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

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Abstract—The question whether the carcinogen *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA) was affected by the microbial flora in the intestinal tract was investigated by comparing the metabolism of this compound in germ-free and conventional Fischer strain rats, employing isotopic techniques.

Although the amounts of total urinary glucosiduronic acids and sulfuric acid esters were similar in axenic and control rats after an i.p. dose of N-OH-FAA, there were important differences among the individual glucosiduronic acids. Germ-free rats excreted considerably larger amounts of the glucuronide of N-OH-FAA and appreciably less of the conjugates from the ring-hydroxylated metabolites. Furthermore, the cecal and fecal metabolites in the conventional rats were mostly free, unconjugated materials, wherease in axenic rats the major fraction was conjugated with sulfuric and glucuronic acid. Injection of N-OH-FAA into the cecum, or intraluminal administration of glucosiduronic acids of N-OH-FAA or of phenolphthalein showed that these materials could be absorbed readily from the gastrointestinal tract. In the cecum of germ-free rats, β-glucuronidase activity was low and had an optimum at a pH characteristic of mammalian enzyme, but in conventional rats it was higher and with a pH optimum more like that of the bacterial enzyme.

The data indicate that N-OH-FAA is metabolized differently in germ-free rats: (1) because they lack the bacterial flora of the intestinal tract in conventional rats, which can hydrolyze glucosiduronic acids excreted into the gut by bile; and (2) because N-OH-FAA is a substrate for a bacterial *N*-dehydroxylase, also absent in axenic rats. In part, the enterohepatic cycle undergone by N-OH-FAA may be related to the liberation in the gut of readily absorbed metabolites, which are then further modified and excreted in urine or in bile.

ACTIVATION of aromatic amines by biochemical hydroxylation on the nitrogen is required to produce carcinogenic intermediates for certain target organs.[1–3] In studies on the metabolism of a typical compound of this series, *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA; *N*-2-fluorenylacetohydroxamic acid), it was found that after a single dose of this compound rats excreted only a small amount in the urine as such or as the glucuronic acid conjugate.[4] On the other hand, the glucuronic acid conjugate of the administered compound was virtually the sole metabolite found in bile.[5] The difference between biliary and urinary metabolites suggested that the microbial flora in the intestinal tract played a role in the transformations, a point which was examined by comparing the metabolism of N-OH-FAA in germ-free and control animals.

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MATERIALS AND METHODS

Chemicals. Unlabeled N-OH-FAA was prepared by reduction of the corresponding nitro compound.[6] Radioactive N-OH-FAA, labeled with ^{14}C on the 9-carbon atom, was purchased from Tracerlab, Inc. (Walthan, Mass. 02154). Bacterial β-glucuronidase, type II, was acquired from Sigma Chemical Co. (St. Louis, Mo. 63118), as was phenolphthalein glucuronide, which was used to standardize the enzyme. The glucuronide of N-OH-FAA was kindly made available by Dr. C. Irving (Veterans Administration Hospital, Memphis, Tenn. 38104). Ring-hydroxylated metabolites of *N*-2-fluorenylacetamide (FAA) such as 7-, 5- and 3-OH-FAA were prepared in connection with earlier research.[3]

Assay of β-glucuronidase. Phenolphthalein glucuronide was incubated in the presence of a few drops of chloroform at 37° for 30 min followed by addition of 0·2 M glycine buffer, pH 10·4, and determination of the extinction at 540 nm. In one series of tests, incubations were performed with 0·1 M buffers at various pH values to determine the pH optimum of the preparation under study.

Treatment of germ-free animals. Male, germ-free Fischer rats, originally derived by Caesarian section from the NIH conventional Fischer strain, were used in this study. The germ-free rats were reared under sterile conditions within a plastic isolator and allowed free access to steam-sterilized diet L-356[7] and drinking water. The procedure for monitoring germ-free animals for bacterial and fungal contamination has been described previously.[8] When the rats were 10 weeks of age, they were transferred to individual metabolism cages (Acme Metal Products, Chicago, Ill. 60619) on stands within a Reyniers germ-free isolator. After conditioning in this environment for 48 hr, they were injected i.p. with 10 mg/kg of labeled N-OH-FAA dissolved in ethanol and then suspended in sterile steroid suspending vehicle.[9] The suspension was checked and found sterile. One group of animals was removed after 24 hr, the other after 48 hr and both groups were killed by exsanguination from the abdominal aorta under light ether anesthesia. The heparinized blood was centrifuged to separate the plasma. The livers, perfused through the portal vein with 0·9% sodium chloride solution, were minced. Portions of 2 g were homogenized and aliquots were counted to obtain total isotope in liver. The homogenate was diluted with 4 vol. of ethanol and the resulting precipitate was extracted with a series of solvents to remove unbound metabolites.[10] The radioactivity remaining in the residual material was a measure of bound metabolites. Although it is realized that the extraction procedure did not remove nucleic acids and similar macromolecules, the name "protein-bound" is used according to antecedent practice. Binding to macromolecules appears to be proportional to total tissue activity in most instances.[11]

Urinary metabolites. Urines were collected in a receiver at room temperature. Preservatives were not added since the environment was sterile. However, urinary β-glucuronidase was expected to hydrolyze in part glucuronic acid conjugates. Therefore, the normal procedure[4] for the isolation of metabolites was modified, omitting the separate extraction of free, unconjugated compounds. After counting aliquots of the urines to determine total urinary metabolites, 1 ml of 1 M sodium acetate buffer, pH 6, was added per 10 ml of urine, and the samples were incubated with β-glucuronidase (400 units/ml) for 2 hr. Extraction with ether removed the sum of the total unconjugated metabolites and those liberated from the hydrolyzed glucosiduronic acids. The aqueous phase was adjusted to pH 1·5, refluxed for 15 min, made to pH 6

with sodium bicarbonate solution, and extracted with ether to remove the metabolites freed from the sulfuric acid esters by this procedure. The residual aqueous phase contained unknown water-soluble conjugates.

The ether extracts were taken to dryness by a stream of nitrogen. The residue, dissolved in a small volume of ethanol, was analyzed by paper chromatography in the solvent system cyclohexane–*tert.*-butanol–acetic acid–water (16:4:2:1) or by thin-layer chromatography on silicic acid in the solvents petroleum ether–acetone (6:4) or chloroform–methanol (97:3). Standard known metabolites were chromatographed at the same time. In addition, spots on the paper and thin-layer chromatograms, located by autoradiography on Kodak Royal Blue film, were cut out or removed and the materials were further identified by colorimetric or spectroscopic means.

Metabolites in cecum. The contents of the cecum were removed and extracted four times with 80% aqueous ethanol. After centrifugation and counting an aliquot, the supernatant fraction was concentrated on a rotary evaporator. Extraction of the concentrate with ether yielded the free, unconjugated metabolites. The aqueous phase was then processed as described for urine to identify and quantitate the glucuronic acid and sulfuric acid conjugates.[12] The final aqueous residue contained unknown water-soluble metabolites. The various ether extracts were chromatographed to determine the specific metabolites present.

Metabolites in feces. The feces were extracted four times with 80% aqueous ethanol. After centrifugation, the residual pellet was further extracted with 0·5 M sodium hydroxide solution. The extracts and residual pellet were counted.

The aqueous ethanol solution was concentrated on a rotary evaporator *in vacuo* and analyzed for free and conjugated metabolites as described for cecal and urinary materials.

Absorption of metabolites from gastrointestinal tract of conventional rats. In one series the portal veins of male adult rats on a diet of Wayne laboratory Blox were cannulated. After intraluminal administration of phenolphthalein glucuronide, the plasma obtained from the portal vein blood was examined for free phenolphthalein and for the glucuronic acid conjugate. By the same technique, the absorption of the glucuronide of N-OH-FAA was studied.

In another series of tests, the absorption of N-OH-FAA from the cecum was determined, using small doses to simulate the conditions resulting from the liberation of compound in the gut. The ceca of young adult male Fischer rats were exposed while the animals were under ether anaesthia. With a 27 gauge needle a fine, sterile suspension of 333 μg of the labeled compound in 0·2 ml of steroid suspending vehicle was injected under sterile conditions. After closing the peritoneal cavity, the animals were placed in metabolism cages. Urine was collected for 24 and 48 hr in ice-cold receivers. Feces for the 48-hr period were combined. The metabolites of N-OH-FAA in urine and feces were analyzed by the procedures described above.

Determination of radioactivity. Aliquots of organic or aqueous solutions were incorporated into a toluene–methanol base scintillation mixture. Solid materials or tissues were first solubilized in 1–3 ml Hyamine solution on a shaking incubator at 60° for 4–6 hr after which a toluene-based scintillation mixture was added. Counting was performed on a liquid scintillation spectrometer operating at an efficiency of 65 per cent. Corrections were made for background and quenching (internal standard).

RESULTS

Tissue metabolites. In relation to conventional controls there was more radioactivity in the liver or bound to liver "protein" in the germ-free groups 24 hr after dosing. However the situation was reversed in respect to activity in the plasma (Fig. 1).

Urinary metabolites. The germ-free and control groups were similar in the excretion of total metabolites in urine at 1 and 2 days as well as in excretion of the glucosiduronic acids and the sulfuric acid esters taken as a group (Table 1). However examination of the individual metabolites revealed important differences particularly among glucuronic acid conjugates. As a function of dose the germ-free group had less of all of the ring-hydroxylated metabolites and more of the *N*-hydroxy derivative of FAA than did the controls (Fig. 2). The relative values found were similar when urines collected for 0–24 or 24–48 hr were examined.

Metabolites in cecum. There was considerably more radioactivity after 1 and 2 days in the ceca of the germ-free rats than in the controls (Table 2). In part this

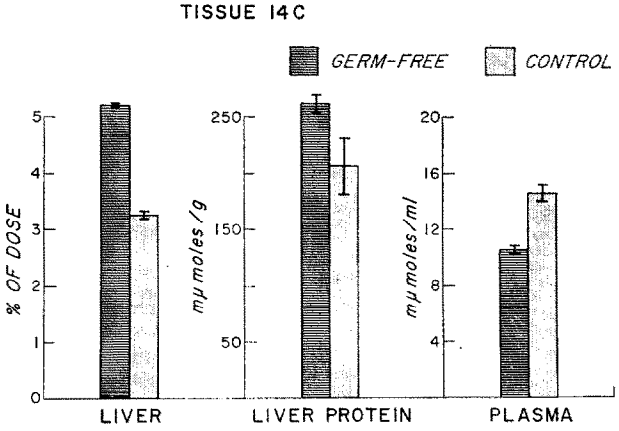

FIG. 1. Metabolites in tissues. Distribution of isotope in the liver after 24 hr is shown as percentage of dose. Liver protein-bound isotope is expressed as millimicromoles of metabolite per gram dry weight; plasma ^{14}C is expressed as millimicromoles of metabolite per milliliter.

TABLE 1. BODY WEIGHTS AND URINARY EXCRETION OF RADIOACTIVITY FROM GERM-FREE AND CONVENTIONAL RATS INJECTED WITH 9-^{14}C-*N*-HYDROXY-*N*-2-FLUORENYL-ACETAMIDE*

Group	No. of rats	Time (hr)	Body wt. (g)	Liver wt. (g)	Urinary metabolites (% of dose)		
					Total	Free + glucuronides	Sulfates
Germ-free	4	24	251 ± 4·6†	8·5 ± 0·4	21·3 ± 0·9	5·9 ± 0·6	6·9 ± 0·3
	3	48	248 ± 3·7	7·8 ± 0·4	25·8 ± 4·6	7·0 ± 1·7	8·6 ± 1·7
Control	4	24	254 ± 4·2	8·5 ± 0·3	23·5 ± 1·7	6·8 ± 0·5	5·4 ± 0·4
	4	48	242 ± 7·2	8·1 ± 0·3	23·9 ± 2·3	7·6 ± 0·8	5·6 ± 0·5

*The germ-free or conventional male Fischer rats each received (i.p.) 0·8 ml of a suspension of 2·2 mg of 9-^{14}C-N-OH-FAA (sp. act., 3·3 × 10^6 cpm/mg) in steroid suspending vehicle.
†Standard error.

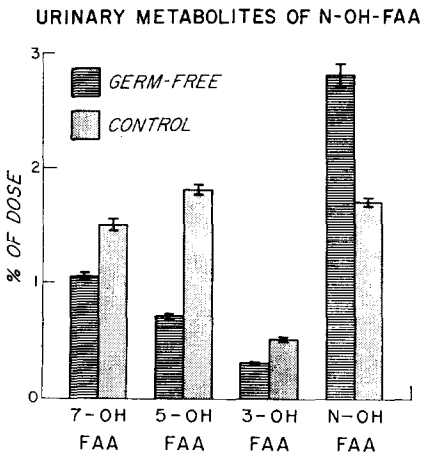

FIG. 2. The major metabolites in 24-hr urine in the free plus glucuronide fractions, expressed as percentage of dose, determined by paper chromatography. In earlier studies, urines were collected in refrigerated flasks and only small amounts of free, unconjugated metabolites were found. In the present tests, the flasks could not be cooled inside the Reyniers isolators. Urinary β-glucuronidase would liberate free metabolites from glucosiduronic acids during the 24-hr collection period. Therefore, the free fraction was not separately estimated.

increased amount of isotope may have been due to the sizable enlargement of the cecum.[13] At both time periods, the major portion of the metabolites from the control group was in the form of free, unconjugated compounds. In contrast, in the germ-free, group the major portion was in the form of conjugates of the glucuronide or sulfate type or as highly water-soluble unknown metabolites. In the control animals, the major cecal metabolite was the reduction product of N-OH-FAA, namely FAA (Fig. 3), but smaller amounts of the 7-, 5- and 3-hydroxy derivatives of FAA were also observed. However, in the germ-free group, N-OH-FAA was the major metabolite both in the free and glucuronic acid fractions. Lesser quantities of 3-OH-FAA, 5-OH-FAA and 7-OH-FAA were also seen as glucuronides. Although the principal component of the sulfuric acid esters was the 7-hydroxy derivative, small amounts of the 5- and 3-hydroxy derivatives were also identified. Paper chromatograms of this fraction indicated that there were additional minor metabolites with a mobility like that of N-OH-FAA and some which moved faster. These were seen also in the corresponding fractions of urinary metabolites (cf. reference 4).

TABLE 2. 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	23·9 ± 1·1*	1·5	3·7	4·0	14·7
	2	48	6·9 ± 3·0	0·3	0·7	1·1	4·8
Control	2	24	8·7 ± 0·1	4·2	0·1	0·7	3·7
	2	48	2·4 ± 0·2	0·8	0·03	0·2	1·4

* The tatol radioactivity of each cecum with contents was determined separately, but the cecal contents of the two rats were pooled for the fractionation.
† Standard error.

Fecal metabolites. After 1 and 2 days, the amounts of radioactivity in the feces and the proportion of material extractable from feces by 80% ethanol were similar in the germ-free and control groups (Table 3). However, while most of the fecal metabolites in the control animals were unconjugated, they were divided about evenly between the free fraction and the glucuronic acid and sulfuric acid conjugates in the germ-free group. A major metabolite, FAA, amounted to about 6 per cent of the dose in the free

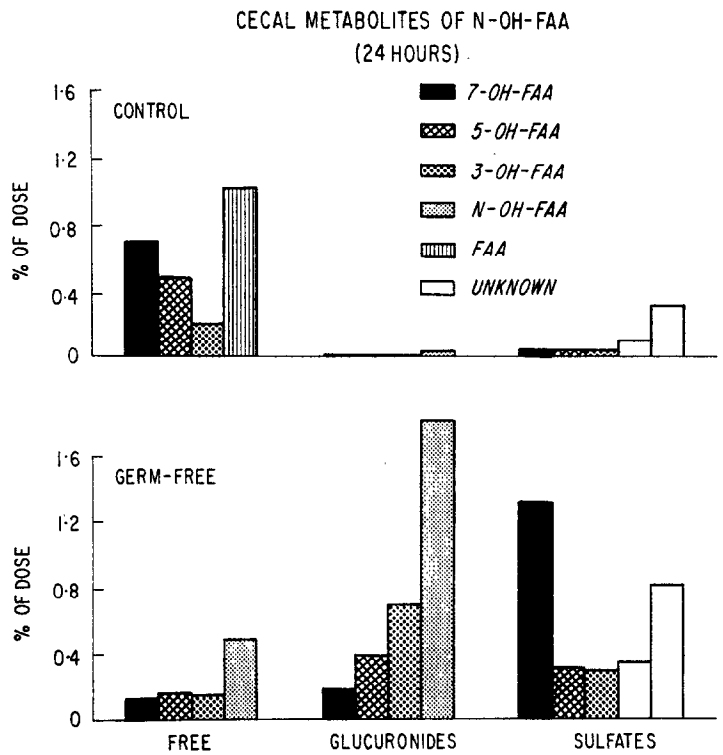

FIG. 3. Metabolites of *N*-hydroxy-*N*-2-fluorenylacetamide in the cecum of germ-free and control rats 24 hr after i.p. injection. Unknown metabolites in the "sulfate" fraction constituted distinct areas with isotope on the paper chromatograms, with a mobility faster than that of FAA or N-OH-FAA.

fraction of the control animals (Fig. 4). In the germ-free group, the main compound noted in the free metabolites was also FAA, and in the glucuronic acid fraction N-OH-FAA was present in relatively large amounts. The chief component of the sulfuric acid esters was the conjugate of 7-OH-FAA.

β-Glucuronidase in cecum. While the optimum pH for glucuronidase from bovine liver is 4·5 to 5 and for bacterial enzyme is pH 7, the optimum for the enzyme present in the cecal contents of germ-free rats was at pH 5, whereas for that in the conventional animals it was at pH 6 (Fig. 5). It seemed therefore that the enzyme present in germ-free rats corresponded more to that of the mammalian enzyme, whereas that in the conventional animals was related more closely to the bacterial enzyme.

Excretion of metabolites after intracecal injection of conventional rats. About 20

TABLE 3. EXCRETION OF RADIOACTIVITY IN THE FECES OF GERM-FREE OR CONVENTIONAL RATS INJECTED WITH 9-^{14}C-*N*-HYDROXY-*N*-2-FLUORENYLACETAMIDE

Group	No. of rats	Time (hr)	Total excretion	Extractable*	Metabolites (% of dose)		
					Free	Glucuronides	Sulfates
Germ-free	2	24	17·5 ± 0·2†	12·5 ± 0·1	1·6 ± 0·4	1·7 ± 0·2	2·5 ± 0·02
	2	48	30·1 ± 5·3	21·7 ± 2·5	2·2 ± 0·2	2·7 ± 0·1	3·9 ± 0·5
Control	2	24	15·5 ± 3·7	11·9 ± 4·1	10·1 ± 4·1	0·14 ± 0·01	0·11 ± 0·08
	2	48	33·9 ± 3·0	19·3 ± 0·5	12·8 ± 1·0	0·09 ± 0·01	0·12 ± 0·04

* Extractable into 80% ethanol.
† Standard error.

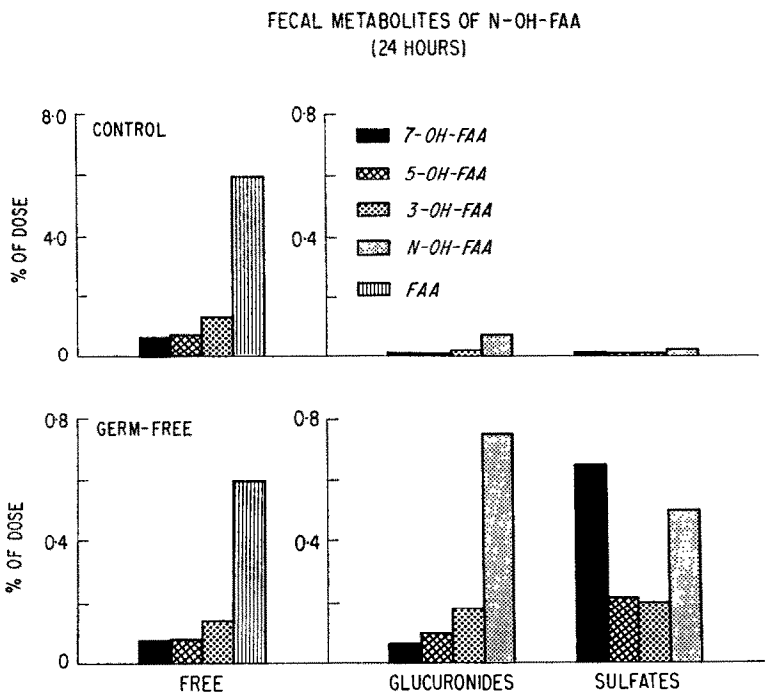

FIG. 4. Fractionation of fecal metabolites of *N*-hydroxy-*N*-2-fluorenylacetamide in germ-free and control rats. Note change in scale in data of control group. The metabolite in the free fraction of the germ-free group shown as FAA is at least 74 per cent this compound by eluting chromatograms containing N-OH-FAA plus FAA, taking up in ethyl ether and extracting with 0·5 N NaOH. The ether layer retained 74 per cent of activity and, upon thin-layer chromatography in petroleum ether–acetone (6 : 4) on silicic acid, gave a spot with the mobility of FAA.

per cent of the dose appeared in urine in 24 hr, while about 10 per cent was excreted during the second day (Table 4). Glucosiduronic acids and sulfuric acid esters accounted for 5·4 and 5·8 per cent of the dose on the first day, and 3·3 and 1·4 per cent, respectively, on the second day. There were only small amounts (0·4 per cent of dose) of free, unconjugated metabolites, mostly in the form of 7- and 5-OH-FAA (24 and 17 per cent of fraction respectively). FAA and 3-OH-FAA accounted for

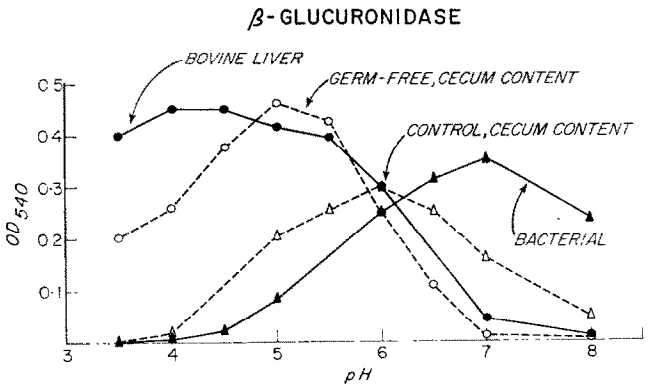

FIG. 5. Activity of β-glucuronidase from various sources as a function of pH. Enzyme activity was determined with phenolphthalein glucuronide as substrate. Bacterial β-glucuronidase, type II, and bovine liver β-glucuronidase, type B-1, were from Sigma Chemical Co. The cecal contents were diluted with water (1 : 4, w/v), stirred and filtered through four layers of cheese-cloth. Aliquots of the filtrate, 0·5 ml and 0·025 ml, were used in the assay for germ-free and control groups respectively.

only a small amount of this fraction. Among the glucosiduronic acids, the major component was 5-OH-FAA; N-OH-FAA as well as 7-OH-FAA were seen in small amounts. The sulfuric acid ester fraction contained in decreasing order: 7-OH-FAA, 5-OH-FAA and 3-OH-FAA.

In the 2-day experiment, the rats excreted 21 per cent of the dose in the stools. Of this amount, about 12 per cent was soluble in 80% ethanol. Analysis of this soluble fraction showed that most was in the form of free compounds (3·4 per cent of dose) and only minor amounts of conjugates (glucosiduronic acids, 0·4 per cent; sulfuric acid esters, 0·6 per cent) were found. The major metabolites in the free fraction were 7- and 5-OH-FAA. The conclusion can be drawn from the combined data that N-OH-FAA is resorbed readily from the cecum. Moreover, the distribution of urinary and fecal metabolites, high in ring-hydroxylated derivatives, suggests that rather extensive *N*-dehydroxylation and further metabolism occurred. It is to be noted that a low dose of N-OH-FAA was injected.

DISCUSSION

In axenic animals, the metabolism of N-OH-FAA differed from controls with respect to liver and plasma levels, the quantitative composition of urinary metabolites, and significantly in the composition of metabolites in the intestine or excreted in the feces. The conclusion was that, after a single dose of N-OH-FAA in axenic animals, there was less conversion of this compound to other metabolites. Consesequently, higher levels were available for reaction in tissues and also for elimination as such or as the glucosiduronic acid.

The question can be asked: What is the relative importance of enzymes, in particular β-glucuronidase and *N*-dehydroxylase, present in liver and in cecum, as regards the overall metabolism of N-OH-FAA? In liver, glucuronidase is localized in lysosomes and, under generally prevailing unfavourable conditions of pH, may not be readily available to the substrate except under unusual intracellular conditions.

TABLE 4. EXCRETION OF METABOLITES IN URINE AND FECES AFTER INJECTION OF *N*-HYDROXY-*N*-2-FLUORENYLACETAMIDE INTO CECUM OF YOUNG ADULT MALE RATS

	Urinary metabolites (% of dose)				Fecal metabolites* (% of dose)				
Time (hr)	Total	Free	Glucuronides	Sulfates	Total	Free	Glucuronides	Sulfates	Others
0—24	20·4	0·3	5·4	5·8	21	3·4	0·4	0·6	7·4†
24—48	10·2	0·06	3·3	1·4					
Major metabolites (0—24 hr)									
7-OH-FAA‡ (R_f 0·09–0·18)		0·07	0·9	2·0		0·5			
5-OH-FAA (R_f 0·24–0·33)		0·05	2·1	0·06		0·5			
3-OH-FAA (R_f 0·50–0·58)		0·02	0·02	0·5		0·2			
N-OH-FAA, FAA (R_f 0·68–0·82)		0·03	0·9	0·3		0·5			

* Only 62% of the fecal-^{14}C was extractable into 80% 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.

On the other hand, conjugation with glucuronic acid in the liver cells appears to be an efficient and fast reaction, which at the same time removes free N-OH-FAA as substrate for the hepatic *N*-dehydroxylase, a soluble and perhaps also a microsome-bound system. Thus, in the liver the equilibrium is on the side of conjugation reactions, followed by secretion into blood and bile of the conjugates. In the cecum, on the other hand, bacterial glucuronidase and perhaps other enzymes exist in solution at a suitable pH favoring the splitting of substrate, as a comparison of the metabolites in germ-free and control rats showed. The N-OH-FAA thus liberated becomes available immediately for the action of *N*-dehydroxylase, so that FAA or other metabolites resulting from the effect of such further transformations can be excreted in feces or can be resorbed and metabolized.

In order to be subject to metabolism by the microflora in the gut, a drug will have to reach the area containing microorganisms in the intestinal tract. As discussed in a superb review by Smith,[14] in most species the upper part of the small intestine is virtually sterile, the lower part begins to have a select population, but the main area of bacterial colonization is the cecum. Therefore, drugs entering the gastrointestinal tract can be absorbed in the upper portion before exposure to enzymes from the microflora. Materials entering the gut via the bile are often in the form of conjugates. As they migrate downward, they are almost completely hydrolyzed by bacterial enzymes. The products are either reabsorbed, constituting an important part of the enterohepatic cycle, or are excreted chiefly as free, unconjugated materials. In the germ-free system, these enzymes in the gut are absent, leading to a different metabolic pattern.

An extension of the results obtained to other drugs or to other species may be of interest. Whether or not like differences between control and germ-free animals would be found depends on whether or not the drug and its metabolites, in particular conjugates, would be secreted into the gut by the bile. This process is a function of the structure of the drug and also of the species.[14–16] Even though reabsorption from the lower intestinal tract occurs, an estimate of biliary secretion can be had from the ratio of fecal to urinary excretion under a given experimental situation. The fecal metabolites usually are the results of biliary transfer to the gut.

There have not been many studies on the fate of other drugs in germ-free animals. Some steroids,[17] lipids or lipid derivatives,[18–22] and also other chemicals[23, 24] are handled somewhat differently in germ-free animals as compared to controls. Germ-free rats injected with a carcinogenic azo dye appear to have lower levels of serum γ-globulins, although the effect on the liver was rather similar.[25] An interesting difference between germ-free and conventional rats was discovered by Laqueur *et al.*,[26, 27] who studied the carcinogenicity of a plant product, cycasin, the β-glucoside of methylazoxymethanol. In germ-free rats this compound was innocuous, since β-glucosidase to liberate the aglycone necessary for hepatotoxicity and tumor induction was lacking. Infection with a strain of *Escherichia coli* again restored the pathological effect of this carcinogen.[28]

With the carcinogenic hydrocarbons, Pollard *et al.*,[29] Salomon[30] and Kelly *et al.*[31] reported a more uniform expression of the carcinogenicity in germ-free as compared to conventional animals, but no relevant metabolism studies under these conditions have been performed. We have not yet examined the carcinogenicity of N-OH-FAA in germ-free rats. After a single dose, as noted above, higher levels of the active

carcinogen are seen. One would thus predict that this agent should be more effective in germ-free rats. However, as we have shown in related studies, under conditions of continuing intake of this compound there is a shift in bacterial flora in the lower intestinal tract, particularly with respect to an elimination of coliform organisms.[32] Thus, the difference in carcinogenicity between conventional and germ-free rats fed N-OH-FAA under chronic conditions might be less pronounced than would be expected on the basis of the single dose experiments.

Note added in press—Short and Davis[33] recently reported that enzyme activities in the 9000 *g* supernatant fraction of rat liver of germ-free and conventional rats towards a number of substrates were similar. Also, Grant and Roe[34] noted that germ-free male C3H mice developed fewer or no liver tumors under conditions where conventional mice did have such tumors.

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