3.8 ± 0.7
Boiled microsomes	None		9.1 ± 0.3
Complete system‡	None		42.3 ± 3.4
Complete system	$NADP^+$	12.5	296.1 ± 46.1§
Complete system	NAD^+	15.0	314.0 ± 29.1§
Complete system	NADH	14.0	265.0 ± 17.3§
Complete system	NADPH	11.3	126.1 ± 19.1

* Microsomes were prepared from the pooled livers of two to three rats by differential centrifugation in 0.25 M sucrose–0.05 M KH_2PO_4–0.025 M KCl buffer, pH 7.5 [6]. The complete incubation system contained microsomes (11–12 mg protein), 9-hydroxy-3-[^{14}C-acetyl]FAA (0.5 μmole in 0.1 ml dimethyl sulfoxide, sp. act. = 0.10 mCi/m-mole) in 0.05 M KH_2PO_4–0.025 M KCl buffer, pH 7.4. The total volume of the incubation system was 6.0 ml and the incubations were performed in air for 30 min at 37° in a rotary shaker [1]. The incubations were terminated by the addition of 1 N HCl (0.5 ml). The non-phenolic metabolites were isolated from the incubation system by solvent extraction [1, 9]. 9-Oxo-3-[^{14}C-acetyl]FAA was separated by h.p.l.c. and quantified as described previously [1].

† Values are the means ± the average deviations of two to four separate experiments each consisting of two incubation mixtures.

‡ Complete system corresponds to the incubation mixture described in the first footnote (*).

§ Differences between these values were statistically not significant ($P > 0.1$).

We had shown previously that CO inhibits the hydroxylation of 3-FAA at C-9 by 70–80 per cent [1]. In contrast, no inhibitory effect of CO on the dehydrogenation of 9-hydroxy-3-FAA to 9-oxo-3-FAA was seen in the present study (Table 3). This would appear to be conclusive evidence that, unlike the hydroxylation of 3-FAA to 9-hydroxy-3-FAA [1], the subsequent oxidation of the alcohol to the ketone is not catalyzed by a microsomal heme protein and that the two oxidative reactions are attributable to two separate enzymes.

Consideration of the effect of changes in pH on the hydroxylation of 3-FAA and on the dehydrogenation of 9-hydroxy-3-FAA (Fig. 4) led to the same conclusion. There was a narrow range of pH near 7.0 at which hydroxylation of 3-FAA to 9-hydroxy-3-FAA proceeded optimally (curve I, Fig. 4). By contrast, dehydrogenation of 9-hydroxy-3-FAA to 9-oxo-3-FAA occurred at nearly equal rates over a fairly broad range of pH extending from 7.0 to 10.0 (curve III, Fig. 4). The pH optimum for the formation of 9-oxo-3-FAA from 3-FAA was identical with that found for the hydroxylation of 3-FAA to 9-hydroxy-3-FAA (curves II and I, Fig. 4, respectively). This observation further supports the concept that 9-hydroxy-3-FAA is an intermediate in the multi-step reaction (3-FAA $\xrightarrow{(1)}$ 9-hydroxy-3-FAA $\xrightarrow{(2)}$ 9-oxo-3-FAA) and that the rate of reaction 1 determines the rate of the overall reaction (3-FAA ⟶

Table 2. Microsomal oxidation of 3-[^{14}C-acetyl]FAA to 9-hydroxy-3-[^{14}C-acetyl]FAA and to 9-oxo-3-[^{14}C-acetyl]FAA in the presence of reduced and oxidized nicotinamide adenine dinucleotides *

Cofactor in incubation system	Amount of cofactor added (μmoles)	9-Hydroxy-3-[^{14}C-acetyl]FAA found† (nmoles)	9-Oxo-3-[^{14}C-acetyl]FAA found† (nmoles)
NADPH	11.3	91.2 ± 2.2	15.3 ± 0.8
$NADP^+$	12.5	1.6 ± 0.1	9.3 ± 1.0
NAD^+	15.0	1.7 ± 0.2	6.2 ± 1.5
NADH	14.0	3.6 ± 0.3	9.3 ± 0.3

* Microsomes were prepared from the pooled livers of three rats by differential centrifugation [6]. The incubation system was that described in Table 1. The substrate was 3-[^{14}C-acetyl]FAA (0.5 μmole in 0.05 ml dimethyl sulfoxide, sp. act. = 0.10 mCi/m-mole). 9-Hydroxy-3-[^{14}C-acetyl]FAA and 9-oxo-3-[^{14}C-acetyl]FAA were separated by h.p.l.c. [4] and quantified as described previously [1].

† Values are the means ± the average deviations of single experiments each consisting of two incubation mixtures.

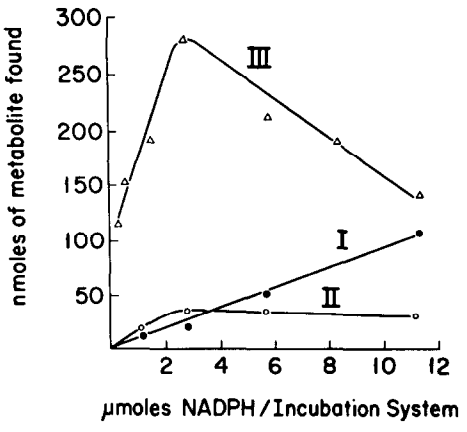

Fig. 3. Formation of 9-hydroxy-3-[^{14}C-acetyl]FAA (curve I) and of 9-oxo-3-[^{14}C-acetyl]FAA (curve II) from 3-[^{14}C-acetyl]FAA as a function of the concentration of NADPH. Formation of 9-oxo-3-[^{14}C-acetyl]FAA from 9-hydroxy-3-[^{14}C-acetyl]FAA as a function of the concentration of NADPH is shown in curve III. Preparation of the microsomes, conditions of incubation, and method of quantitation of the two metabolites are indicated in the text and in Table 1.

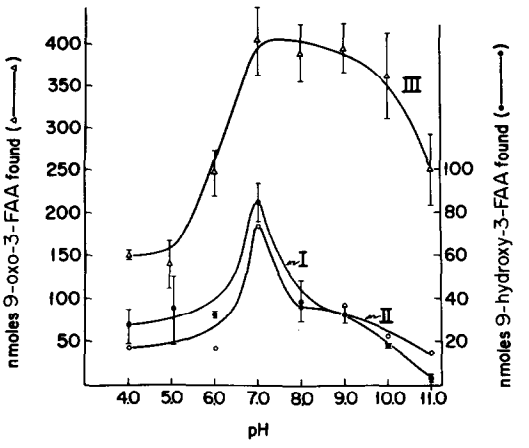

Fig. 4. Effect of pH on the formation of 9-hydroxy-3-[^{14}C-acetyl]FAA from 3-[^{14}C-acetyl]FAA (curve I) and 9-oxo-3-[^{14}C-acetyl]FAA from 3-[^{14}C-acetyl]FAA (curve II) or from 9-hydroxy-3-[^{14}C-acetyl]FAA (curve III). The preparation of the microsomes, conditions of incubation, and the method of quantitation of the two metabolites were those described in the text and in Table 1. The pH of the phosphate buffer in the medium ranged from 4.0 to 11.0.

9-oxo-3-FAA) and thus the extent of formation of the ketone.

Final evidence for two distinct enzymes concerned with the oxidation of 3-FAA at C-9 came from the determination of the intracellular distribution of the hydroxylating and of the dehydrogenating activities (Table 4). These experiments showed that the dehydrogenating activity was nearly equally distributed throughout the microsomal and the soluble fractions, whereas 85–90 per cent of the hydroxylating activity was concentrated in the microsomal fraction. The high level of the ketone-forming activity in the soluble fraction made it unlikely that the enzyme(s) in the soluble fraction was due to microsomal contamination. At present, we do no know whether the soluble and the microsomal ketone-forming enzymes are identical. The resistance of the microsomal oxidase to CO inhibition indicated that the enzyme is not a heme protein. On the other hand, susceptibility of the microsomal hydroxylating enzyme to CO classified it as a heme protein

Table 3. Effect of CO on the formation of 9-oxo-3-[^{14}C-acetyl]FAA from 9-hydroxy-3-[^{14}C-acetyl]FAA by hepatic microsomes of the rat*

Exposure of incubation system to gas mixture†,‡	Cofactor in incubation system	Amount of cofactor added (μmoles)	9-Oxo-3-[^{14}C-acetyl]FAA found† (nmoles)§
A	None		47.1 ± 3.1‖
B	None		51.6 ± 5.5‖
A	$NADP^+$	12.0	318.8 ± 8.2‖
B	$NADP^+$	12.0	298.8 ± 6.7‖
A	NAD^+	15.0	314.0 ± 29.7‖
B	NAD^+	15.0	344.6 ± 2.2‖

* Microsomes were prepared from the pooled livers of three rats by differential centrifugation [6]. The incubation conditions were those described in Table 1. 9-Oxo-3-[^{14}C-acetyl]FAA was separated by h.p.l.c. [4] and quantified as described previously [1].

† A = 90% N_2: 10% O_2; B = 90% CO: 10% O_2.

‡ Gas mixtures A and B were prepared with a gas-mixing pump, type SA 18-2a (Wösthoff, 463 Bochum, West Germany). The incubation medium was equilibrated with the gas mixtures for 20 min. Following equilibration of the medium, 9-hydroxy-3-[^{14}C-acetyl]FAA and the microsomes were added. Then the 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 water bath at 37°.

§ Values are the means ± the average deviations of two separate experiments each consisting of two incubation systems.

‖ Differences were statistically not significant ($P > 0.1$).

Table 4. Intracellular distribution of the rat liver enzymes which oxidize 9-hydroxy-3-[^{14}C-acetyl]FAA to 9-oxo-3-[^{14}C-acetyl]FAA and hydroxylate 3-[^{14}C-acetyl]FAA to 9-hydroxy-3-[^{14}C-acetyl]FAA*

Expt. No.	Cell fraction	Cytochrome P-450† (nmoles/mg protein)	9-Oxo-3-[^{14}C-acetyl]FAA found‡,§ (nmoles)	Specific activity‖	9-Hydroxy-3-[^{14}C-acetyl]FAA found§,¶ (nmoles)	Specific activity‖
I	Microsomes	1.30	277.5 ± 1.5	23.0 ± 0.1	98.1 ± 5.9	8.2 ± 0.5
	Soluble fraction	N.D.**	215.9 ± 5.7††	17.0 ± 0.5	20.8‡‡	1.3
II	Microsomes	0.79	298.3 ± 4.1	24.9 ± 0.3	91.2 ± 2.2	7.6 ± 0.6
	Soluble fraction	N.D.**	244.9 ± 11.3††	20.4 ± 0.9	9.9 ± 0.9	0.8 ± 0.1

* Microsomes and soluble fraction were prepared from the pooled livers of three rats by differential centrifugation [6]. The incubation system and conditions of incubation were those described in Table 1. The amount of substrate was 0.5 μmole, sp. act. = 0.1 mCi/m-mole. The amounts of protein derived from the soluble fraction or microsomes were 12 mg/incubation system. The separation of the metabolites by h.p.l.c. and their quantitation were carried out as described previously [1, 4].

† Cytochrome P-450 was assayed by the method of Omura and Sato [8].

‡ The substrate in these experiments was 9-hydroxy-3-[^{14}C-acetyl]FAA and the cofactor was NADP$^+$ (12.5 μmoles/incubation system).

§ Values are the means ± the average deviations of two incubation systems.

‖ Specific activity is defined as the nmoles of metabolite found/mg of protein.

¶ The substrate in these incubations was 3-[^{14}C-acetyl]FAA and the cofactor was NADPH (11.3 μmoles/incubation system).

** Not detected.

†† Differences were statistically not significant ($P > 0.1$).

‡‡ This value was obtained from a single incubation system.

which resembles cytochrome P-450 [1]. Ketone-forming enzymes in the soluble fraction of the livers of the rabbit and the rat have been described previously [12–14]. Thus, hydroxynicotine is oxidized to cotinine by an oxidase of rabbit liver in the presence of NADP$^+$ or NAD$^+$ [13]. In an analogous fashion, 5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid was dehydrogenated to the corresponding ketone, 5-(3-oxo-1-cyclohexenyl)-3,5-dimethylbarbituric acid, by an oxidase requiring NADP$^+$ or NAD$^+$ as cofactor [14]. Similar to our results on the intracellular distribution of the oxidase converting 9-hydroxy-3-FAA to 9-oxo-3-FAA, Billings *et al.* [12] reported that an NADP$^+$- or NAD$^+$-requiring oxidase which converted indanol to indanone was demonstrable in the microsomes, as well as in the soluble fraction.

The cytosolic enzyme oxidizing 9-hydroxy-3-FAA to 9-oxo-3-FAA may be described as a dehydrogenase which converts an alcohol to a ketone. The evidence at hand permits no definite conclusion as to whether this enzyme is the same as the cytosolic enzyme in rat liver which oxidizes ethanol. It should be noted that 9-oxo-3-FAA was obtained from 9-hydroxy-3-FAA in good yield (~ 50 per cent) in the presence of NADP$^+$ (Table 4), whereas the oxidation of ethanol by rat liver alcohol dehydrogenase was poorly supported by NADP$^+$ [15]. Since the dehydrogenation of indanol to indanone likewise utilized NADP$^+$ and since the reaction was not catalyzed by purified horse liver alcohol dehydrogenase [12], the cytosolic enzyme which forms 9-oxo-3-FAA appears more closely related to the cytosolic enzyme which forms indanone than to the dehydrogenase specific for ethanol.

In summary, the results of the present study, in conjunction with the previous investigation [1], lead us to conclude that oxidation of the arylamide, 3-FAA, consists of the two sequential reactions depicted as pathways 1 and 2 in Fig. 1. Our previous data had already suggested that the initial step consists of the hydroxylation of 3-FAA to 9-hydroxy-3-FAA [1]. Since it has been shown that fluorene hydroperoxide is an intermediate in the metabolism of fluorene to 9-fluorenol [10], the possibility should be borne in mind that hydroxylation of 3-FAA to 9-hydroxy-3-FAA proceeds in an analogous manner through 3-acetamido-9-fluorene hydroperoxide. In the final step, the alcohol is dehydrogenated to the ketone, 9-oxo-3-FAA. The two reactions are catalyzed by two different enzymes as indicated by cofactor requirements, susceptibility to CO, pH optimum, and intracellular distribution.

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