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Metabolism of the Carcinogen 2-Acetylaminofluorene by Rainbow Trout.* (31690)

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2-Acetylaminofluorene (AAF) is a carcinogen of wide species and tissue range(1). In most species AAF is metabolized extensively to monophenolic derivatives which have not been found to be carcinogenic(1-4). In the susceptible species which have been studied AAF is also *N*-hydroxylated (reviewed in 5, 6). This metabolic reaction converts AAF to *N*-hydroxy-AAF which is more carcinogenic than the parent amide in the rat, mouse, and hamster. Furthermore, *N*-hydroxy-AAF is carcinogenic in the guinea pig while the amide is not. The guinea pig apparently can not *N*-hydroxylate AAF(2), although Kiese *et al* (7) report that it does form the carcinogen *N*-hydroxy-2-aminofluorene from 2-aminofluorene (AF) which is not carcinogenic in this species(8).

Recently Halver(9) observed a low incidence of liver tumors when rainbow trout (*Salmo gairdnerii*) were fed AAF as 0.015 or 0.060% of the dry diet. Surgical examination indicated the presence of gross liver tumors in 6 of 101 fish fed the lower dose and 7 of 99 fed the higher dose for 1 year. In 2 cases these tumors were confirmed histologically as trabecular parenchymal cell carcinomas. The present studies were undertaken to investigate the metabolism of AAF in this species. Metabolism to the 5- and 7-hydroxy derivatives was observed both *in vivo* and with liver preparations *in vitro,* but no *N*-hydroxylation was demonstrated. The ring-hydroxylation of AAF by trout liver was in contrast to the earlier suggestion of Brodie and Maickel(10) that fish and amphibia are unable to metabolize foreign compounds, but

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consistent with recent reports by Creaven *et al*(11) and Buhler(12) on the hydroxylation of biphenyl and aniline by trout livers.

Materials and methods. Male rainbow trout (*Salmo gairdnerii*), about 2 years old and weighing 300-400 g, were administered AAF (Mann Research Laboratories, New York), 7-fluoro-AAF (kindly supplied by Dr. T. Lloyd Fletcher, Dept. of Surgery, University of Washington Medical School, Seattle), or *N*-hydroxy-AAF(13) synthesized in this laboratory. These compounds (10-30 mg) were administered orally in a gelatin base diet(14) or by intraperitoneal injection. Injections were either made by direct insertion of crystals through an incision in the body wall or by injection of a suspension in corn oil. Urine (by cannulation(15)) and feces were collected separately for 72 hours; the samples were collected under toluene and frozen immediately after collection at the Western Fish Nutrition Laboratory for transport to the University of Wisconsin.

For analysis(16) urine or feces samples were incubated with β-glucuronidase and Taka-diastase, after which the ether-extractable material was washed successively with 10% Na_2CO_3(17), water, 0.5 N HCl and water, and then chromatographed on Whatman No. 1 filter paper. After elution with ethanol the ultraviolet spectra of the metabolites were determined from 270-350 mμ with a Beckman model DB spectrophotometer equipped with a recorder.

For *in vitro* studies rainbow trout were obtained locally through the courtesy of the Madison Fish Hatchery of the Wisconsin Conservation Commission. Ten percent homogenates were prepared from the pooled livers from 4-6 fish in ice-cold 0.25 M sucrose solution. For one experiment the microsomal pellet was obtained by sedimenting the mitochondrial supernatant (obtained at 8000 × *g*) at 105,000 × *g* for 60 minutes. The surface of the microsomal pellet was washed twice with 0.25 M sucrose solution before it was resuspended in the same medium. The incubation medium was identical to that described by Cramer *et al*(18) for ring-hydroxylation of AAF except that KF to a final concentration of 0.1 M was added where indicated. DPNH and TPNH (500 μg of each per flask) were substituted for DPN and TPN in the experiment with liver microsomes. The reaction flasks were incubated in air at 15° or 37°C for 20 minutes, after which the flasks were chilled and 4 ml of ice-cold 1 M sodium acetate buffer (pH 6) was added per flask. The contents of 36 flasks were combined for each analysis, the medium was extracted immediately with ethyl ether, and the ether extract was fractionated into acidic, basic and neutral fractions by extractions with acid and alkali. The acidic fraction was chromatographed on Whatman No. 1 paper with solvent systems composed of cyclohexane:t-butanol:acetic acid:water in ratios of 16:4:2:1(19) or 18:2:2:1 by volume. The AF in the basic fraction was quantitated directly by spectrophotometry.

All of the metabolic data presented in this paper were corrected by the following recovery data obtained by analyzing the compounds added to normal urine samples: *N*-hydroxy-AAF, 75%; 5-hydroxy-AAF, 96%; 7-hydroxy-AAF, 63%; AF, 100%. Since about 15% of *N*-hydroxy-AAF is reduced to AAF during chromatography, the AAF analyses were corrected by deducting 15% of the corrected *N*-hydroxy-AAF excretion; the recovery of added AAF is 100%.

Results. The detectable urinary metabolites of AAF and *N*-hydroxy-AAF accounted for 0.4-3% of the dose; smaller amounts were excreted in the feces, except that 15% of orally administered AAF or *N*-hydroxy-AAF was excreted as such (Table I). 7-Hydroxy-AAF, 5-hydroxy-AAF, and AAF were excreted both in the urine and feces of trout administered AAF orally or by injection; the urinary excretion of 7-hydroxy-AAF was about 10 times that of 5-hydroxy-AAF. No *N*-hydroxy-AAF was detected when AAF was administered. When *N*-hydroxy-AAF was administered, *N*-hydroxy-AAF, 7-hydroxy-AAF, and, in one case, 5-hydroxy-AAF were excreted in the urine and feces. The levels of the phenolic derivatives were lower when *N*-hydroxy-AAF was administered rather than AAF. 3-Hydroxy-AAF (0.1% of the dose) was detected in the urine of one fish which was injected intraperitoneally with 10

mg of AAF; 1-hydroxy-AAF was not detected in any of the excreta. In all cases the hydroxylated metabolites were excreted primarily as conjugates released by β-glucuronidase and Taka-diastase. As indicated by the standard deviations the variations in the levels of metabolites excreted by similarly treated fish were large.

Feeding or injection of 7-fluoro-AAF did not yield detectable amounts of *N*-hydroxy or phenolic metabolites in the urine or feces.

Trout liver homogenates or hepatic microsomes formed similar amounts of 7-hydroxy-AAF to those produced by rat liver homogenates(18); 5-hydroxy-AAF, when it could be detected, was formed in smaller amounts (Table II). In the absence of KF a large amount of AAF was deacetylated to AF; 0.1 M KF inhibited this reaction by 90-95%. No *N*-hydroxylation of AAF was detected.

Discussion. The data reported here together with the recent data of Creaven *et al* (11) and Buhler(12) clearly demonstrate that trout liver contains at least some of the microsomal oxygenases which have been widely studied in mammalian species. In fact *in vitro* the formation of 7-hydroxy-AAF is

TABLE I. Urinary and Fecal Metabolites of AAF and *N*-Hydroxy-AAF in the Rainbow Trout.

Treatment	Sample	Excretion (% of dose)*			
		N-hydroxy-AAF	5-hydroxy-AAF	7-hydroxy-AAF	AAF
AAF fed	Urine(6)†	n.d.‡	.21 ± .20	3.0 ± 2.1	.09 ± .08
" "	Feces(3)	"	.10 ± .10	.40 ± .40	14 ± 6.3
" injected	Urine(8)	"	.16 ± .09	2.1 ± .98	.03 ± .02
" "	Feces(6)	"	.20 ± .17	.28 ± .23	.07 ± .07
N-hydroxy-AAF fed	Urine(6)	.16 ± .16	n.d.§	.10 ± .06	.06 ± .04
Idem	Feces(1)	15.2	.02	.06	.25
N-hydroxy-AAF injected	Urine(3)	1.2 ± .87	n.d.	.57 ± .29	.09 ± .05

* Samples were collected for 72 hr after administration of 10-30 mg of compound.
Values expressed as mean ± standard deviation.
† Numbers in parentheses denote total No. of fish studied; 1 collection was made from each fish.
‡ n.d. signifies that the compound could not be detected. A small amount of ultra-violet light-absorbing material with a non-characteristic spectrum was usually eluted from the paper and would have obscured the presence of less than 0.08% of *N*-hydroxy-AAF or 0.01% of 5-hydroxy-AAF.
§ Of 6 fish 1 excreted 0.02% of the dose as 5-hydroxy-AAF; none could be detected in the urine of the other 5 fish.

TABLE II. Metabolism of AAF by Rainbow Trout Liver Homogenate and Microsome Preparations.

Exp No.	Preparation	Incubation temp	0.1 M KF	mμmoles of metabolite/g liver/20 min*			
				AF	*N*-hydroxy-AAF	5-hydroxy-AAF	7-hydroxy-AAF
1	Homogenate	37°C	—	1700	n.d.	n.d.	110
	"	"	+	57	"	"	73
	Microsomes	"	—	340	"	"	36
	"	"	+	37	"	"	33
2	Homogenate	"	+	57	"	5	67
	"	15°C	+	27	"	7	110

* In all cases 36 identical flasks, each of which contained 30 mg of liver or equivalent amounts of microsomes and 225 mμmoles of AAF, were incubated under each condition. In Exp 1 the metabolites from 30 flasks were chromatographed in cyclohexane:*t*-butanol:acetic acid:water (18:2:2:1 by volume) for optimal resolution of *N*-hydroxy-AAF and the contents of 6 flasks were chromatographed in a mixture of the same solvents in a ratio of 16:4:2:1 for optimal resolution of 5- and 7-hydroxy-AAF. In Exp 2 the metabolites from 36 flasks were chromatographed together in the 18:2:2:1 system, and the metabolites in the 5- and 7-hydroxy-AAF region were then rechromatographed in the 16:4:2:1 system. Under these conditions the least amount of *N*-hydroxy-AAF which gave a definitive spectrum was 5-10 mμmoles/g liver/20 minutes; for 5-hydroxy-AAF the least detectable amounts were 5 and 1-2 mμmoles/g liver/20 minutes for Exp 1 and 2, respectively. The abbreviation n.d. (not detected) indicates that a metabolite could not be definitively estimated and characterized, even though a low amount of ultra-violet light absorbing material with non-characteristic spectrum was usually eluted from the paper chromatograms.

similar to that of rat liver homogenates(18).

Our inability to demonstrate the formation of *N*-hydroxy-AAF *in vivo* is consistent with the low carcinogenicity of AAF in the rainbow trout. However, in view of the low recoveries of the administered compounds as known metabolites in the urine and feces of the trout, these data do not preclude the excretion of *N*-hydroxylated metabolites in other forms or through another excretory path. Likewise, the inability of the trout liver preparations to *N*-hydroxylate AAF *in vitro* in these studies is difficult to interpret. Even in the rat and rabbit, species which excrete large amounts of *N*-hydroxy-AAF in the urine(16,17), this reaction proceeds poorly *in vitro* with liver preparations(20).

Summary. Administration of the carcinogen 2-acetylaminofluorene (AAF) orally or by injection into rainbow trout resulted in the excretion of 5- and 7-hydroxy-AAF in the urine and feces. These metabolites were also formed *in vitro* from AAF by trout liver homogenates or the microsomal fraction in the presence of TPNH or a TPNH-generating system. *N*-Hydroxylation of AAF was not demonstrated either *in vivo* or *in vitro*.

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Effect of Cocaine on Vagal Escape in the Open-Chest Dog.* (31691)

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It has been well established that cocaine can potentiate the cardiovascular responses to exogenously administered norepinephrine(1). Recent studies made in this laboratory have shown that cocaine can potentiate significantly the cardiac responses to endogenous norepinephrine released both by submaximal and supramaximal stimulation of the cardiac sympathetic nerves(2). The present investigation was conducted to explore further the

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