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PARTICIPATION OF LIVER FRACTIONS AND OF INTESTINAL BACTERIA IN THE METABOLISM OF *N*-HYDROXY-*N*-2-FLUORENYLACETAMIDE IN THE RAT*

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Abstract—The level of the active carcinogenic intermediate, *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA) in rats is controlled in part by enzymic reduction to *N*-2-fluorenylacetamide (FAA). These reactions are performed not only by enzymes in liver but also by systems from the microbial flora in the gut.

N-OH-FAA was secreted via the bile into the intestinal tract as a glucuronic acid conjugate. Cecum contents contained β-glucuronidase of a bacterial type with maximal activity at pH 6. Lower glucuronidase activity was present in the cecum of germ-free rats with an optimum at pH 5, typical of mammalian β-glucuronidase. Rat cecal contents readily hydrolyzed glucosiduronic acids and reduced N-OH-FAA to FAA. The *N*-dehydroxylation was faster after preincubation of an extract of cecal contents with N-OH-FAA. Strain K 32 of *Escherichia coli* contained the enzyme required for this reduction, The microflora in the gut, in particular coliform organisms, was inhibited by N-OH-FAA.

N-dehydroxylase activity in liver was chiefly in the soluble fraction, although a small amount was also associated with the microsome fraction.

On chronic feeding of 160 ppm of N-OH-FAA for 8 weeks, there was a progressive shift in microbial flora in the gut with a reduction in coliform type organisms and an increase in yeast cells. As a result there was a lowering in cecal β-glucuronidase and in *N*-dehydroxylase. Soluble liver *N*-dehydroxylase increased slightly at the 4-week point, then decreased. During the same period, urinary excretion of the glucuronide of N-OH-FAA increased from 1·8 per cent of the dose to a peak of 19·4 per cent after 4 weeks. In part, the alterations in intestinal microflora with consequent shifts in enzyme content may account for the increased excretion of this metabolite of N-OH-FAA. Other factors play a role, since alteration of the microbial flora in the cecum by administration of Neomycin failed to yield a proportional increase in the excretion of the glucuronide of N-OH-FAA.

A new analytical method to determine the substrate N-OH-FAA was devised. Oxidation of solutions of N-OH-FAA by potassium permanganate was followed by the colorimetric assay of the intermediate product with trisodium pentacyanamino ferrate at 560 nm. Concentrations of 5–100 μg/ml of N-OH-FAA could thus be determined readily.

IT HAS been established that carcinogens of the aromatic amine type require biochemical activation by hydroxylation on the nitrogen, a reaction which proceeds via

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the endoplasmic reticulum or the microsomal fraction of liver and some select other organs.[1–6] Thus, in the initiation of the processes leading to neoplasia by such compounds, one important reactive intermediate is the *N*-hydroxy derivative.

In particular, the well known wide-spectrum carcinogen, *N*-2-fluorenylacetamide (FAA), is converted, probably chiefly in the liver, to *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA; *N*-2-fluorenylacetohydroxamic acid). After a single intraperitoneal injection of N-OH-FAA, the major biliary metabolite in the rat was the corresponding glucosiduronic acid.[7] However, under the same conditions, the urine contained only a small proportion of the dose as this conjugate, but a number of other metabolites of FAA were excreted.[8] Furthermore, Grantham *et al.*[9, 10] noted that the major fecal metabolite after a single dose of N-OH-FAA was FAA. It seemed, therefore, that the glucosiduronic acid of N-OH-FAA, entering the intestinal tract via the bile, was subject to metabolic conversion in the gut, presumably by the microflora therein. The present paper deals with an examination of this problem.

It was also found that under conditions of chronic intake of N-OH-FAA the bacterial flora in the intestinal tract underwent quantitative and qualitative changes which resulted in sizable alterations in enzyme levels concerned with the metabolism of N-OH-FAA derivatives. The shifts in metabolic pattern seen during chronic carcinogen administration[1, 11] may be ascribed in part to such modifications in the microbial flora.

In order to facilitate this study a new method for the analysis of N-OH-FAA in tissue and body fluids was developed. Based on an oxidative step followed by colorimetric estimation of the product, it was considerably simpler than the prior solvent partition or paper and column chromatographic techniques[1,8,11] and was more suitable for the analysis of numerous samples.

MATERIALS AND METHODS

Chemicals. Unlabeled N-OH-FAA (m.p. 145–147°,[12]) migrated as a single spot upon thin-layer chromatography (TLC) in petroleum ether–acetone (6:4) or on Whatman 3 MM paper in cyclohexane–*tert.*-butanol–acetic acid–water (16 : 4 : 2 : 1).[8] N-OH-FAA-9-^{14}C was purchased from Tracerlab (Waltham, Mass. 02154). The labeled compound was diluted with unlabeled N-OH-FAA to a specific activity of $1{\cdot}89 \times 10^6$ cpm/mg. The glucosiduronic acid of N-OH-FAA was kindly contributed by Dr. C. Irving (Veterans Administration Hospital, Memphis, Tenn. 38104). Bacterial β-glucuronidase, type II, and phenolphthalein glucuronide came from Sigma Chemical Company (St. Louis, Mo. 63118). Trisodium pentacyanamino ferrate (TPF) and Folin reagent were from Fisher Scientific Company (Silver Spring, Md. 20910). Strain K-32 of *E. coli* was acquired from the American Type Culture Collection (Rockville, Md. 20852). All other chemicals were reagent grade. Paper and thin-layer chromatographic separations were performed as described.[7, 8] Spectrophotometric analyses were done with a Beckman model DB spectrophotometer. High speed centrifugations were carried out in an International Equipment Company ultracentrifuge, model B60.

Treatment of animals. Acute or short-term studies were performed with male weanling or young adult Fischer strain rats which had free access to Wayne Lab Blox (Allied Mills, Chicago, Ill. 60606) and water. Chronic carcinogen feeding studies employed male animals fed a semi-synthetic diet (A)[13] from weaning until they were

10 weeks of age. At that time a group was placed on the same diet containing 160 ppm of N-OH-FAA (diet K). Another group received 1000 ppm of Neomycin (diet AN), and another both 160 ppm of N-OH-FAA and 1000 ppm of Neomycin (diet KN). Control rats were continued on diet A.

At intervals, groups of rats were selected at random from the animals fed control or experimental diets and injected with a sterile suspension of 20 mg/kg of N-OH-FAA-9-^{14}C. The animals were placed in pairs in metabolic cages (Acme Metal Company, Chicago, Ill. 60619). Urine was collected in ice-cold flasks for 24 hr. Urinary metabolites were analyzed by the previously employed methods.[8, 14] The animals were killed under carbon dioxide narcosis by withdrawal of blood from the abdominal aorta.

Treatment of tissues.

Livers. Non perfused livers were removed *in toto* and minced with scissors in ice-cold containers. After manual mixing, aliquots were removed and homogenized with 4 : 1 (v/w) 0·1 M Tris buffer, pH 7·4, in a Potter–Elvehjem Teflon-glass homogenizer. Where applicable, homogenates were centrifuged at 4° at 10,000 *g* for 15 min to remove nuclei and mitochondria. The 10,000 *g* supernatant fraction was centrifuged at 200,000 *g* for 2·5 hr. The resulting soluble fraction was reserved for enzyme assays. Microsomal pellets were resuspended in an equal volume (to soluble fraction) of Tris buffer and recentrifuged before assay.

Cecal contents. The entire cecum was removed and the contents were expressed, weighed, and suspended in 4 vol. per g weight of 0·14 M sodium phosphate buffer, pH 7·6. The suspension was filtered through four layers of cheesecloth to remove large particulate matter. Results of studies on the number and type of bacteria, *N*-dehydroxylase activity and β-glucuronidase activity in the cecal contents are expressed in terms of activity per milliliter of the resulting coarse filtrate.

Assay method for N-OH-FAA

The basic procedure was as follows: A mixture of 1·0 ml of a 0·01% aqueous $KMnO_4$ solution and 3·0 ml of an ethanol–water (1 : 1) or pure ethanol solution of N-OH-FAA was allowed to stand at 22° for 10 min. Then 1·0 ml of a freshly prepared 0·25% aqueous solution of TPF reagent was added, and after 15 min the extinction at 560 nm was determined. The maximum in the absorption spectrum of the colored complex was relatively broad (545–575 nm. A standard curve at the selected wavelength of 560 nm showed that Beer's law was obeyed throughout the range of 5–100 μg/ml (Fig. 1). Within the limits of the concentration range and the reaction time specified, the presence of *N*-2-fluorenylacetamide (FAA), of phenolic derivatives of FAA or of 2-fluorenamine (FA) did not interfere with the determination of N-OH-FAA.

Metal ions that complex with the TPF reagent and some lipids did interfere. Reaction mixtures containing these substances, virtually all crude samples of biological material, were extracted with ether, transferring N-OH-FAA to the organic phase. The ether was evaporated under nitrogen and the residue was dissolved in ethanol, typically 3·0 ml, after which the assay was performed. However, in the case of whole liver homogenates there was a transfer of reducing substances into the ether layer. These interfered with the assay because they used up permanganate (characterized by immediate decolorization) and resulted in an incomplete oxidation of N-OH-FAA.

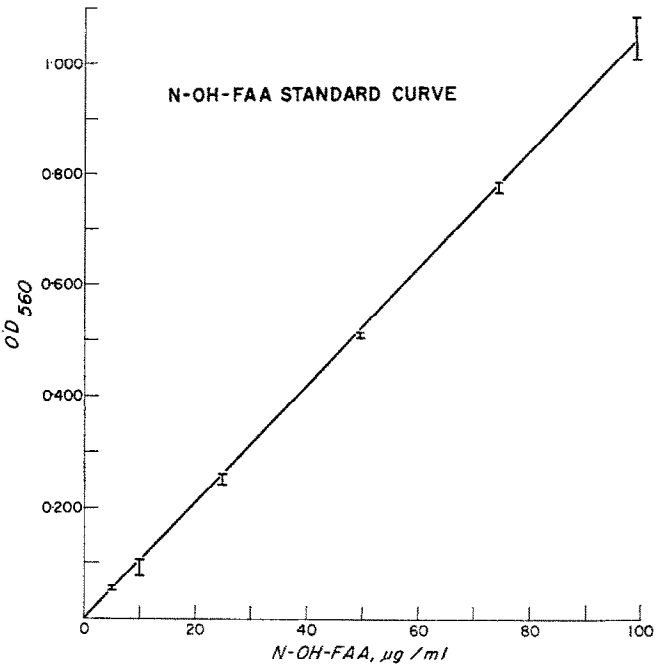

FIG. 1. N-OH-FAA standard curve: extinction at 560 nm versus concentration of N-OH-FAA in ethanol. See text for experimental details.

With such samples, it was necessary to dilute the residue, after evaporation of the ether, with a larger volume of ethanol, usually 6·0 ml, and to continue the assay with 3·0 ml aliquots.

Recovery of substrate in zero time samples was 90 per cent with liver fractions or cecal contents.

The method could not yet be applied to urine fractions because ether extracted large amounts of reducing substances which decolorized permanganate and thus gave incomplete oxidation of N-OH-FAA.

The addition of TPF reagent to certain ethanol-treated reaction mixtures prior to permanganate oxidation did not reveal the prescence of detectable amounts of *N*-2-fluorenylhydroxylamine or of 2-nitrosofluorene. Permanganate oxidized these substrates in part to 2-nitrofluorene, which is not detected by the TPF reagent.

Enzyme assays

N-*dehydroxylase.* The colorimetric method was used to assess the amount of N-OH-FAA remaining in a homogenate or in an extract after incubation. Standard solutions of N-OH-FAA were analyzed as controls. With liver fractions, the decrease in substrate concentration in a sample incubated in the presence of 0·1 M NaF was taken as a measure of *N*-dehydroxylase activity, the fluoride blocking deacylase action.[15] Fluoride was not necessary for the assay of *N*-dehydroxylase in cecal contents.

Assays were run on 1·0 ml samples representing 250 mg wet weight of liver per ml

n the case of whole homogenate. With liver fractions, assays were on 1·0-ml aliquots of samples derived from the original homogenate. Protein concentrations were determined in each case and used in calculating specific activities.

N-OH-FAA (300 μg/ml) was introduced into the liver fractions by addition of either 50 μl of an ethanol solution (6 mg/ml) or 100 μl of a fine suspension (3 mg/ml) in 0·06 N NaOH. Duplicate 1·0-ml aliquots were incubated at 37° in glass-stoppered tubes on a mechanical shaker. Zero time samples were extracted immediately after substrate introduction. After 30 min, the reaction was stopped by extracting three times with 5 vol. of diethyl ether. The combined ether extracts were blown to dryness by a stream of nitrogen. The residues were dissolved in 3·0 ml ethanol and N-OH-FAA was determined colorimetrically by the permanganate oxidation method. Aliquots of extracts were resolved by TLC to examine the nature of the products.

N-dehydroxylase in bacterial systems was followed by introducing N-OH-FAA into the buffer-diluted filtrate of cecal contents and incubating at 30°. Duplicate aliquots were withdrawn periodically, usually up to 8 hr, or until the N-OH-FAA concentration approached zero. The aliquots were extracted with diethyl ether and treated as described for liver fractions.

Protein concentration in the liver fractions was determined by the Fiszer[16] modification of the method of Lowry *et al.* [17]

β-Glucuronidase. Cecal β-glucuronidase was estimated by incubating an aliquot of the buffer-diluted filtrate of cecal contents with phenolphthalein glucuronide for 30 min at 37° and determining the amount of phenolphthalein liberated. The data were expressed as Sigma units per ml with reference to a standard bacterial β-glucuronidase preparation.

Microbiological assays

Total bacterial counts of buffer-diluted filtrates of cecal contents were estimated from multiple log dilution pour plates. Each aliquot was inoculated onto the following: blood agar plate; blood agar plate with phenylethyl alcohol; eosin–methylene blue; mannitol salt; and Sabaraud's with chloramphenicol. Semiquantitative estimation of the amount of each type of microorganism present was determined by intercomparison of the battery of media. Organisms were identified by current standard bacteriological techniques.

Germ-free rats

Rats were secured by Caesarean section from pregnant female Fischer strain rats and reared under sterile conditions in plastic isolators.

RESULTS

Localization in subcellular fractions of liver of enzyme converting N-OH-FAA to FAA. The enzyme activity was measured from the decrease of substrate N-OH-FAA added. In the absence of fluoride ion, the specific enzyme activity was higher in the microsomal fraction than in other subcellular elements, although the total amount of enzyme per gram of liver was higher in the soluble fraction than in the microsomes (Table 1). The decrease in substrate under these conditions represents not only dehydroxylation, but also deacylation, [15, 18–20] which can be inhibited substantially by fluoride ion. With this addition, the specific activity of the microsomal fraction was similar to that

of the soluble fraction, but the latter contained much more of the total enzyme activity, as units per milliliter.

Additional confirmation for the inhibition of deacylation was adduced from the results of TLC. FAA was the sole product visible when 0·1 M fluoride ion was added to the incubation mixture with a 10,000 *g* supernatant or a soluble fraction. By means of 9-^{14}C-labelled N-OH-FAA, it was established that the disappearance of substrate in the presence of fluoride yielded only the equivalent amounts of FAA. In the absence of fluoride, the chromatograms showed 2-fluorenamine as well as FAA, providing further evidence for a deacylase which can be inhibited by fluoride ion. With the microsome fraction alone, there was little product seen, particularly with added fluoride. Thus, in the presence of fluoride ion the reduction in the amount of N-OH-FAA added represented a measure of the *N*-dehydroxylase.

TABLE 1. METABOLISM OF N-OH-FAA BY FRACTIONS OF RAT LIVER*

	Decrease in N-OH-FAA /mg protein/30 min (nmoles)		Total activity /5 g liver (μmoles)	
Whole homogenate	13	2·4	22·3	4·1
10,000 *g* Supernatant	24	12	17·0	8·9
Soluble fraction	30	26	15·3	13·4
Microsomes	36	24	5·3	3·6

*N-OH-FAA concentration: 300 μg/ml, added in 50 μl ethanol. Reaction time, 30 min; sodium fluoride (where indicated), 0·1 M; temp, 37°. The reaction was stopped by ether extraction and N-OH-FAA was assayed by the permanganate oxidation method. Four young adult male Fischer rats were utilized. Assays were run on 1·0-ml aliquots of whole homogenate or on fractions obtained from whole homogenate with a concentration of 250 mg wet weight of liver/ml.

TABLE 2. AGE, SEX AND SPECIES DIFFERENCES IN LIVER SOLUBLE FRACTION *N*-DEHYDROXYLASE ACTIVITY*

Species	No. of animals	Age (wks)	Sex	Average decrease in N-OH-FAA (μg/ml/30 min)	Sp. act. (nmoles/mg protein/30 min)	Soluble fraction (mg protein/ml)
Rat	6	4	♂	76†	17 ± 1·5‡	19
	6	4	♀	63	14 ± 1·5	19
	6	14	♂	138	27 ± 3·8†	22
	6	14	♀	85	17 ± 3·9	21
	6	24	♂	103	21 ± 3·6	20
	6	24	♀	76	17 ± 1·6	19
Mouse	3	weanling	♂	50	15	14
Syrian hamster	6	weanling	♂	225	56 ± 1·1	17
Hartley guinea pig	6	weanling	♂	37	9 ± 0·5	18
Rabbit	3	weanling	♂	224	42 ± 5·0	20

*N-OH-FAA: 300 μg/ml starting concentration, added in 50 μl ethanol; 0·1 M NaF present in all cases; incubation time, 30 min; temp, 37°. N-OH-FAA assayed by permanganate oxidation method. TLC of ether extract of products showed only N-OH-FAA and FAA.

†Addition of cofactors (1 μmole of ATP, NAD, NADPH, G-6-P, G-6-PD per ml soluble fraction) did not increase dehydroxylation of N-OH-FAA in these preparations.

‡Standard error.

A comparison was made of the *N*-dehydroxylase activity of the soluble fraction of livers from weanling and young adult rats of both sexes. Male and female weanling rats exhibited almost the same enzyme activities. However, in young adults of two different ages, 14 and 24 weeks, males had appreciably higher levels than females (Table 2). Mouse and guinea pig fractions had lower activities and those of hamster and rabbit were higher than those of the rat.

Introduction of the substrate in ethanol solution into the soluble fraction of rat liver yielded higher levels of enzyme activity, measured by disappearance of N-OH-FAA, than when the substrate was introduced as the water-soluble sodium salt without ethanol (Table 3). These reactions were performed in the presence of 0·1 M fluoride

TABLE 3. EFFECT OF VEHICLE USED IN ADDITION OF SUBSTRATE ON *N*-DEHYDROXYLASE ACTIVITY OF SOLUBLE FRACTION OF RAT LIVER*

Group	Conditions	Decrease in N-OH-FAA/ml/30 min (nmoles)	Specific enzyme activity (nmoles/mg/ml/hr)
I	N-OH-FAA in 50 μl EtOH	490	64
II	N-OH-FAA in 100 μl 0·1 N NaOH, 50 μl EtOH added	471	60
III	N-OH-FAA in 100 μl 0·1 N NaOH	144	19

*The protein concentration was 15·2 mg/ml; 0·1 M NaF was present. The decrease in substrate was measured after incubation at 37° for 30 min, with substrate added in ethanol (EtOH), in 0·1 M NaOH with ethanol added separately or in NaOH alone.

so that the reaction measured was chiefly the dehydroxylase activity. Also, upon addition of ethanol, supplements of NADPH as cofactor did not further increase the specific or total enzyme activity in the soluble fraction of rat liver. Thus, the soluble fraction of livers from 4 male weanling rats reduced 60 μg N-OH-FAA in 30 min per ml of soluble fraction of liver (200 mg wet weight or 19 mg protein). In the presence of 1 μmole per ml of ATP, NADH, NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase, the amount reduced under similar conditions was 57 μg. Likewise, with young adult rats the corresponding values were 111 and 110 μg. These results suggest that ethanol stimulated the production of NADPH in the soluble fraction. In all these tests (fluoride present) thin-layer chromatograms showed only N-OH-FAA, the starting material, and FAA, the dehydroxylated product, and no 2-fluorenamine.

Presence of N-dehydroxylase activity in the microflora of intestinal tract. Incubation of N-OH-FAA with cecal contents of conventional rats led to a progressive reduction in the amount of substrate over a 24-hr period (Fig. 2). Thin-layer chromatography showed that the only product was FAA, in the presence or absence of fluoride ion. Thus, the decrease in the amount of N-OH-FAA represented mainly dehydroxylase. In contrast, there was no disappearance of substrate with cecal contents of germ-free

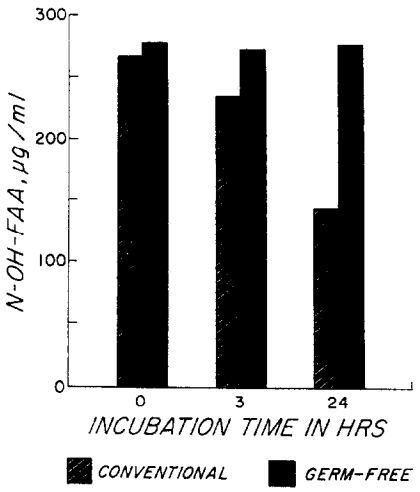

FIG. 2. Dehydroxylation of N-OH-FAA by cecal contents of conventional and germ-free rats. Samples were withdrawn at 0, 3 and 24 hr and extracted with ether for assay of remaining N-OH-FAA. Temp 30°. No fluoride was added. Note absence of reductive activity in cecal contents of germ-free rats.

TABLE 4. EFFECT OF STARTING CONCENTRATION OF SUBSTRATE ON RATE OF METABOLISM BY RAT CECAL CONTENTS*

Starting concn. N-OH-FAA (μg/ml)	N-OH-FAA metabolized (μg)
168	154
187	86
283	42

*Temperature, 30°; incubation period 4 hr. Note inverse relationship between starting concentration of N-OH-FAA and rate of metabolism.

Because of apparent inhibition by substrate of the reductive enzyme system and the consequent lag period, data on linearity versus enzyme concentration cannot be generated. Under our conditions with a 4:1 dilution of cecal contents, a starting concentration of about 170 μg/ml of N-OH-FAA led to a minimal lag period and yielded substrate decreasing as a function of time. Bacterial composition of microbial flora in cecum, and thus enzyme activity, depended in part on diet of rats used.

rats. We concluded that the enzyme activity noted resided in the microflora of the gut in the conventional rats.

As had been found with the parent compound, 2-fluorenamine,[21–23] N-OH-FAA did, however, appear to exert a bacteriostatic effect in high concentrations. Thus, with larger amounts of material present initially, the amount of N-OH-FAA metabolized in 4 hr was decreased (Table 4). With 168 μg/ml of N-OH-FAA, complete reduction

took place in 6–10 hr, but with higher concentrations longer periods of incubation were required (see also Fig. 2).

Examination of the type and quantity of microorganism present at various times during the incubation with 168 μg/ml of N-OH-FAA initially revealed no change in the type or in the number of organisms present over the first 6 hr after incubation (Table 5).

Incubation in the presence of substrate led to an increasing rate of *N*-dehydroxylase activity when additional substrate was introduced at subsequent time intervals (Fig. 3). A pure strain of *E. coli* developed the capability to convert N-OH-FAA to FAA, but prolonged incubation was also required (Table 6).

TABLE 5. BACTERIOLOGICAL STUDIES AT VARIOUS TIMES DURING DEHYDROXYLATION

Incubation time (hr)	N-OH-FAA (μg/ml)	Colony count* (bacteria/ml)
0	168	39×10^6
3	48	
4	14·5	30×10^6
6	0	32×10^6

*No significant changes in species were observed. Temp, 30°. Fluoride was not present.

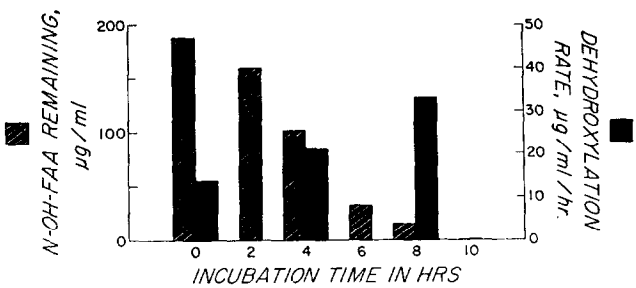

FIG. 3. Dehydroxylation of N-OH-FAA by buffered filtrate of rat cecal contents at 30°. Substrate was reintroduced at 0, 4 and 8 hr after ether extraction for measurement of 1-hr rate changes. Note progressive increase in 1-hr rate of dehydroxylation as exposure time increases.

TABLE 6. DEHYDROXYLATION OF N-OH-FAA BY *E. coli* (STRAIN *K*-32) AT 30°*

Time (hr)	N-OH-FAA remaining (μg/ml)
0	109
1	96·5
3	80
5	80
24	25

* *E. coli* on tryptone broth were incubated in presence of N-OH-FAA for 24 hr. Samples were withdrawn periodically and assayed for remaining N-OH-FAA. Fluoride was not present.

β-Glucuronidase in microorganisms of intestinal tract. Inasmuch as the main metabolite of N-OH-FAA produced in the liver and secreted in the bile is the corresponding glucosiduronic acid, the presence of β-glucuronidase in the microflora of the gut was assessed. Hydrolysis of the β-glucuronide linkage would make available free N-OH-FAA as substrate for the subsequent action of *N*-dehydroxylase. Determination of β-glucuronidase, utilizing phenolphthalein glucuronide as substrate, showed that the cecal contents of conventional rats possessed appreciable β-glucuronidase activity with an apparent optimum activity at pH (Fig. 4). Under the same conditions, germ-free rats exhibited much lower β-glucuronidase activity with an optimum at

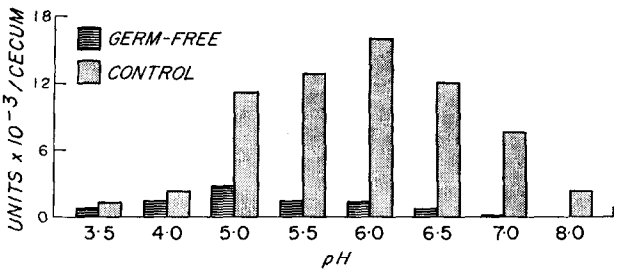

FIG. 4. β-Glucuronidase activity in cecum of germ-free or conventional control rats. Cecum contents diluted with phosphate buffer at different pH values and filtered through cheesecloth. Substrate, phenolphthalein glucuronide, incubation time, 30 min; temperature 37°.

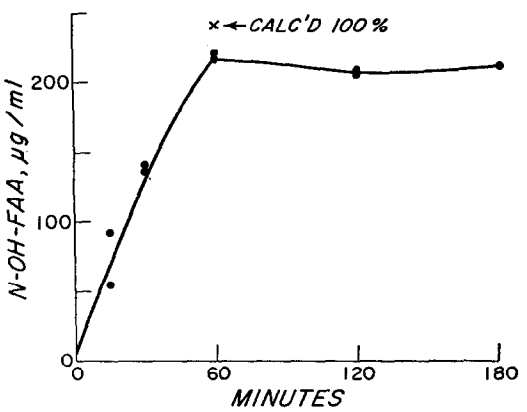

FIG. 5. Hydrolysis of N-OH-FAA glucuronide by rat cecal contents at 30°. Aliquots were extracted with ether and assayed for liberated N-OH-FAA by the permanganate oxidation method.

pH 5.[9] In conventional rats, therefore, the activity is most likely due to bacterial enxyme whereas in the germ-free rats the pH optimum was characteristic of that of the mammalian enzyme.

Rat cecal contents readily hydrolyzed the glucosiduronic acid of N-OH-FAA giving nearly the theoretical amount of product (Fig. 5).

The enzymes of present concern, namely β-glucuronidase and *N*-dehydroxylase, were limited chiefly to the cecum. There were no detectable levels of these enzymes in the upper, middle and lower third of the small intestine.

TABLE 7. INFLUENCE OF INTESTINAL FLORA ON THE METABOLISM OF N-OH-FAA DURING CHRONIC FEEDING IN THE PRESENCE AND ABSENCE OF NEOMYCIN AND/OR CARCINOGEN*

		Cecal contents					
Diet†	Day	Dehydroxylation of N-OH-FAA (μg/ml/4 hr)	β-Glucuronidase (Sigma units/ml)	Approximate colony count (organisms/ml × 10^6)	Predominant organisms	Liver dehydroxylase (nmoles/ml/30 min)	24-hr Urinary N-OGl-FAA‡ (% of dose)
K	0	170	5600 ± 60	2·6	Coliforms, yeast, strep	4·8 ± 0·4	1·8 ± 0·1
K	7	120	4800 ± 300	3·3	Coliforms, strep	6·5 ± 1·3	2·9 ± 0·1
K	14	15	3800 ± 100	0·75	Coliforms, strep, pseudomonas	7·4 ± 0·7	8·4 ± 1·8
K	21	57	3900 ± 40	0·25	Coliforms, yeast, pseudomonas	7·2 ± 0·2	12·5 ± 3·7
K	28	68	1600 ± 10	0·29	Coliforms, strep	11·5 ± 0·7	19·4 ± 4·7
K	42	110	1800 ± 200	0·56	Yeast	7·4 ± 0·0	18·4 ± 7·6
K	56	120	6060 ± 400	0·38	Yeast, coliforms, strep	3·8 ± 1·8	11·3 ± 1·3
K	140		1100 ± 20			2·1 ± 1·0	
KN	7	150	200 ± 10	3·2	Yeast	6·7 ± 0·7	3·3 ± 1·3
KN	14	90	400 ± 10	1·0	Yeast	6·5 ± 0·9	6·6 ± 0·9
KN	28	100	850 ± 10	0·2	Coliforms, proteus, strep	14 ± 2·2	11·0
A	14	165	5560 ± 60	2·6	Coliforms, yeast, strep, proteus	4·8 ± 0·4	1·76 ± 0·1
A	28	83	5520 ± 60	2·4	Coliforms, yeast strep, proteus	8·2 ± 1·9	
AN	7	168	264 ± 10	4·0	Yeast	13·7 ± 4·0	2·6 ± 0·2
AN	21	111	865 ± 30	4·0	Yeast	6·4 ± 1·3	2·4 ± 0·1

* Groups of randomized animals on various diets were sacrificed at intervals, 24 hr after i.p. injection with N-OH-FAA-9-^{14}C (20 mg/kg). Cecal contents were studied for dehydroxylation of N-OH-FAA (incubation for 4 hr), β-glucuronidase activity and composition of microflora. Urinary metabolites were determined by our standard methods.[8, 11, 14] Each value represents the average of determinations on 4 animals (2 groups of 2 each), with the exception of the KN-28 group.

† Diet A, semipurified control diet; K, diet containing 160 ppm of N-OH-FAA; AN, 1000 ppm of Neomycin sulfate; KN, 160 ppm of N-OH-FAA plus 1000 ppm of Neomycin sulfate.

‡ N-OGl-FAA is glucosiduronic acid of N-OH-FAA.

Changes in microflora and enzyme activity in cecum and carcinogen metabolism during chronic N-OH-FAA intake. Examination at weekly intervals of the number and type of microorganisms present during feeding of a diet containing 160 ppm of N-OH-FAA showed a progressive decline beginning at the second week (Table 7). After the fourth week, the organisms present were chiefly yeast and there was virtually complete disappearance of coliform type bacilli.

β-Glucuronidase also tended to decrease to a minimum after approximately 4 weeks. There appeared to be a resurgence at the eighth week, but its significance is not clear. After 20 weeks there was relatively little β-glucuronidase activity.

N-dehydroxylase levels decreased to a minimum after 2 weeks, after which there was a slight increase. The heightened activity may have been due to the presence of yeast type organisms.

N-dehydroxylase in the soluble fraction of liver during the entire feeding period underwent only minor alterations, with an apparent peak 4 weeks after initiation of the chronic N-OH-FAA intake. At the latter time periods, the activity was below that of untreated control rats, possibly owing to morphological changes seen in the liver at that time. After the eighth week on carcinogen diet, there were progressive development of cirrhosis, hydropic degeneration and hyperplastic areas and nodules.

Simultaneous analysis of the 24 hr urinary metabolites revealed a continuing increase in the amount of N-OH-FAA glucuronide excreted in urine. Whereas only 1·8 per cent of the dose was present in urine as this metabolite at the beginning of the feeding period, it amounted to almost 20 per cent by the fourth week, as already reported.[1, 11]

Effect of chronic Neomycin and N-OH-FAA intake on cecal and liver enzyme activities and on urinary metabolites of the carcinogen. Examination of the microbial flora in the ceca of rats fed diet AN with 1000 ppm of Neomycin for 7 days revealed no bacterial growth in the standard microbiologic assay after 24 hr of incubation, although at 48 hr substantial growths of yeast were noted (Table 7). After 21 days on the Neomycin diet, 4×10^6 organisms per ml, predominantly yeast cells, were counted. In agreement with these findings, β-glucuronidase was very low, but *N*-dehydroxylase was present in the cecal contents and also in the soluble fraction of liver. After 7 and 21 days on the Neomycin diet, the level of urinary N-OH-FAA glucuronide was only slightly changed from that of 0 day or diet A controls.

Administration of a combination of Neomycin and N-OH-FAA (diet KN) also reduced the number of microorganisms in the cecum and gave a qualitatively different population of cells, consisting chiefly of yeasts. The cecal β-glucuronidase was low at all three time periods examined, namely on days 7, 14 and 28. The *N*-dehydroxylase was decreased from control levels at the later time periods.

Urinary excretion of N-OH-FAA glucuronide increased progressively but did not reach the same levels, in percentage of dose, as compared to rats fed carcinogen alone (diet K).

DISCUSSION

The impetus to this series of studies was the report that after a single i.p. dose of FAA an appreciable amount of the activity in bile was in the form of the glucuronide of N-OH-FAA. Also, after a dose of N-OH-FAA, virtually the sole metabolite

excreted in bile was the glucuronide.[7] On the other hand, after a single dose of either compound, only a small amount of N-OH-FAA glucuronide was found in urine. It seemed, therefore, that the metabolite in bile underwent further changes in the gut. In a separate paper it is reported that germ-free rats injected with N-OH-FAA did excrete larger amounts of N-OH-FAA glucuronide than did conventional controls.[9]

The techniques generally used to study the metabolism of N-OH-FAA, namely solvent partitions and paper, thin-layer and column chromatography, spectrophotometric and isotopic analyses, were too cumbersome for convenient study of the effect of mammalian and bacterial enzymes on N-OH-FAA. Thus, a procedure was developed which was amenable to serial analysis of many samples in a reasonable time. It is based on the oxidation of N-OH-FAA to an intermediate which reacts with trisodium pentacyanoamino ferrate in a linear fashion over a range of at least 5–100 μg/ml of N-OH-FAA. Stepwise examination of the reaction indicated that a yield of about 35 per cent of 2-nitrosofluorene, as established by thin-layer chromatography, resulted from the permanganate oxidation step, although other products such as azoxyfluorene were present.

By means of this assay procedure, it was shown that the bacterial flora in the intestinal tract contained both a glucuronidase which hydrolyzed the glucosiduronic acid of N-OH-FAA and also an enzyme which could reduce N-OH-FAA to FAA. The relationship of the latter enzyme and the known hydroxylamine reductases[24–27] or the connection between this type of bacterial enzyme and the corresponding liver enzyme, which can also reduce N-OH-FAA to FAA, remains to be established. There are cofactor requirements for the enzymes which appear to be identical, i.e. they need NADPH. The cofactor itself does not reduce N-OH-FAA. Additional comparative studies will be necessary.

There are other reductive enzymes which bear relationship to *N*-dehydroxylase. For example, certain microorganisms can reduce an activated carbon-bound hydroxy group, like that present in caffeic acid[28, 29] and in proto- and homoprotocatechuic acid.[30, 31] In addition there are systems which reduce azo dues,[32, 33] although their cofactors are based on flavin adenine dinucleotide. The intestinal enzymes of this class may well play an insufficiently recognized role in the detoxification of carcinogenic as well as non-carcinogenic azo dyes.[34–39] For example, Childs *et al.*[37] showed that subcutaneous injection of an azo dye, 1-phenylazo-2-naphthol, led to liver tumours. However, oral intake gave none, suggesting a rapid detoxification, presumably by reduction of the azo dye.

During chronic feeding of N-OH-FAA, there was a progressive increase in urinary excretion of the glucuronide of N-OH-FAA.[1, 11] It has been proposed that possibly increasing liver damage led to progressively lower elimination of metabolites in the bile.[1, 7] In turn, this would lead to higher blood levels and consequent elimination via the kidneys. In addition to strict morphological evidence in favour of this thesis, it has been demonstrated that complete abolition of clearance through the bile led to an increased elimination of N-OH-FAA glucuronide in urine.[7] It has not been shown that biliary excretion of N-OH-FAA was reduced or lacking during chronic feeding of N-OH-FAA. If such were the case, then other glucuronides might likewise be excreted in lesser amounts in the bile. When urinary N-OH-FAA glucuronide was elevated at 42 days, we found 0·1 mg per 100 ml of bilirubin glucuronide in the serum and no free bilirubin. Thus, by this criterion there appeared to be no alteration in

the excretion of bilirubin metabolites in bile or in the ability of the liver to conjugate bilirubin.

There may be participation by factors other than the partition between bile and blood. Larger molecular weight compounds such as fluorene derivatives are more likely to be secreted in bile than are derivatives of acetanilide or 4-acetylaminobiphenyl (4-phenylacetanilide).[40–42] Interestingly, there was no increase in the urinary excretion of the glucuronide of *N*-hydroxy-4-acetylaminobiphenyl upon chronic intake of 4-acetylaminobiphenyl.[43] Thus, with the bacteriostatic fluorene derivatives [21–23] being secreted in the bile of rats in substantial amounts, there was a shift in intestinal flora, which in turn affected the metabolic pattern of the carcinogen during continuing feeding. There was virtually complete loss of coliform type organisms from the cecum, which resulted in a substantial alteration in the enzymic potential of the microflora in the gut. These changes may well play a role in the increased excretion of the glucuronide of N-OH-FAA in urine upon chronic intake of N-OH-FAA. Since there is less destruction of the glucuronide in the gut, it was necessary to demonstrate also that this material can be resorbed. By sampling the portal vein blood we have satisfied ourselves that the glucuronides of phenolphthalein and N-OH-FAA are absorbed after their intraduodenal administration. It is not known whether these glucuronides traverse intestinal membranes by an active transport mechanism or by passive diffusion. The latter process is more probable for free, lipophilic compounds such as N-OH-FAA or FAA.

The data obtained when the gut flora was altered by the administration of the intestinal antibiotic, Neomycin, suggest that the mechanism of the increased excretion of N-OH-FAA glucuronide during continuing feeding of the carcinogen is actually rather complex. Thus, whereas Neomycin feeding changed the microflora in the cecum resulting in low levels of β-glucuronidase, the excretion of N-OH-FAA glucuronide was scarcely changed. In part, this might have been due to a problem of absorption of the glucosiduronic acid from the gut. Neomycin does alter the permeability of the intestinal membranes to certain materials.[44] When the diet included not only Neomycin but also N-OH-FAA, there was a similar rapid alteration of the intestinal flora and consequent change in enzyme composition, particularly with respect to β-glucuronidase. Yet, in this case there was some increased excretion of N-OH-FAA glucuronide in urine, although at the 4-week period it was not as high as was seen in the absence of Neomycin. The entire question will require further study, especially as regards: (1) the possible increased level of hydroxylating and conjugating systems in the liver; and (2) the clearance of metabolites from the liver of rats via blood or bile under chronic conditions.

In conclusion it would appear that the intestinal microflora participates to a significant degree in the metabolic pattern of N-OH-FAA. After oral intake the free compound probably enters the enterohepatic circulation by absorption from the proximal portion of the small bowel. Metabolic conversion occurs in the liver, particularly conjugation to the corresponding glucuronide, N-OGl-FAA, which is concentrated and secreted in bile, thus finding its way back to the small intestine. During its transit toward the cecum, a portion of the glucuronide is probably reabsorbed from the small intestine and thus is recycled. A fraction of this material is cleared by the kidneys. Little, if any, glucuronide hydrolysis occurs in the small intestine. However, in the cecum, N-OGl-FAA is rapidly hydrolyzed by bacterial

β-glucuronidase, resulting in N-OH-FAA which is in turn reduced by bacterial *N*-dehydroxylase to FAA, the major fecal metabolite. Either N-OH-FAA or FAA can be absorbed from the cecum, entering the portal blood for further hepatic metabolism and recirculation. A small fraction of the free compound is cleared by the kidneys, thus entering the urine.

Consideration of the dynamics of drug–host interaction in this case must include the fact that the host depends in part upon its intestinal microflora for detoxification of the carcinogenic metabolite, N-OH-FAA. Furthermore, this compound exerts a toxic effect on the very bacterial populations responsible for production of metabolizing enzymes, particularly the production of β-glucuronidase by coliforms.

ADDENDUM

Additional papers dealing with the metabolism of exogenous compounds by intestinal microflora have appeared recently.[45–48] A review by Dacre is in press.[49]

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