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METABOLISM OF THE CARCINOGEN *N*-2-FLUORENYLACETAMIDE IN GERM-FREE AND CONVENTIONAL RATS

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Abstract—Because an appreciable difference was observed in the metabolism of *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA) in germ-free and conventional control rats, the metabolism of the parent compound, *N*-2-fluorenylacetamide (FAA), was examined in this system. Germ-free and conventional rats injected i.p. with a single dose of FAA excreted similar levels of hydroxylated metabolites in urine, chiefly as conjugates with glucuronic acid. By quantitative rank, decreasing amounts of the 7- 5- 3- and *N*-hydroxy derivatives of FAA were present in both groups of rats. On the other hand, there were significant differences between the germ-free and control rats in metabolites in the cecum and in the feces. Substantial amounts of conjugated metabolites, glucosiduronic acids and sulfuric acid esters were found in the germ-free group. In the controls, the major portion of the metabolites occurred as free, unconjugated materials. Intracecal injection of FAA led to virtually complete absorption and metabolism similar to what was found after i.p. injection. The bacterial flora in conventional rats affects the metabolism of FAA by converting conjugates to free compounds in the lower portion of the intestinal tract; these in turn are reabsorbed in part and undergo additional metabolism.

THE CARCINOGEN *N*-2-fluorenylacetamide (FAA) undergoes many biochemical transformation steps. Hydroxylation at various carbons of the fluorene ring leads to phenolic derivatives, which when tested were found not carcinogenic.[1] Therefore, such reactions may be considered detoxification steps. On the other hand, this compound is also hydroxylated on the nitrogen leading to the *N*-hydroxy derivative. Several lines of evidence suggest that this material, *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA; *Chemical Abstracts*' name, *N*-2-fluorenylacetohydroxamic acid), and select esters are more active than FAA or are active under conditions where the parent compound is not.[2] Thus, *N*-hydroxylation of FAA is a key activation step.

It has been known for some time that a single dose of FAA gives rise to only small amounts of N-OH-FAA, excreted as conjugates in urine. However, a sizable proportion of a single dose is carried by the bile from liver to intestinal tract as the glucosiduronic acid of N-OH-FAA.[3] This discrepancy between levels recovered from bile and urine suggested that the intestinal tract contained enzyme systems, presumably derived from the microflora, which resulted in the conversion of the glucuronide of N-OH-FAA. This and related points were examined and the results form the subject of this series of papers.[4,5]

MATERIALS AND METHODS

Chemicals. FAA was purchased from Aldrich Chemical Company (Milwaukee, Wis, 52210); N-OH-FAA was prepared from 2-nitrofluorene,[6] ^{14}C-labeled FAA was in part prepared as described[7] or purchased from Tracerlab, Inc. (Waltham, Mass. 02154). The ring-hydroxylated metabolites of FAA such as 7-, 5- and 3-OH-FAA were prepared in this laboratory in earlier studies.[7]

Bacterial β-glucuronidase was procured from Sigma Chemical Co. (St. Louis, Mo. 63118). All the other materials were reagent quality chemicals.

Animals. Male Fischer rats (germ-free and conventional) were used in the study. The source and methods of maintaining the germ-free group of rats have been described in another publication.[4] At 10 weeks of age, each germ-free rat was transferred to an individual metabolism cage (Acme Metal Products, Inc., Chicago, Ill. 60619) suspended by a stand inside a Reyniers germ-free isolator; the conventional rats were place in similar cages, but housed in a standard animal room. Both germ-free and conventional groups of rats were given free access to water and diet L-356,[8] but water and diet were not steam-sterilized for the conventional rats.

After a 2-day conditioning period in the metabolic cages, the rats were injected i.p. with a dose of 50 mg/kg of labeled FAA as a sterile fine suspension in steroid suspending vehicle.[9] Half of the animals were removed and killed under light ether anaesthesia after 24 hr; the remainder were killed after 48 hr by withdrawal of blood into a heparinized syringe through the abdominal aorta. Blood was separated into red cells and plasma by centrifugation.

Metabolites in urine, cecum and feces. Urinary metabolites were assessed by sequential enzymic or acid hydrolysis followed by ether extraction and paper or thin-layer chromatography of the compounds liberated from conjugates, as described.[4] The contents of cecum or the feces were extracted 4 times with 80% aqueous ethanol, separating the residue by centrifugation. The combined extracts were concentrated to a small volume *in vacuo*. The residue, diluted to a convenient volume with 0·1 M acetate buffer, pH 6, was processed as described for urine to identify and quantitate individual metabolites.

Metabolites of FAA after intracecal injection in conventional rats. The peritoneal cavity was opened under light ether anaesthesia and 0·296 mg of labeled FAA, finely suspended in 0·2 ml of sterile, steroid suspending vehicle was injected into the cecum. A small amount of compound of high specific radioactivity ($5{\cdot}3 \times 10^7$ cpm/mg) was used to examine the capability of the cecum to absorb quantities such as might become available by biotransformation of precursors. The peritoneal cavity was closed under aseptic conditions and the rat was placed in a metabolism cage for collection of urine and feces for a 48-hr period. Metabolites in the excreta were analyzed by the procedures described above.

Determination of radioactivity. Organic or aqueous solutions were counted in the conventional way in a toluene–methanol scintillation mixture on a liquid scintillation spectrometer with an efficiency of 65 per cent correcting for background and quenching (internal standard). Aliquots of solids such as cecum contents or feces were digested in Hyamine solution on a shaking water bath at 60° for 4–6 hr followed by decolorization with H_2O_2 as required.

RESULTS

Blood and urinary metabolites after injection of FAA into germ-free and control rats. Table 1 details the body and liver weights and the radioactivity from FAA in blood and plasma found in germ-free and control rats. There was little difference in the activity in blood between germ-free and control groups.

The urinary metabolites in the germ-free rats were not separated into free, unconjugated materials and glucosiduronic acids as is customary,[1] because the collected urine could not be cooled within the germ-free isolator. Thus, during the 24- or 48- hr

TABLE 1. BODY WEIGHTS AND BLOOD RADIOACTIVITY OF GERM-FREE OR CONVENTIONAL RATS INJECTED WITH 9-^{14}C-*N*-2-FLUORENYLACETAMIDE*

Group	No. of rats	Time (hr)	Body wt. (g)	Liver wt. (g)	Specific activity	
					Blood (mμmoles/ml)	Plasma (mμmoles/ml)
Germ-free	3	24	263 ± 6·3†	8·3 ± 0·4	60·0 ± 7·4	26·2 ± 1·4
	3	48	249 ± 9·6	8·2 ± 0·1	44·3 ± 7·0	14·8 ± 1·1
Control	4	24	227 ± 3·0	7·7 ± 0·3	43·3 ± 5·8	39·5 ± 2·7
	4	48	223 ± 6·0	7·7 ± 0·3	41·1 ± 3·3	18·1 ± 3·3

* Germ-free or conventional male Fischer rats each received (i.p.) 1 ml of a suspension of 12·2 mg 9-^{14}C-FAA (sp. act., 2·0 × 10^6 cpm/mg) in steroid suspending vehicle.
† Standard error.

TABLE 2. URINARY EXCRETION OF RADIOACTIVITY FROM GERM-FREE AND CONVENTIONAL RATS INJECTED WITH 9-^{14}C-*N*-2-FLUORENYLACETAMIDE

Group	No. of rats	Time (hr)	Metabolites (% of dose)		
			Total	Free + glucuronides	Sulfates
Germ-free	3	24	31·7 ± 1·7*	11·6 ± 1·2	10·8 ± 0·1
	3	48	37·5 ± 2·1	16·0 ± 0·9	10·5 ± 1·5
Control	4	24	43·6 ± 5·7	13·8 ± 1·6	15·3 ± 1·2
	4	48	49·9 ± 2·2	20·7 ± 0·85	12·9 ± 2·2

* Standard error.

experimental period, urinary glucuronidase was expected to hydrolyze some of the conjugates, and it was deemed unwise to add preservatives or to adjust the pH as a preventive measure. Ordinarily, free compounds amount to only a small fraction of the total urinary radioactivity, whereas the glucosiduronic acids constitute a major portion.[10–12] The germ-free groups excreted somewhat less of the total dose in urine than did controls. In the first day, approximately equal amounts were found in the free plus glucosiduronic acid fraction and in the sulfuric acid esters in germ-free and control groups. During the second day, there was somewhat less of the latter fraction (Table 2).

Paper chromatography of the metabolites in the free plus glucosiduronic acid fraction showed that the germ-free groups tended to excrete more of the 7-hydroxylated

derivative of FAA and somewhat less of the 5-hydroxy derivative than the conventional controls (Fig. 1). However, the differences were not great with the 3- and *N*-hydroxylated metabolites. Expressed as percentage of the dose, there were decreasing amounts of 7-, 5-, 3- and *N*-hydroxylated derivatives in both groups of rats. As reported previously,[12] the major metabolite found in the sulfuric acid ester fraction was 7-OH-FAA.

Metabolites in cecum. In contrast to control animals, the cecum of the germ-free rats was characteristically enlarged and filled with considerable volumes of fluid.[13] One

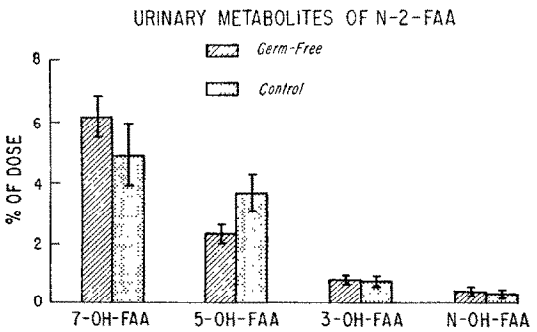

FIG. 1. Major metabolites of *N*-2-fluorenylacetamide excreted in 24 hr as glucosiduronic acids in urine of germ-free and control rats. 7-OH-FAA and the like stand for *N*-(7-hydroxy-2-fluorenyl)-acetamide, etc.

TABLE 3. FRACTIONATION OF CECAL CONTENTS FROM RATS INJECTED WITH 9-^{14}C-*N*-2-FLUORENYLACETAMIDE*

Group	No. of rats	Time (hr)	Metabolites (% of dose)				
			Total	Free	Glucuronides	Sulfates	Others
Germ-free	2†	24	18·3 ± 1·3	0·59	5·95	5·20	6·77
	2†	48	8·2 ± 2·5	0·18	1·90	2·71	3·44
Control	2	24	7·3 ± 1·7	4·4 ± 0·9	0·2 ± 0·03	0·4 ± 0·1	2·4 ± 0·6
	2	48	0·6 ± 0·1	0·29 ± 0·05	0·025 ± 0·007	0·03 ± 0·0	0·25 ± 0·01

* The fractionation technique employed with the cecal contents was similar to that used for fractionation of the feces.

† The cecal contents from the two rats were pooled.

day after injection the cecum contents in the germ-free rats contained 18 per cent of the dose versus 7 per cent in controls (Table 3). Even after the 2-day period, the germ-free group retained considerably more radioactivity in the cecum than did the controls. In addition, the metabolites exhibited an entirely different type of distribution. In the control rats, virtually all of the known metabolites were in the free, unconjugated form. There were only small amounts of glucosiduronic acids or sulfuric acid esters. Some of the activity present was in the form of water-soluble, unknown metabolites other than glucuronides or sulfates. In the germ-free group, on the other hand, a substantial portion of the metabolites present was conjugated with glucuronic acid or sulfuric acid and only a small part was unconjugated at 1 and 2 days.

In the control group, the chief metabolite was 7-OH-FAA and there were smaller amounts of the 5- and 3-hydroxylated metabolites as well as of FAA itself (Fig. 2). In the germ-free group, 7-OH-FAA was the main component of the small amount of unconjugated metabolites. The glucosiduronic acid fraction contained approximately equal amounts of the 7-, 5-, 3- and *N*-hydroxy derivatives, and the 5-hydroxy derivative was the predominant metabolite. The sulfuric acid ester fraction showed mainly the conjugate of 7-OH-FAA, but there were also present some 5- and 3-OH-FAA.

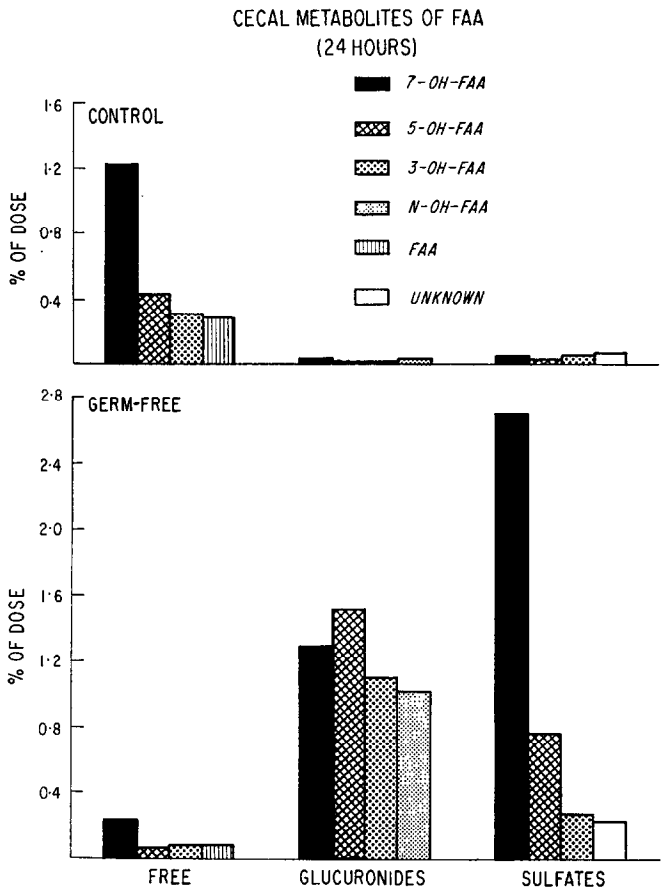

FIG. 2. Fractionation of metabolites of *N*-2-fluorenylacetamide present in the cecum of germ-free and control rats at 24 hr.

Metabolites in feces. In contrast to the situation in the cecum, the control rats excreted more of the dose in the feces than did the germ-free groups (Table 4). However, addition of the amounts of isotope in the cecum contents and feces at 1 and 2 days in the conventional and germ-free groups yielded roughly the same percentage of the dose. The retention of metabolites in the cecum of the germ-free group may have been due to the enlarged condition of this segment of the gastrointestinal tract.

As with the cecum, metabolites in the stools of the conventional rats were mostly in

the unconjugated form, whereas in the germ-free group the major part of the metabolites occurred as conjugates. Even the individual metabolites in the feces paralleled what was found in cecum, namely the presence of 7-OH-FAA as free compound and smaller amounts of the 5- and 3-hydroxy derivatives of FAA and of FAA itself in the control animals (Fig. 3). The germ-free groups exhibited mostly glucuronic acid conjugates of 7-, 5-, 3- and N-OH-FAA, and sulfuric acid esters, mainly of the 7-hydroxylated derivative.

TABLE 4. EXCRETION OF RADIOACTIVITY IN THE FECES OF GERM-FREE AND CONVENTIONAL RATS INJECTED WITH 9-^{14}-C-*N*-2-FLUORENYLACETAMIDE

Group	No. of rats	Time (hr)	Metabolites* (% of dose)				
			Total	Free	Glucuronides	Sulfates	Others
Germ-free	2	24	6·4 ± 0·6†	0·4 ± 0·14	1·6 ± 0·1	1·9 ± 0·1	1·9 ± 0·2
	2	48	16·7 ± 1·7	0·7 ± 0·04	3·8 ± 0·4	5·3 ± 0·7	5·4 ± 0·3
Control	2	24	18·7 ± 3·6	13·3 ± 2·8	0·5 ± 0·2	0·4 ± 0·03	2·2 ± 0·3
	2	48	28·9 ± 0·2	17·5 ± 1·0	1·2 ± 0·5	0·6 ± 0·05	4·0 ± 0·6

* The amounts of ^{14}C extractable were 91 and 80% of the total for germ-free and control groups respectively. An additional 8·5 and 16·4% were extracted from the residual pellets with 0·5 N NaOH solution.
† Standard error.

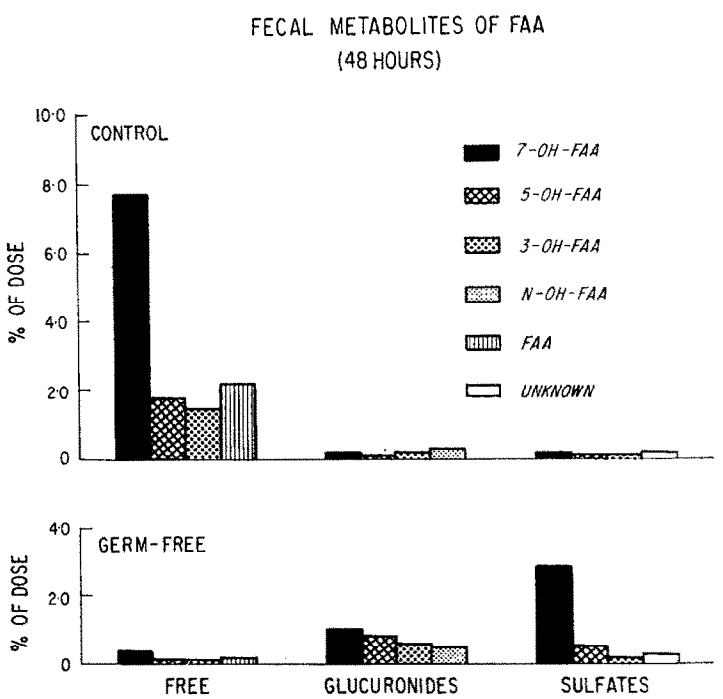

FIG. 3. Fractionation of metabolites of *N*-2-fluorenylacetamide found in the feces of germ-free and control rats during a 48-hr collection period.

TABLE 5. EXCRETION OF METABOLITES IN URINE AND FECES AFTER INJECTION OF *N*-2-FLUORENYLACETAMIDE INTO CECUM OF YOUNG ADULT MALE CONTROL RATS

	Urinary metabolites (% of dose)				Fecal metabolites* (% of dose)				
Time (hr)	Total	Free	Glucuronide	Sulfates	Total	Free	Glucuronide	Sulfates	Others
0—24	15·4	0·3	4·5	4·7	24·6	9·0	0·6	0·5	8·3†
24—48	7·7	0·1	2·4	1·2					
Major metabolites (0—24 hr)									
7-OH-FAA‡ (R_f 0·09–0·18)		0·1	1·2	2·6		2·4			
5-OH-FAA (R_f 0·24–0·33)		0·05	1·9	0·4		1·4			
3-OH-FAA (R_f 0·51–0·68)		0·02	0·1	0·2		0·5			
N-OH-FAA, FAA (R_f 0·68–0·82)		0·05	0·1	0·1		0·6			

* Only 75% of the fecal-^{14}C was extractable into 80% aqueous ethanol.
† A relatively large percentage of the extractable metabolites in the feces were highly polar, water-soluble compounds.
‡ 7-OH-FAA and the like are *N*-(7-hydroxy-2-fluorenyl)acetamide, etc.

Metabolites in urine and feces after intracecal injection of FAA into conventional rats. After the single intracecal injection of a tracer dose of FAA, almost 15 per cent of the dose was excreted in urine in 24 hr and an additional 7·7 per cent was found during the 24–48 hr period (Table 5). Of the activity in urine in the first and second day, respectively, 0·3 and 0·1 per cent of the dose were in the fraction of free compounds, 4·5 and 4·7 per cent in the glucosiduronic acids, and 4·7 and 1·2 per cent in the sulfuric ester fraction.

The major metabolite among the unconjugated compounds was 7-OH-FAA. Also present in somewhat lesser amounts were 5-OH-FAA, FAA and 3-OH-FAA. Among the glucuronides, 5-OH-FAA was the main metabolite, but 7-OH-FAA represented a sizable portion. 7-OH-FAA was virtually the only compound conjugated with sulfuric acid.

In 48 hr the feces contained 24·6 per cent of the dose, of which a large portion (9%) was as unconjugated metabolites. Thus represented were 7-OH-FAA and 5-OH-FAA and also small amounts of 3-OH-FAA and FAA.

The glucuronide and sulfate fractions were relatively minor, constituting only 0·6 and 0·5 per cent of the dose respectively. A large percentage of the dose, 8·3 per cent was of a highly polar nature, which failed to extract into ether after enzymic or acid hydrolysis.

DISCUSSION

In a previous paper we reported that germ-free rats excreted in the urine larger amounts of a single i.p. dose of *N*-hydroxy-*N*-2-fluorenylacetamide than did conventional rats.[4] Irving *et al.*[3] noted that after a single dose of N-OH-FAA the bile contained virtually exclusively the corresponding glucosiduronic acid, and also, that a single dose of FAA resulted in the biliary excretion of a sizable amount of the glucuronide of N-OH-FAA. Thus, a difference in excretion of metabolites might likewise be expected between germ-free and conventional animals given FAA. An analysis of the metabolites present in cecum and in the feces indeed disclosed such a difference, indicating that the intestinal flora did participate in further transformations of the carcinogen entering the gut via the bile. However, the urinary metabolites were rather similar. Perhaps, after a single relatively small dose, the urinary metabolites observed resulted from a direct clearance by the renal system of metabolites in the blood.[14] Although some N-OH-FAA glucuronide entered the gastrointestinal tract with the bile in the germ-free rats, the amounts reaching the urine were relatively small after undergoing a cycle of reabsorption and further metabolism. Therefore, similar levels of this important metabolite were excreted in urine by axenic and control animals. It is only after the larger amounts of N-OH-FAA glucuronide, resulting from the administration of this compound itself, that sufficient amounts were reabsorbed from the gut of germ-free rats to appear in urine in excess of the level observed in controls.

In addition, it should be mentioned that the cecum and feces of germ-free rats contained not only glucosiduronic acids, but also sulfuric acid esters and indeed some unknown, water-soluble conjugates. The control rats exhibited low levels of glucuronides and sulfate esters, suggesting that, in addition to β-glucuronidase, the bacterial flora in the cecum possessed a sulfatase. The latter enzyme may have been less specific than the sulfatase from Takadiastase, for this has been shown to hydrolyze only the sulfuric acid ester of *N*-(7-hydroxy-2-fluorenyl) acetamide and not those of the other

ring-hydroxylated metabolites of FAA.[15, 16] It is also noteworthy that the unknown, water-soluble metabolites do appear in similar amounts in germ-free and control rats. Their structures might be akin to reaction products of the carcinogen with nucleic acids, nucleosides, proteins or peptides, and carbohydrates.[17, 18]

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