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N-(9-HYDROXY-9H-FLUOREN-2YL)-ACETAMIDE AND *N*-(9-OXO-9H-FLUOREN-2YL)-ACETAMIDE: METABOLITES OF *N*-(9H-FLUOREN-2YL)-ACETAMIDE

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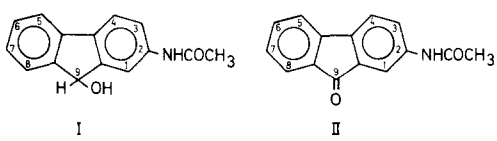
Abstract—*N*-(9-Hydroxy-9H-fluoren-2yl)-acetamide (9-OH-FAA*), *N*-(9-oxo-9H-fluoren-2yl)-acetamide (9-O-FAA), and *N*-(9H-fluoren-2yl)-glycolamide (FGA) were found in the neutral fraction of microsomal metabolites of *N*-(9H-fluoren-2yl)-acetamide (FAA). 9-OH-FAA and 9-O-FAA are new metabolites of FAA. The former was identified by R_f value, by u.v.- and n.m.r.-spectroscopy, the latter by R_f value and u.v.-spectroscopy. 9-OH-FAA was also found in the urine of rats and guinea-pigs injected with FAA. Treatment of rabbits and guinea-pigs with 3-methylcholanthrene diminished microsomal 9-hydroxylation of FAA and stimulated the production of FGA, whereas treatment with phenobarbital did not do so.

Biotransformation of *N*-(9H-fluoren-2yl)-acetamide (FAA) yields a variety of hydroxylation products. All the phenolic metabolites except the 4-hydroxy derivative have been found in urine by Weisburger *et al.* [1–3]. Cramer *et al.* [4] isolated the *N*-hydroxy derivative of FAA from urine of rats, and recently Fries *et al.* [5] discovered *N*-(9H-fluoren-2yl)-glycolamide (FGA) in the urine of rabbits injected with FAA. Products of the oxygenation of FAA at the 9-carbon atom have been sought, but not detected so far [2, 6, 7].

In a study of the transformation of FAA to FGA in a variety of species and by isolated liver microsomes we discovered that the neutral fraction of metabolites produced by rabbit or guinea-pig liver microsomes contains two unknown metabolites in addition to a substantial proportion of FGA. The new metabolites were isolated and identified as *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide (9-OH-FAA) and *N*-(9-oxo-9H-fluoren-2yl)-acetamide (9-O-FAA). The formulae are shown in Fig. 1.

The metabolites were also found in urine and in incubates with liver microsomes of other species. The biochemical transformation of the metabolites to 2-biphenyl carboxylic acids is under investigation.



I II

Fig. 1. Formulae of *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide (I) and *N*-(9-oxo-9H-fluoren-2yl)-acetamide (II), new metabolites of *N*-(9H-fluoren-2yl)-acetamide.

* Abbreviations: FAA, *N*-(9H-fluoren-2yl)-acetamide; 9-OH-FAA, *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide; 9-O-FAA, *N*-(9-oxo-9H-fluoren-2yl)-acetamide; FGA, *N*-(9H-fluoren-2yl)-glycolamide; MC, 3-methylcholanthrene, 1,2-dihydro-3-methyl-benz[j]aceanthrylene; PB, phenobarbital.

MATERIALS AND METHODS

2-Aminofluorene was a commercial product obtained from Fluka AG. FAA was prepared by acetylation of sublimed, colorless 2-aminofluorene with acetic anhydride, m.p. 193–194°; Diels *et al.* [8] gave 191°. FAA, ^{14}C-labeled in the acetic acid residue, was prepared by acetylation of 2-aminofluorene with [1-^{14}C]acetic anhydride; specific activity of the product was 516 μCi/m-mole.

[9-^{14}C]FAA, sp. act. 10·15 mCi/m-mole, was purchased from NEN Chemicals GmbH, Dreieichenhain. Synthetic 9-OH-FAA was a gift from Dr. T. L. Fletcher [9]. 9-O-FAA was prepared by oxidation of FAA with chromic acid in acetic acid. After purification by t.l.c. using chloroform–methanol (98:2) as developing fluid, the compound crystallized from ethanol as bright-red needles, m.p. 227–228° (corr.); O. Gerhardt [10] gives 227–228°. N.m.r.-, i.r.-, and u.v.-spectra of the 9-hydroxy- and 9-oxo-derivatives of 2-fluorenylacetamide are given below. N-OH-FAA was synthetized according to Miller *et al.* [11].

Methods

Urines collected during 24 hr after administration of FAA were incubated with glucuronidase and extracted with ether, as described earlier [5]. Incubates of microsomes with FAA were directly extracted three times with equal volumes of ether. The extracts of 100 or more ml of incubate were reduced to 250 ml and extracted three times with 30 ml of 1 N NaOH to remove acidic metabolites. After drying (Na_2SO_4) the ether was evaporated. The residue was dissolved in a small volume of chloroform–methanol (80:20, v/v) and applied to t.l.c. plates of silica gel Merck 60 PF_{254}. Authentical compounds were applied alongside in each case. Chloroform–methanol (95:5 or 97:3, v/v) was used as developing solvent. For u.v. spectroscopy the metabolites were eluted with methanol. N.m.r. spectra were recorded with a Varian HA-100 spectrometer. FGA was identified and measured as described by Fries *et al.* [5].

For determination of N-OH-FAA by its u.v. absorbance, the acid metabolites of FAA were separated on precoated t.l.c. plates cellulose F (Merck) with cyclohexane–glacial acetic acid (92·5:7·5, v/v) as developing fluid.

Liver microsomes were prepared according to Jagow *et al.* [12]. Protein contents of microsome suspension were determined according to Szarkowska and Klingenberg [13]. Incubation mixtures contained 1 mg microsomal protein per ml of 0·15 M phosphate pH 7·4 and 0·12 mM NADP, 2·4 mM glucose-6-phosphate, 1400 IU glucose-6-phosphate dehydrogenase per liter, 70 mM KCl, 80 mM saccharose, 40 mM nicotinamide [14], and 0·1 mM FAA. In the experiments for biochemical preparation of 9-OH-FAA, however, 3 mg protein per ml was used. In order to prevent deacetylations of the substrate and metabolites, incubates were made 0·1 M in NaF [15]. After 30 min incubation at 37° under air the reactions were stopped by extraction of the incubates with ether.

Animals used for the production of urinary metabolites of FAA were housed in stainless steel metabolic cages. They were fed a standard laboratory diet (Altromin GmbH, 4937 Lage/Lippe, Germany) and water *ad lib.* FAA dissolved in olive oil was applied intraperitoneally.

For pretreatment with 3-methylcholanthrene (MC) four doses of 10 mg in 1 ml olive oil per kg i.p. were injected in 2 weeks. Sodium phenobarbital dissolved in water and adjusted to pH 7·4 was injected daily s.c. for 2 weeks. The first three doses were 25 mg/kg each and the following eleven doses 50 mg/kg each.

Statistics

Since normal distribution could not be proven for the small samples and because the variances were not equal, parametric tests could not be applied. Therefore, nonparametric tests were used. To prove the hypothesis that three samples (controls, PB- and MC-stimulated microsomes) do not belong to the same population, the Kruskal–Wallis test [16] for independent sample groups was applied. The following statistic has been computed,

$$\hat{H} = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N+1)$$

where $\hat{H}$ is the test statistic, N the number of cases in all samples combined, k the number of samples, R_i the sum of ranks in the i-th sample, and n_i the number of cases in the i-th sample. The computer printed the test statistic $\hat{H}$ or, in the presence of ties, the corrected test statistic $\hat{H}$, the number of degrees of freedom and the test statistics G (gamma-distribution) and B (beta-distribution). If all $n_i < 5$, the values of H were then compared with those of $H_{(n_1,n_2,n_3,\,5\%)}$ taken from tables [17]. If at least one $n_i > 5$, the printed values of G were compared with those of $\chi^2_{(k-1,\,5\%)}$ taken from tables. If $\hat{H}(G)$ was found to be equal or larger than $H(\chi^2)$, the three samples did not belong to the same population, i.e. they were significantly different.

To evaluate differences between two specific groups, e.g. controls and PB- or controls and MC-stimulated microsomes, the Wilcoxon test for independent samples was applied. Three test statistics (Wilcoxon's T, Mann–Whitney's U, and an approximately normally distributed statistic) have been computed considering possible ties. Because of the small size of sample ($n_1 < 5$, $n_2 < 5$), Mann–Whitney's U-test has been used for the evaluation of the probability of statistical significance for the one-sided test problem. The printed value of $\hat{U}$ has been compared with $U_{(n_1,n_2,5\%)}$ taken from tables [18]. Values of $P_{(u_1,u_2,U)}$ 0·05 indicate significance on the 5% level.

RESULTS

(a) *Isolation and identification of new metabolites.* 9-OH-FAA was found in incubates of FAA with liver microsomes of all species tested. Hamster liver microsomes showed the highest yield. The metabolite was isolated in the early experiments with guinea-pig liver microsomes. From the chromatograms of a total of 2200 ml of incubates with 10^{-3} M FAA the metabolite showing the R_f value and u.v. spectrum of 9-OH-FAA was collected. Its n.m.r. spectrum was identical to that of the synthetic compound and is shown in Table 1. The identity of the u.v. spectra of the metabolite and of synthetic 9-OH-FAA is demonstrated in Fig. 2. The molar absorbance coefficients in methanol were as follows:

$$\epsilon_{233\,nm} = 16{,}425; \epsilon_{240\,nm} = 14{,}930;$$
$$\epsilon_{293\,nm} = 26{,}770; \epsilon_{307\,nm} = 19{,}330.$$

The u.v. spectra did not change on the addition of NaOH. The R_f value in t.l.c. with chloroform–methanol (95:5) was 0·046 and with benzene–dioxane–glacial acetic acid (90:24:4) 0·17. The following i.r. bands were found with synthetic 9-OH-FAA in KJ: 3300 cm^{-1} (broad): $\nu_{NH,OH}$; 1650 cm^{-1}: $\nu_{-CO-\underset{|}{N}-}$.

The rates of production of the metabolite by liver microsomes of various species are shown in Tables 2 and 3. Liver microsomes from hamsters were found to be ten times more active than microsomes from rabbits.

9-O-FAA was detected in incubates with liver microsomes of rats, hamsters, and of guinea-pigs. The evidence, in addition to the R_f value in t.l.c., for its

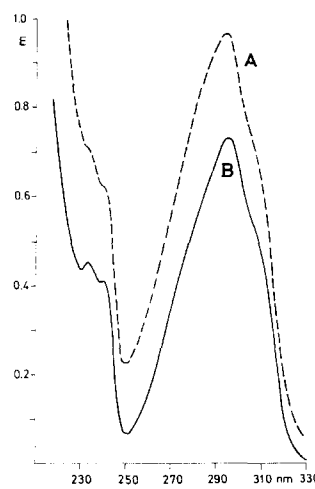


Fig. 2. Absorbance in methanol of metabolite isolated from incubates of guinea-pig liver microsomes with 10^{-3} M FAA (A), and of $2{\cdot}75 \times 10^{-5}$ M synthetic *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide (B); optical path 1 cm.

Table 1. N.m.r.-spectra of *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide and of *N*-(9-oxo-9H-fluoren-2yl)-acetamide in d_6-dimethylsulfoxide

Compound	δ (ppm)	No. of protons	Assignment
Synthetic *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide	2·08 s	3	$CH_3—CO—N—$
	5·44 d	1	ar—C(H)(O—)—ar
	J ~ 8 cps, after exchange against D:s		
	5·81 d	1	ar—C(OH)—ar
	J ~ 8 cps, exchange against D		
	between 7·23 and 7·94	7	aromatic protons
	10·0 exchange against D	1	—CO—NH—ar
Metabolite of FAA	2·07 s	3	$CH_3—CO—N—$
	5·44 s	1	ar—C(H)(O—)—ar
	between 7·21 and 7·93	7	aromatic protons
	10·09	1	—CO—NH—ar
Synethetic *N*-(9-oxo-9H-fluoren-2yl)-acetamide	2·08 s	3	$CH_3—CO—N—$
	between 7·20 and 7·93	7	aromatic protons
	10·18	1	—CO—NH—ar

Table 2. Rates of production of 9-OH-FAA, nmoles/min per mg microsomal protein, from 10^{-4} M FAA in incubates with liver microsomes of various species

Species	9-OH-FAA (nmoles/min/mg)
Rat	0·026 ± 0·02
Guinea-pig	0·033 ± 0·01
Rabbit	0·015 ± 0·01
Hamster	0·171 ± 0·04

The data are the means and standard deviation of the results of 4 experiments.

presence in incubates of FAA with rat liver microsomes is presented in Fig. 3. The R_f value in t.l.c. with chloroform–methanol (95:5) was 0·17 and with benzene–dioxane–glacial acetic acid (90:24:4) was 0·31. The yield of 9-O-FAA was lower than the yield of 9-OH-FAA. About 0·01 nmoles/min/mg protein were found to be produced. The amounts of 9-O-FAA isolated were too small for n.m.r. or i.r. spectrometry.

As a further proof of the identity of the synthetic 9-O-FAA its i.r. spectrum in KJ may be added:

1688 cm^{-1} (very broad) $\nu_{ar-CO,-CO-N-}$

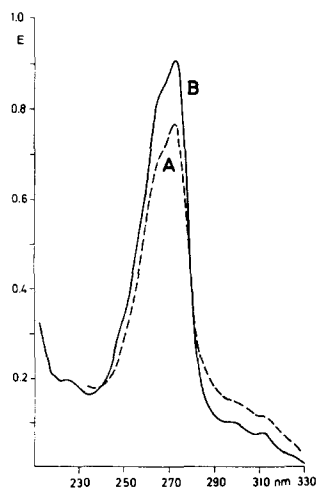


Fig. 3. Absorbance in methanol of metabolite isolated from incubates of rat liver microsomes with 10^{-3} M FAA (A), and of $1{\cdot}35 \times 10^{-5}$ M synthetic *N*-(9-oxo-9H-fluoren-2yl)-acetamide (B); optical path 1 cm.

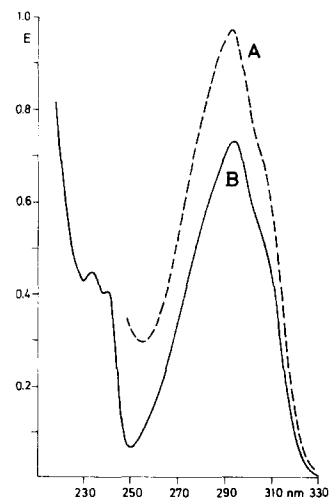


Fig. 4. Absorbance in methanol of metabolite isolated from urine of guinea-pigs injected with FAA, 100 mg/kg i.p., (A) and of $2{\cdot}75 \times 10^{-5}$ M synthetic *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide (B); optical path 1 cm.

Table 3. Production of *N*-(9H-fluoren-2yl)-glycolamide and *N*-(9-hydroxy-9H-fluoren-2yl)-acetamide from *N*-(9H-fluoren-2yl)-acetamide by liver microsomes of guinea-pigs or rabbits. Effect of treatment of the animals with phenobarbital or 3-methylcholanthrene

					Kruskal–Wallis test			Wilcoxon test			
								Control–PB		Control-3-MC	
		Controls	PB	3-MC	Computed Test statistic	Degrees of freedom	$H_{(n_1,n_2,n_3,5\%)}$ or $\chi^2_{(k-1,5\%)}$	Computed U	$P_{(n_1,n_2,U)}$	Computed $\hat{U}$	$P_{(n_1,n_3,U)}$
Rabbits	FGA	0·016 ± 0·0033 (4)	0·015 ± 0·0017 (4)	0·097 ± 0·016 (3)	$\hat{H}$ 6·07	2	H 5·58	7·0	0·443	0·0	0·028
	9-OH-FAA	0·016 ± 0·0053 (4)	0·013 ± 0·0023 (4)	0·006 ± 0·0013 (3)	$\hat{H}$ 4·25	2	H 5·58	—	—	—	—
	N-OH-FAA	0·077 ± 0·015 (5)	0·09 ± 0·016 (7)	0·677 ± 0·132 (3)	G 8·73	2·5	χ^2 6·90	15·0	0·378	0·0	0·018
Guinea-pigs	FGA	0·14 ± 0·019 (4)	0·09 ± 0·0077 (6)	0·29 ± 0·015 (3)	G 12·03	2·6	χ^2 7·09	2·0	0·019	0·0	0·028
	9-OH-FAA	0·04 ± 0·0067 (4)	0·08 ± 0·0047 (5)	0·01 ± 0·001 (3)	$\hat{H}$ 9·73	2	H 5·63	0·0	0·008	0·0	0·028

Microsomal protein 1 mg/ml of incubate; FAA 10^{-4} M. In each experiment extracts from 6–18 incubates were pooled for analysis. The figures indicate nmoles metabolite/min per mg of microsomal protein and standard error. Columns 6–12 show the results of statistical test for significance.

The molar absorbance coefficients in the u.v. of synthetic 9-O-FAA in methanol were found as follows:

$$\epsilon_{266{\cdot}5\,nm} = 60{,}860; \epsilon_{273\,nm} = 67{,}620;$$
$$\epsilon_{313\,nm} = 5670; \epsilon_{325\,nm} = 1920;$$
$$\epsilon_{300\,nm} = 6760;$$
$$\epsilon_{428\,nm} = 503.$$

(b) *Urinary excretion of* N-*(9-hydroxy-9H-fluoren-2yl)-acetamide.* After i.p. injection of guinea-pigs or rats with FAA only very small proportions of the dose were excreted as 9-OH-FAA. The presence of the metabolite in guinea-pig urine is demonstrated in Fig. 4. Ten guinea-pigs had been injected with ^{14}C-labeled FAA, 100 mg/kg i.p. From the urine produced during the first day, which contained 73% of the radioactivity injected, 0·078 mg of 9-OH-FAA was isolated. This amount corresponded to about 0·01% of the dose of FAA.

A similarly small proportion of a dose of FAA, 0·008%, was found in the urine of rats. For other purposes, 30 rats had been injected with 3,4-benzpyrene, 100 mg/kg i.p., and the following 3 days with [9-^{14}C]FAA, 50 mg/kg i.p. In the chromatograms of ether extracts from urine collected from the day after the first injection of FAA until the 3rd day after the last injection, the metabolite was detected. The amount of the chromatographically isolated metabolite was determined to be 0·13 mg.

(c) *Effect of treatment of rabbits and guinea-pigs with phenobarbital or 3-methylcholanthrene on microsomal production of* N-*(9-hydroxy-9H-fluoren-2yl)-acetamide.* Results of a vast number of experiments have shown that hydrocarbons, such as MC and PB stimulate microsomal hydroxylations differently and that certain hydroxylations may be connected with a special oxygenating system. Therefore, we tried the effect of treatment of rabbits and guinea-pigs with MC or PB on the microsomal 9-hydroxylation of FAA. The results are shown in Table 3. For comparison, the other non-aromatic hydroxylations, *N*-hydroxylation and formation of FGA, were measured. Significant differences were assumed on the 5% level.

In guinea-pig liver microsomes, pretreatment with PB increased 9-hydroxylation and diminished production of FGA. On the other hand, MC pretreatment strongly diminished 9-hydroxylation and increased production of FGA. In no case was *N*-OH-FAA detected in incubates of FAA with guinea-pig liver microsomes. Data presented in Table 4 show that there is no substantial difference between the rates of disposal of *N*-OH-FAA in guinea-pig and rabbit liver microsomes. The loss of *N*-OH-FAA during incubation could not be accounted for by the recovery of small amounts of FAA and FGA. Concentrations of *N*-OH-FAA as low as 4×10^{-6} M were determined under the conditions of our experiments. Thus guinea-pig liver microsomes *N*-hydroxylate FAA very slowly, if at all.

Table 4. Disposal of *N*-OH-FAA during 30 min incubation with fortified liver microsomes of rabbits and guinea-pigs

N-OH-FAA added (M)	% *N*-OH-FAA recovered after 30 min incubation	
	Rabbit	Guinea-pig
2×10^{-5}	24	18
10^{-4}	52	50
10^{-3}	96	92

Microsomal protein 1 mg/ml. As with the incubates with FAA as substrate 120 ml of incubate was analyzed.

In rabbit liver microsomes, pretreatment with PB affected none of the three hydroxylations. As with guinea-pig liver microsomes, MC pretreatment increased formation of FGA and diminished 9-hydroxylation. Furthermore, it increased *N*-hydroxylation nearly 10-fold.

DISCUSSION

The data reported above present unequivocal evidence of the biochemical oxygenation of FAA at C-9 yielding 9-OH-FAA and, by dehydrogenation, 9-O-FAA. They also show that FGA, which so far is known only as urinary metabolite of FAA [5], is a microsomal metabolite. Thus all non-aromatic atoms of FAA, the N [4, 15, 19, 20], the acetic acid residue [5] and C-9 are accessible to microsomal oxygenation. The experiments of enzyme induction with PB or MC show that microsomal hydroxylation at the three non-aromatic atoms is stimulated differently. This points to different enzymes or enzymic sites involved in the hydroxylation. Failure of PB treatment of rabbits to stimulate microsomal hydroxylation of the acetic acid residue and strong stimulation of this reaction by MC treatment has been found with another *N*-arylacetamide, *p*-chloroacetanilide [21]. In rabbit liver microsomes, hydroxylation of the acetic acid residue seems to be related to *N*-hydroxylation of FAA, which also is stimulated by MC treatment. The decrease in C-9-hydroxylation by MC treatment was also found with guinea-pig liver microsomes. Differential stimulation of hydroxylations of FAA by PB or MC has also been found with the microsomal production of phenols from FAA [20, 22, 23]. A comprehensive discussion of the problem of differential stimulation by inducers of microsomal enzymes and of involvement of cytochromes in microsomal oxygenation may be found in Conney *et al.* [24]. More recent contributions to the problem have been made by Lu *et al.* [25], Alvares *et al.* [26], Berg and Gustafsson [27] and Kiese and Lenk [21].

In accordance with Irving's [15] results, our experiments with guinea-pig liver microsomes demonstrated lack of, or extremely slow, *N*-hydroxylation of FAA by these microsomes. This illustrates a very important species difference in microsomal hydroxylations, which is the more remarkable as guinea-pig liver microsomes *N*-hydroxylate non-acetylated aminofluorene quickly [28].

The quantitation of 9-OH-FAA as urinary metabolite of normal rats awaits further investigation. Our results demonstrate only that 9-OH-FAA truly is a urinary metabolite of FAA. At the time we found 9-OH-FAA in rat urine it was not known that treatment of rats with MC, and probably also with benzpyrene, diminishes microsomal 9-hydroxylation of FAA. Therefore the proportion of FAA excreted by normal rats as 9-OH-FAA may be different from our result.

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