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Uptake by Dietary Exposure and Elimination of Aflatoxins in Muscle and Liver of Rainbow Trout (*Oncorhynchus mykiss*)

Hirotaka Nomura,[†] Motoki Ogiso,[‡] Michiaki Yamashita,[§] Hiroshi Takaku,[†] Ayako Kimura,[‡] Masato Chikasou,[‡] Yukio Nakamura,[†] Seiji Fujii,[†] Masatoshi Watai,[‡] and Hisashi Yamada*[,†]

[†]Marine Ecology Research Institute, Towaedogawabashi Building, 347 Yamabukicho, Shinju-ku, Tokyo 162-0801, Japan

[‡]Tama Laboratory, Japan Food Research Laboratories, 6-11-10 Nagayama, Tama, Tokyo 206-0025, Japan

[§]National Research Institute of Fisheries Science, Fisheries Research Agency, 1-12-4 Fukuura, Kanazawa, Yokohama 236-8648, Japan

ABSTRACT: Uptake and elimination of aflatoxins (AFs) by rainbow trout (*Oncorhynchus mykiss*) during a long-term (21 days) dietary exposure were studied to assess contamination by AFs in aquaculture fish fed AF-containing feed. The uptake factor (UF) of aflatoxin B_1 (AFB_1) in muscle ranged from 0.40×10^{-3} to 1.30×10^{-3}. AFB_1 concentrations in liver were 165−342 times higher than in muscle. AFs from feed were more highly accumulated in liver than in muscle. Aflatoxicol (AFL) and aflatoxin M_1 (AFM_1) were detected in muscle and liver and also in the rearing water. AFL concentrations were higher than AFM_1 by 2 orders of magnitude in muscle, and AFL was a major metabolite of AFB_1. The elimination rate constants (α) of AFB_1 and AFL in muscle (1.83 and 2.02 day^{-1}, respectively) and liver (1.38 and 2.41 day^{-1}, respectively) were very large. The elimination half-life ($t_{1/2}$) of AFB_1 was 0.38 days (9.12 h) in muscle and 0.50 days (12.00 h) in liver. The elimination half-life of AFL in muscle and liver was 0.34 day (8.16 h) and 0.29 day (6.96 h), respectively. These data show that AFs are eliminated rapidly and are not biomagnified in fish. Thus, AFB_1 concentration in muscle of fish fed AFB_1-containing feed (ca. 500 μg/kg) decreased to below the detection limit (20 ng/kg) of the most sensitive analytical method at 1.54 days (36.96 h) after the change to uncontaminated feed.

KEYWORDS: aflatoxins, rainbow trout, aquaculture, liver, muscle, aflatoxicol, aflatoxin B_1

INTRODUCTION

Aflatoxins (AFs) are potent toxins produced as secondary metabolites by fungi of the genus *Aspergillus*. With regard to carcinogenesis, a mixture of naturally occurring AFs (AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2)) is classified as group 1 (carcinogenic for human), and AFM_1 (metabolite of AFB_1) is classified as group 2B (possibly carcinogenic for human) by the International Agency for Research on Cancer (IARC). Halver[1] reported that AFB_1 and AFG_1 caused hepatomas to develop in rainbow trout during long-term (1 year) dietary exposure experiments.

Fungi of the genus *Aspergillus* proliferate on the surface of grain and seeds, such as corn, rice, wheat, and peanuts, and produce AFs on the surface of these materials. If contaminated grain is present in formula feed for aquaculture fish, there is a fear that AFs will be transferred to the fish and produce harmful effects.

Several papers have investigated the harmful effects of AFs in fish species. Acute toxicity was studied by several researchers,[1−3] and a 10 day median lethal dose (10 d-LD_{50}) was determined. These data showed that the toxicity of AFs was especially high in rainbow trout. Metabolism and excretion of AFs were studied by Loveland et al.,[4] Plakas et al.,[5] Toledo et al.,[6] Troxel et al.,[7] and Shelton et al.[8] during short-term experimental periods after a single dose by oral or intraperitoneal administration. They clarified that AFB_1 is metabolized to aflatoxicol (AFL) and eventually excreted in the rearing water. Carcinogenesis mechanisms were studied by Lee et al.,[9] and Couch and Harshbarger[10] examined carcinogenic potencies of AFs to fish species by reviewing published research. However, uptake of AFs in fish from contaminated feed (ratio of AF concentration in fish to concentration in feed) has not been fully clarified by rearing experiments in which the experimental fish are fed AF-containing feed during long-term exposure. The elimination of AFs from fish after dietary exposure has also not been studied over an extended time period. From the information available in the literature, it is considered that the characteristics of oral uptake and elimination of AFs from fish species have not been determined in sufficient detail.

In this study, changes in AF concentrations in fish (muscle and liver) were studied over a long-term (21 days) rearing experiment using rainbow trout (*Oncorhynchus mykiss*) to determine the extent of uptake (uptake factor, UF), elimination rate constant, and elimination half-life. On the basis of data from the present study, we consider measures against the contamination of aquaculture fish by aflatoxins.

MATERIALS AND METHODS

Chemicals. AFB_1, AFB_2, AFG_1, and AFG_2 for chemical analysis reference were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), as were AFM_1 and AFL. The purity of AFB_1, AFB_2, AFG_1, and AFG_2 was >99.0%, and these chemicals were used without further purification.

Test Fish. Juvenile rainbow trout (*O. mykiss*) were purchased from Fuji Trout Hatchery (Fujinomiya, Japan). They were spawned, hatched artificially, and fed formula feed (Nisshin-Marubeni, Tokyo, Japan; type 2P)

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Table 1. Rearing Experiments of Rainbow Trout Fed Feed Containing AFs

			av fish wt (g)		
expt	AF	AF concn in feed (μg/kg)	initial	after	exptl period (days)
A		<1[a]	13.99 ± 2.60 (n = 36)	18.97 ± 3.33 (n = 3)	23
B	AFB_1	115 ± 1[b]	14.13 ± 2.98 (n = 36)	18.74 ± 3.90 (n = 27)	uptake, 21; elimination, 2
C	AFB_1	347 ± 4	13.76 ± 3.03 (n = 36)	17.94 ± 3.82 (n = 26)	uptake, 21; elimination, 2
D	AFB_1	1190 ± 10	14.15 ± 2.82 (n = 36)	18.80 ± 3.89 (n = 24)	uptake, 21; elimination, 2
E	AFB_1	6276 ± 72	13.08 ± 1.47 (n = 18)	14.7 ± 1.71 (n = 19)	uptake, 7
F	AFB_1	219 ± 7	14.46 ± 2.15 (n = 18)	15.7 ± 2.32 (n = 17)	uptake, 7
	AFB_2	306 ± 4			
	AFG_1	321 ± 6			
	AFG_2	270 ± 3			

[a] Detection limit. [b] Mean ± standard deviation (n = 3).

Table 2. AF Concentrations in Experimental Feed Contaminated with Four Kinds of AFs and Changes during Storage at −20 °C[a]

		AFs concn in feed (μg/kg)				
expt	AF	nominal	3 days[b]	80 days[c]	CV[d] (%)	difference between 3 and 80 days (%)
B	AFB_1	100	115 ± 1[e]	102 ± 3	0.9	11.3
C	AFB_1	300	347 ± 4	290 ± 6	0.9	16.4
D	AFB_1	1000	1190 ± 10	1050 ± 150	0.7	11.8
E	AFB_1	5000	6276 ± 72		1.0	
F	AFB_1	250	219 ± 7		2.2	
	AFB_2	250	306 ± 4		1.0	
	AFG_1	250	321 ± 6		1.6	
	AFG_2	250	270 ± 3		1.1	

[a] Formula feed for trout (Nisshin-Marubeni Co., 2P) was used in the control experiment, and concentrations of AFB_1, AFB_2, AFG_1, and AFG_2 were below the detection limit (1 μg/kg). [b] Determined at 3 days after preparation. [c] Determined at 80 days after preparation. [d] Coefficient of variation calculated by using the concentration at 3 days after preparation. [e] Mean ± standard deviation (n = 3).

prior to the experiments. Initial body weight is shown in Table 1. AFs in the control fish were analyzed at the beginning and end of the exposure experiment. AFs were not detected in muscle or liver of the control trout by the analytical procedures used (detection limits in muscle and liver were 20 and 100 ng/kg, respectively).

Preparation of AF-Containing Feed. Appropriate amounts of AFs were dissolved in 50 mL of chloroform. AF solution was gradually added to commercially available formula feed (Nisshin-Marubeni, type 2P) and mixed. The addition and mixing procedure was repeated, and the feed was dried under vacuum. As shown in Table 2, several kinds of feed containing different amounts of AFB_1 and AFs (AFB_1, AFB_2, AFG_1, AFG_2) were prepared. AF concentrations in the spiked feed were decided by considering acute toxicity (10 d-LD_{50}),[1−3] results of previous research on carcinogenesis,[10] and regurgitation of stomach content caused by AFB_1.[3] The concentration of AFs in the control feed was below the detection limit of 1 μg/kg.

Apparatus for Rearing Experiments. As the AFs are carcinogenic compounds, the rearing experiments were carried out in a semistatic system. Six aquaria (L = 591 mm, W = 293 mm, H = 380 mm) were placed in a large container for adjustment of the rearing water temperature. Tap water from Yokohama City was introduced into the large container, and water temperature was adjusted to 15 °C by a thermocontroller (Aqua Co., type HC401A-10). Yokohama City tap water, previously dechlorinated by passing through an activated carbon column and adjusted to 15 °C, was used in each aquarium for the rearing experiments. Water temperature was maintained at 15 °C by the water in the large container. Air was pumped into the rearing water to maintain an adequate DO level.

Rearing Experiment. *Experimental Group.* Six experimental groups were prepared as shown in Table 1. Experiment A (expt A) is the control experiment, and these rainbow trout were fed the control feed (Nisshin-Marubeni, type 2P) for 23 days.

Fish in experiments B (expt B), C (expt C), and D (expt D) were fed the experimental feed containing AFB_1 during the uptake experiment of 21 days and then fed control feed during the elimination experiment of 2 days. AFB_1 concentrations in the contaminated feed used in expts B, C, and D were 115, 347, and 1190 μg/kg, respectively.

Experiment E (expt E) was performed to determine the optimal time for collecting trout after feeding. Fish were fed the AFB_1-containing feed (6276 μg/kg) for 7 days and collected at 3, 6, 9, 12, and 24 h after the final feeding on day 7 of the uptake experiment.

Experiment F (expt F) was designed to study the difference in uptake factor among AFs, and the fish were fed four kinds of AFs (AFB_1, AFB_2, AFG_1, and AFG_2) for 7 days. The cncentrations of each AF in the feed were 219 μg/kg for AFB_1, 306 μg/kg for AFB_2, 321 μg/kg for AFG_1, and 270 μg/kg for AFG_2. Fish were collected at 6 h after the final feeding on day 7 of the uptake experiment.

Rearing Method. The feeding rate was 15 mg/g fish/day in all experiments. Fish consumed all feed immediately. To prevent deterioration of the water quality, half of the water in the rearing aquarium was exchanged with fresh water twice a day (10:00 a.m. and 4:00 p.m.), and feces were carefully removed by siphoning from the aquarium several times after the exchange of water and feeding. Water used for the exchange was Yokohama City tap water, previously dechlorinated by passing through an activated carbon column, and adjusting the temperature to 15 °C, as described above.

Temperature and dissolved oxygen were measured twice a day (morning and afternoon) by an electrode (model 556MPS, YSI Nanotech Co.). Inorganic nitrogen (NH_4-N, NO_2-N, and NO_3-N) was determined in expt A before and after the exchange of rearing water in the afternoon on days 3, 7, 14, and 21 of the uptake experiment to determine whether levels of inorganic nitrogen from excrement were harmful to the experimental fish. AFB_1 and its metabolites (AFM_1 and AFL) in the rearing water were measured in expts B, C, and D at the same time as the inorganic nitrogen measurement to consider the metabolic pathways of AFB_1 and percentage of AFB_1 derived from the rearing water in fish muscle.

Fish in the uptake experiment (expt F) were collected at 6 h after feeding on day 7. Fish in expt E were collected at 3, 6, 9, 12, and 24 h after feeding on day 7 of the uptake experiment to determine the changes of the concentration of AFB_1 in muscle with time after feeding. In expts A−D, fish were collected at 6 h after feeding on days 3, 7, and 14 of the uptake experiment. Further fish were collected at 6 and 9 h after feeding on day 21 and afterward every 6 h until 48 h after feeding to investigate the elimination of AFB_1 from muscle and liver. On day 22 of the experiment, the feed was switched to uncontaminated control feed.

For each sampling three fish were collected. Fish were sacrificed by decapitation immediately after sampling, and individual fish were weighed. Muscle and liver were separated and stored in the refrigerator at −20 °C. AFs in muscle were analyzed individually, whereas liver samples were combined for AF analysis.

Analytical Method. *Inorganic Nitrogen.* The concentration of NH_4-N was determined by measuring the absorption of indophenols (blue color) according to the method of Koroleff.[11] NO_2-N and NO_3-N were determined by colorimetry according to the method of Grasshoff.[12] Samples containing excess amounts of ammonium and nitrate were diluted by using distilled water.

AFs in Rearing Water, Formula Feed, and Fish Tissue (Muscle and Liver). AFs were analyzed according to the method of Akiyama et al.[13] However, the procedure was modified slightly for microanalysis. AFs were extracted with dichloromethane from the rearing water. AFs in feed and fish tissues (muscle and liver) were extracted with a mixture of acetonitrile and water (9:1). Extracted AFs were purified by using an immunoaffinity column (AFLAKING, Horiba Co., Kyoto, Japan) and subjected to HPLC equipped with fluorescence detection and post-column photochemical derivatization. AFs were identified by the retention time on a chromatogram and quantified by using a calibration curve.

AFs were separated by using a C18 reverse phase column (TSK gel ODS-100 V, 4.6 mm × 150 mm, TOSO, Osaka, Japan) at 40 °C with a solvent of water, methanol, and scetonitrile (6:2:2). Fluorescent derivatives were detected at a 450 nm (418 nm for AFL) emission wavelength and at a 365 nm (333 nm for AFL) excitation wavelength, as described previously.[14]

Average recoveries of spiked AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFL in muscle were 81.6, 82.2, 77.7, 81.2, 72.7, and 69.1%, respectively, at a concentration of 50 ng/kg. The detection limit of AFs (AFB_1, AFB_2, AFG_1, and AFG_2) and metabolites (AFM_1 and AFL) was 20 ng/kg for muscle and 100 ng/kg for liver. The coefficient of variance (CV) was below 4.6% (AFM_1) for muscle and 6.2% (AFB_1) for liver. Average recovery of spiked AFs from the rearing water was 86−95%, and the detection limit of AFs was 0.1 ng/L.

These results indicated that trace AFs in the muscle and liver of fish, formula feed, and rearing water were analyzed satisfactorily by the present analytical procedures.

Uptake Factor (UF), Elimination Rate Constant, and Biological Half-Life Calculations and Statistics. The UF is defined as the ratio of AF concentration in muscle or liver to that in feed, according to the biomagnification factor.[15] Thus, the UF is an indicator showing the degree of accumulation of AFs in muscle or liver. UFs were calculated by the following equation at the equilibrium period of the dietary exposure experiments:

$$UFn = ((Cen - Cb)/CF) \times 10^{-3} \quad (1)$$

UF*n* is the UF on the *n*th day, Cen is the AF concentration in muscle or liver of fish fed AF-contaminated feed (ng/kg wet weight), Cb is the AF concentration in muscle or liver of the control fish (ng/kg wet weight), and CF is the AF concentration in the contaminated feed (μg/kg wet weight). Because AFs were not detected in the muscle and liver of the control fish at the beginning and the end of the uptake experiment, Cb was assumed to be zero.

Decreases in the concentrations of AFB_1 and AFL in muscle or liver during the elimination experiment were applied to the exponential function of eq 2, according to Connell,[16] and the elimination rate constant (α) was calculated.

$$Cf1 = Cf2\, e^{-\alpha t} \quad (2)$$

Cf1 is the AF concentration in the muscle or liver of the fish at time *t* of the elimination experiment, Cf2 is the AF concentration in the fish (muscle or liver) at the beginning of the elimination experiment, *t* is the period of the elimination experiment, and α is the elimination rate constant.

In this study, decreases in the concentrations in muscle and liver are due to the transfer of both AFB_1 and AFL from muscle and liver to other tissues and organs and excretion.

The elimination half-life ($t_{1/2}$) was calculated by the following equation with α:[16]

$$t_{1/2} = (\ln 2)/\alpha \quad (3)$$

All statistical analyses were performed using StatView 5.0 (SAS Institute Inc., Cary, NC). Differences among groups were analyzed by ANOVA. *P* values of <0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Concentrations of AFs in Control and Experimental Feed. As shown in Table 1, AFs were not detected in the control formula feed. The concentration of AFs in the spiked experimental feed was determined 3 days after preparation (beginning); values are shown in Table 2. Actual concentrations approximated to the nominal concentrations, and CV was between 0.7 and 2.2%. These results showed that AFs were added uniformly to the control formula feed.

AFB_1 concentration was determined 80 days after preparation (end), and the results are also shown in Table 2. During storage for 77 days at −20 °C, the AFB_1 concentration decreased from 115 to 102 μg/kg, from 347 to 290 μg/kg, from 1190 to 1050 μg/kg. However, the difference in the concentrations between the beginning and end ranged from 11.3 to 16.4%. It is considered that the difference in the AFB_1 concentration during the exposure experiment of 21 days was smaller than the above values, due to the storage period (28−49 days after preparation) being shorter. Thus, concentrations at the beginning were used in the analysis of the results obtained in the uptake experiments.

Quality of the Rearing Water. *Temperature, Dissolved Oxygen (DO), and pH.* The water temperature was similar in all experiments and varied from 13.9 to 15.1 °C; thus, temperature showed minimal fluctuation and was maintained at approximately the target temperature of 15 °C. DO was above 7.75 mg/L (DO saturation = 79.3%) in all experiments during the experimental period. These values satisfied the requirement for DO (saturation > 60%) proposed by OECD.[17]

Table 3. Concentration (μmol/L) of Dissolved Inorganic Nitrogen (DIN) in the Rearing Water of the Control Experiment (Experiment A)

date of sampling		NH_4^+-N	NH_3	NO_2^--N	NO_3^--N	DIN
day 3 of uptake expt	before[a]	271	2.32	1.18	76.82	349.00
	after[b]	179	1.54	0.72	68.08	247.80
day 7 of uptake expt	before	243	2.09	1.20	71.73	315.93
	after	136	1.17	0.95	72.59	209.54
day 14 of uptake expt	before	271	2.32	0.84	86.29	358.13
	after	150	1.29	0.78	79.89	230.67
day 21 of uptake expt	before	271	2.32	0.70	83.55	355.25
	after	150	1.29	0.73	71.34	222.07
day 1 of elimination expt	before	200	1.72	0.77	83.93	284.70
	after	114	0.98	0.56	73.30	187.86

[a] Before the exchange of water in the afternoon. [b] After the exchange of water in the afternoon.

Inorganic Nitrogen. As the rearing experiment was conducted under semistatic conditions, inorganic nitrogen (especially toxic ammonia (NH_3)) accumulated in the rearing water due to excretion from the fish. The concentration of dissolved inorganic nitrogen (DIN) in expt A is shown in Table 3. DIN was between 187.86 and 358.13 μmol/L and decreased after water exchange. Ammonium (114−271 μmol/L) was a major constituent in DIN and accounted for 61−78% of DIN. As rearing water was maintained in aerobic conditions by aeration, part of the ammonium was oxidized to nitrate by bacteria. As shown in Table 3, nitrate was a second major constituent, with a concentration between 68.08 and 86.29 μmol/L.

Ammonia (un-ionized form of ammonium; NH_3) is harmful to aquatic organisms. An equilibrium is established between ammonium (NH_4) and ammonia (NH_3), and the percentage of ammonia is dependent on pH and temperature.[18] According to Emerson et al.,[18] 0.859% of ammonium is present as ammonia under the conditions of rearing experiment (15 °C, pH 7.5). The ammonia concentration was calculated from ammonium (114−271 μmol/L) as 0.98−2.32 μmol/L and is shown in Table 3.

Ammonia concentrations were significantly lower than the acute toxicity values (LC_{50} = 11.43−78.57 μmol/L),[19,20] and the highest ammonia concentration was lower than the no-observed-effect concentration (NOEC) (2.86 μmol/L).[20]

Inorganic nitrogen was measured only in expt A, in which AFs were not administered. As the number of experimental fish, feeding rate, and volume of aquariums were similar among the rearing experiments, it is considered that the concentrations of DIN and/or ammonium were similar among the experiments, expts B−D. On the basis of the results that DO and ammonia had negligible effects on the experimental fish, we consider that the fish were reared appropriately during the experiments.

Growth and Survival of the Experimental Fish. As shown in Table 1, the experimental fish grew 1.36 times in expt A, 1.33 times in expt B, 1.30 times in expt C, and 1.33 times in expt D during the uptake experiment. Differences in growth were not seen between the experiments.

No fish died in expts A−C. In expt D, two fish died, the first on day 13 and the second on day 19 of the exposure experiment, and two further fish died on day 21 of the experiment. These results suggested that AFB_1-containing feed of relatively high concentration (1190 μg/kg) was harmful to rainbow trout.

Table 4. Concentration (ng/L) of AFs and Metabolites in the Water during the Rearing Experiment of Rainbow Trout

expt	date of sampling		AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFL
A	day 3 of uptake expt	before[a]	<0.1[c]				<0.1	<0.1
		after[b]	<0.1				<0.1	<0.1
B	day 3 of uptake expt	before	1.9				0.1	5.0
		after	1.2				tr[d]	3.0
	day 7 of uptake expt	before	1.7				0.1	4.9
		after	<0.1				tr	3.0
	day 21 of uptake expt	before	2.0				0.1	3.5
		after	1.1				tr	2.4
	day 1 of elimination expt	before	0.8				tr	2.2
C	day 3 of uptake expt	before	7.6				0.5	16
		after	4.4				0.4	11
	day 7 of uptake expt	before	5.0				0.4	13
		after	2.8				0.3	7.6
	day 21 of uptake expt	before	5.4				0.5	13
		after	0.3				0.2	7.2
	day 1 of elimination expt	before	2.4				0.3	7.8
D	day 3 of uptake expt	before	28				2.0	52
		after	17				1.3	35
	day 7 of uptake expt	before	19				1.3	40
		after	10				0.6	22
	day 21 of uptake expt	before	21				1.2	40
		after	1.3				0.4	20
	day 1 of elimination expt	before	1.9				0.6	24
E	day 7 of uptake expt	before	59				4.7	160
		after	30				2.9	85
F	day 7 of uptake expt	before	2.6	3.5	2.4	3.2	0.2	6.9
		after	1.3	1.7	1.1	1.5	0.1	4.0

[a] Before the exchange of water in the afternoon. [b] After the exchange of water in the afternoon. [c] The detection limit of AFs and metabolites was 0.1 ng/L. [d] Trace. Peak was recognized, but concentration was below the detection limit (0.1 ng/kg).

Bauer et al.[2] studied the acute toxicity of AFB_1 to rainbow trout and reported that the 10d-LD_{50} by oral administration was >6.0 mg/kg body weight. Supposing that the feeding ratio by rainbow trout is 15 mg/g body weight/day, the rainbow trout fed the feed containing AFB1 at the concentration of 400 mg/kg. It is considered from the present research that if AFB_1 was administered continuously, small amounts of AFB_1 (1/330 of 10d-LD_{50}) are harmful to the survival of rainbow trout.

AFs Concentration in Rearing Water. The rearing experiment was carried out in a semistatic system; thus, it was supposed that AFs accumulated in the rearing water from the AF-containing feed, feces, and urine. AFs in the rearing water may be bioconcentrated in the experimental fish. Thus, AF concentrations in the rearing water were determined to clarify the metabolic pathway of AFB_1 and to estimate the percentage of AFs derived from the rearing water to the concentrations in muscle. The concentrations of AFs in the rearing water were determined several times; values are shown in Table 4.

No AFs were detected in the rearing water of the control (expt A) on day 3 of the exposure experiment. AFB_1, AFM_1, and AFL were detected in the rearing water of the exposure experiments (expts B−E). In expt F, in which trout were fed feed containing four kinds of AFs (AFB_1, AFB_2, AFG_1, and AFG_2), AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFL were also detected in the rearing

Table 5. Change of AF Concentration in Muscle from 3 to 24 h after Feeding on Day 7 of the Uptake Experiment (Experiment E)

	AF concn in muscle (ng/kg)		
time after feeding (h)	AFB_1	AFM_1	AFL
3	4100 ± 1100[a] (2600−5300)[b]	50 ± 30 (30−90)	2100 ± 700 (1300−2900)
6	4100 (3100−5100)	30 (20−40)	2900 (2800−3000)
9	3500 ± 800 (2600−4600)	60 ± 30 (20−80)	2300 ± 400 (1700−2800)
12	3900 ± 1300 (2800−5800)	50 ± 20 (20−80)	2000 ± 200 (1800−2200)
24	2200 ± 300 (1800−2600)	30 ± 8 (tr[c]−40)	1600 ± 400 (1000−2000)

[a] Mean ± standard deviation (n = 3). [b] Range of concentration. [c] Trace. Peak was recognized, but concentration was below the detection limit (20 ng/kg).

water. AFB_1 concentrations in expts B, C, and D were <0.1−2.0, 0.3−7.6, and 1.3−28 ng/L, respectively. The AFL concentration changed from 2.2 to 5.0 ng/L in expt B, from 7.2 to 16 ng/L in expt C, and from 20 to 52 ng/L in expt D and was higher than that of AFB_1. AFL was also detected in expt F, with the concentration in the range of 4.0−6.9 ng/L. It was clear from these results that the AFB_1 concentration was higher in the exposure experiment, in which fish were fed feed containing a higher concentration of AFB_1, and that the order of concentration was AFL > AFB_1 ≫ AFM_1.

High concentrations of AFL in the rearing water suggest that rainbow trout metabolize AFB_1 to AFL, and AFL is excreted. Loveland et al.,[4] Shelton et al.,[8] Plakas et al.,[5] Toledo et al.,[6] and Troxel et al.[7] studied the metabolism of AFB_1 using rainbow trout, channel catfish (*Ictalurus punctatus*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*), respectively. They demonstrated that AFB_1 was metabolized to AFL in these species. Thus, the findings of the present research are consistent with these previous studies.[4−8]

AFB_1 is dissolved from the contaminated feed and released from the fish gills in a nonmetabolized form and/or excreted in feces. All of the feed was immediately ingested by the fish; thus, the AFB_1 in the water is derived from excretion.

AFs Concentration in Muscle and Liver of the Experimental Fish. *Changes in Muscle after Feeding.* The water solubility of AFB_1 has been reported as 1.0 g/L by the Food Safety Commission of Japan.[21] High water solubility suggests that the octanol−water partition coefficient (K_{ow}) is very small. K_{ow} was calculated using a mathematical model (KOWWIN ver. 1.66, Syracuse Research Corp., Environmental Science Center, North Syracuse, NY) in cooperation with the Chemical Evaluation and Research Institute (CERI), Japan; the log K_{ow} of AFB_1 was estimated to be 1.23. According to the pharmacokinetic model of Hawker and Connell,[22] it is supposed that AFB_1 having a small log K_{ow} is rapidly excreted from exposed fish. This indicates that it is important for the exact determination of the AF concentration to decide the sampling time after feeding. Change in the AF concentration in muscle with time after feeding was studied in expt E, and the results are shown in Table 5.

AFB_1 was detected at 3 h after feeding on day 7 of the uptake experiment, and the average concentration was 4100 ng/kg. As shown in Table 5, the AFB1 concentrations at 6, 9, 12, and 24 h were 4100, 3500, 3900, and 2200 ng/kg, respectively. The average concentration of AFL changed from 2000 to 2900 ng/kg during the sampling times of 3−12 h. This change in concentration was very small. AFL concentration was lower than that of AFB_1, but higher than that of AFM_1. This again suggested that AFL was a major metabolite of AFB_1. The AFL concentration declined to 1600 ng/kg at 24 h after feeding. These results indicated that the concentrations of AFB_1 and AFL decreased slightly at 24 h after feeding. However, the AFM_1 concentration was very low and did not change after feeding. These results suggested that AFM_1 was a minor metabolite of AFB_1 in rainbow trout. It was decided from the results in expt E that the experimental fish should be sampled at 6 h after feeding in expts B−D and F.

AFB_1 Concentration and Uptake Factor (UF) in Muscle and Liver. AFB_1 concentrations in muscle during the uptake experiment in expts B−F are shown in Table 6.

As described in the footnotes of Table 6, AFB_1 was not detected in the muscle of the control fish. AFB_1 was detected in the experimental fish, and the average concentration in muscle during the uptake experiment of 21 days was 60−100 ng/kg in expt B, 170−230 ng/kg in expt C, and 260−880 ng/kg in expt D. The difference in concentration due to the exposure period was analyzed by one-way ANOVA in expts B−D. No significant difference was recognized among the AFB_1 concentrations for any uptake period (P < 0.05). It was clear from the results that the AFB_1 concentration in muscle reached equilibrium during day 3 of the uptake experiment. The average concentrations of AFB_1 after 7 days of expts E and F were 4100 and 150 ng/kg, respectively. The AFB_1 concentration was higher in fish fed feed containing AFB_1 at higher concentrations.

As described above, AFB_1 was detected in the rearing water (Table 4). AFB_1 in the rearing water is again bioconcentrated in fish. It is important to consider the percentage of AFB_1 derived from rearing water in the AFB_1 concentration in muscle. AFB_1 derived from the rearing water was roughly estimated from the AF concentration in water by using the bioconcentration factor (BCF) of AFs. BCFs of AFs were not found in the literature and were estimated by investigating the BCF of 11 kinds of chemicals published in the literature.[23] BCF is generally dependent on the K_{ow} value of chemicals; 11 chemicals were selected by the log K_{ow}, the log K_{ow} of which was between 1.0 and 1.5. The average BCF value of the 11 chemicals was between 1 and 4.

As shown in Table 4, the average AFB_1 concentration in the rearing water during the uptake experiment was 1.3 ng/L in expt B, 4.3 ng/L in expt C, 16.1 ng/L in expt D, 44.5 ng/L in expt E, and 2.0 ng/L in expt F.

On the basis of the BCF (1−4) and the AFB_1 concentration in the rearing water, AFB_1 derived from water was 1.3−5.3 ng/kg in expt B, 4.0−16 ng/kg in expt C, 16.1−64.4 ng/kg in expt D, 44.5−178 ng/kg in expt E, and 2.0−8.0 ng/kg in expt F. The percentage of AFB_1 derived from water was between 0.9 and 16.9%. Excluding the AFB_1 concentration on day 21 in expt D, most of the percentage values were below 10%.

Table 6. Changes of AF Concentration in Muscle and Uptake Factor (UF) of AFB_1 with the Concentration in Feed

		AFB_1 concn		
expt	period of uptake expt (day)	feed[a] (μg/kg)	muscle[b] (ng/kg)	UF $\times 10^3$
B	3	115 ± 1	90 ± 30 (70−130)[c]	0.78 ± 0.25 (0.61−1.13)
	7		90 ± 5 (90−100)	0.81 ± 0.04 (0.78−0.87)
	14		100 ± 40 (50−150)	0.87 ± 0.36 (0.43−1.30)
	21		60 ± 5 (60−70)	0.55 ± 0.04 (0.52−0.61)
F	7	219 ± 7	150 ± 20 (130−170)	0.70 ± 0.08 (0.59−0.78)
C	3	347 ± 4	200 ± 60 (160−280)	0.58 ± 0.16 (0.46−0.81)
	7		210 ± 30 (170−240)	0.60 ± 0.08 (0.49−0.69)
	14		230 ± 70 (140−290)	0.67 ± 0.19 (0.40−0.84)
	21		170 (150−180)	0.48 (0.43−0.52)
D	3	1190 ± 10	490 ± 90 (380−600)	0.41 ± 0.07 (0.32−0.50)
	7		720 ± 270 (530−1300)	0.66 ± 0.30 (0.45−1.09)
	14		880 ± 310 (460−1200)	0.74 ± 0.26 (0.39−1.01)
	21		260 ± 110 (140−410)	0.21 ± 0.09 (0.12−0.34)
E	7	6276 ± 720	4100 (3100−5100)	0.65 (0.49−0.81)

[a] AFB_1 concentration in the control feed was below the detection limit of 1 μg/kg. [b] The concentration was determined 6 h after feeding. AFB_1 concentration in muscle of the control fish was determined at the beginning and end of the experiment, and the concentration was below the detection limit of 20 ng/kg. [c] Range of concentration.

The concentrations of AFB_2, AFG_1, and AFG_2, derived from the water, were also calculated to be 2.6−10.4 ng/kg for AFB_2, 1.8−7.2 ng/kg for AFG_1, and 2.4−9.6 ng/kg for AFG_2. The percentage of AFs derived from water was 1.6−8.0% for AFB_2, 1.3−8.0% for AFG_1, and 1.2−6.4% for AFG_2.

These results suggest that the percentage of AFs derived from water was small and that the AFs in muscle were derived primarily from the feed. Thus, the AF concentrations were used in the analysis without correcting for AFs derived from the rearing water.

The UF in muscle (ratio of AFB_1 concentration in muscle to feed) was calculated by using eq 1; values are shown in Table 6. UF $\times 10^3$ in expts B−D, the exposure periods in which were relatively long, was 0.43−1.30, 0.40−0.84, and 0.12−1.09, respectively. UF was determined on a dry weight basis by correcting the water content of the fish muscle (76.8%) and the feed (8.5%). It was possible to calculate UF on a dry weight basis by multiplying the UF values by 3.9.

Differences in UF among expts B−D were analyzed statistically by two-way ANOVA. UF in the exposure period of 21 days in expt D was statistically significant ($P < 0.05$). As described above, some fish in expt D died during the latter period of the exposure experiment. Thus, it was considered from these findings that the physiology of fish in expt D was changed to abnormal by AFB_1 and that the metabolism of AFB_1 in fish was influenced by physiological alterations during the latter period of the uptake experiment. Thus, it was considered that the AFB_1 concentration decreased, and a small UF was obtained. However, further research will be necessary to demonstrate the small UF in expt D.

AFB_1 was not detected in the liver of the control fish; the concentration was below the detection limit of 100 ng/kg. However, AFB_1 was detected in the liver of fish in expt C. As shown in Figure 1, the concentration ranged from 24000 to 48000 ng/kg during the uptake experiment, and the concentration in liver was 165−342 times higher than that in muscle. UFs of AFB_1 in liver were between 69.2 $\times 10^{-3}$ and 138.3 $\times 10^{-3}$. These results show that AFB_1 in the feed is more highly accumulated in liver than in muscle.

Difference in UFs by Several AFs. Four kinds of AFs (AFB_1, AFB_2, AFG_1, and AFG_2) were detected in the muscle of fish fed feed contaminated with a mixture of the four kinds of AFs (expt F). As shown in Table 7, the concentrations of AFB_1, AFB_2, AFG_1, and AFG_2 in the muscle were 150 ± 20, 150 ± 10, 120 ± 20, and 180 ± 20 ng/kg, respectively. UF was calculated by dividing the concentration in the muscle by the concentration in the feed (Table 7). UF $\times 10^3$ was 0.70 ± 0.08 for AFB_1, 0.48 ± 0.04 for AFB_2, 0.39 ± 0.08 for AFG_1, and 0.67 ± 0.08 for AFG_2. It was recognized from these results that the UF was higher for AFB_1 and AFG_2 than for AFB_2 and AFG_1. As shown in Table 6, UF $\times 10^3$ of AFB_1 varied by 3-fold (0.43−1.30) in expt B, in which the AFB_1 concentration in feed was similar to that in expt F. Considering the fluctuation in UFs, UFs of the four AFs were not considered to differ from each other. Further research should be carried out using the feed with different concentrations of AFs to assess differences in UFs among several AFs.

Elimination of AFB_1 and AFL from Muscle and Liver. Changes in the AFB_1 concentrations in muscle in expt C are shown in Figure 1A. AFB_1 concentrations in muscle changed from 140 to 290 ng/kg during the exposure experiments (Table 6). Concentrations did not change significantly during the uptake experiment and reached a steady state on day 3 of the experiment, as described previously. AFB_1 concentrations did not change significantly until 24 h after the final feeding on day 21 of the exposure experiment. However, concentrations decreased rapidly with time after feeding of the control feed on day 22 (Figure 1A). Changes in concentrations in the elimination

experiment were applied to eq 2. The correlation coefficient (r) of the regression analysis was 0.824, and the correlation was significant ($P < 0.05$). The slope of the regression line is considered to be the elimination rate constant (α). An elimination half-life ($t_{1/2}$) was calculated by using eq 3 and the determined α value. As shown in Figure 1A, the α and $t_{1/2}$ of AFB_1 in muscle were 1.83 day^{-1} and 0.38 day (9.12 h), respectively.

AFB_1 concentrations were higher in liver than in muscle (Figure 1B). However, the concentrations changed in the same manner observed in muscle and decreased rapidly with time after feeding the control feed. Again, α and $t_{1/2}$ were calculated on the basis of changes of the AFB_1 concentrations in the elimination experiment and are also shown in Figure 1B; the values were 1.38 day^{-1} and 0.50 day (12.00 h), respectively.

Shelton et al.[8] studied the distribution, metabolism, and elimination of AFB_1, which was injected intraperitoneally in rainbow trout, and the half-lives in blood, liver, and residual carcass (including muscle) were determined. The elimination half-lives in blood, liver, and carcass were 15.5, 31.3, and 25.4 h, respectively. The half-life in liver was longest among the three tissues and became short in order: residual carcass > blood. The result described by Shelton et al.[8] was similar to the result obtained here and demonstrates that AFB_1 in liver is not more rapidly eliminated than that in muscle and/or residual carcass.

Elimination half-lives (liver, 31.3 h; carcass, 25.4 h) from Shelton et al.[8] were 2.6 (liver) and 2.8 (carcass) times longer than the $t_{1/2}$ values (liver, 12.00 h; muscle, 9.12 h) determined in the present research. Half-life is determined by relationships

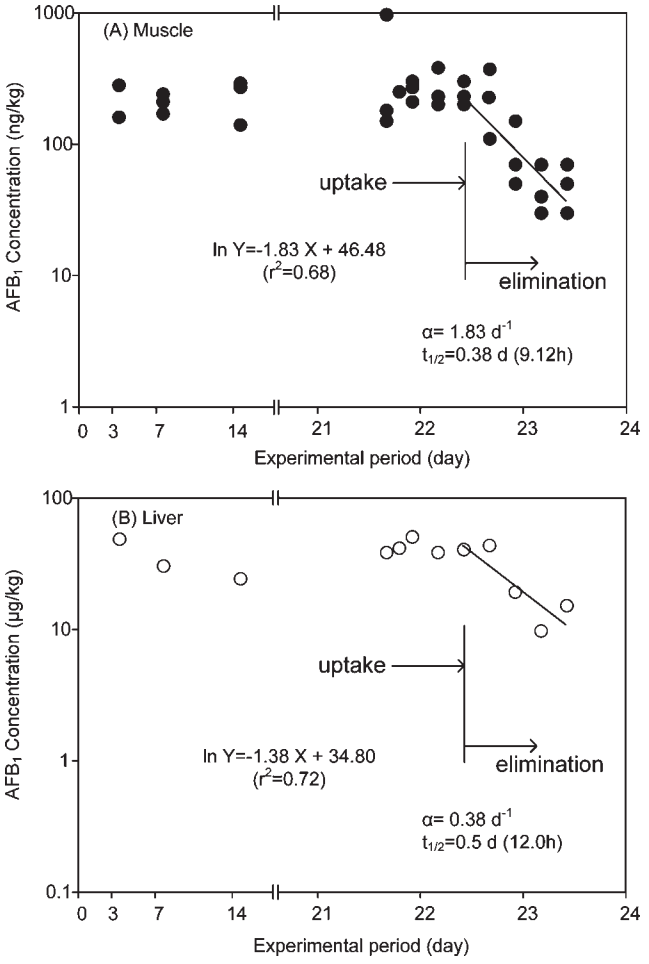

Figure 1. Changes in AFB_1 concentrations in muscle and liver of rainbow trout fed AFB_1-containing feed during the exposure and elimination experiments.

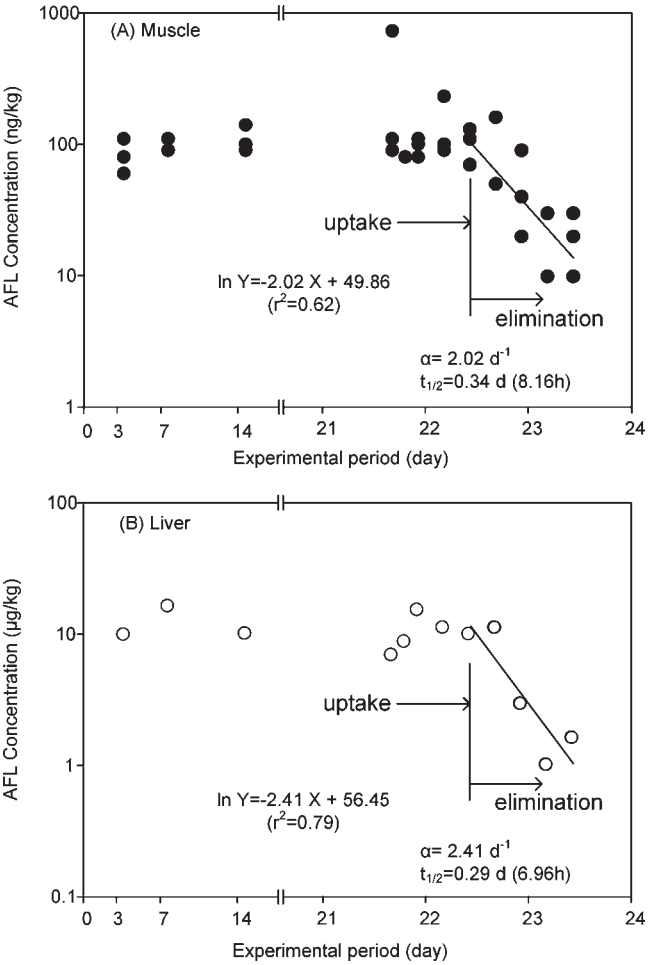

Figure 2. Changes in AFL concentrations in muscle and liver of rainbow trout fed AFB_1-containing feed during the exposure and elimination experiments.

Table 7. AF Concentration in Muscle and Feed Contaminated with AFs and Uptake Factor (UF) of Each AF in Experiment F

	AF			
	AFB_1	AFB_2	AFG_1	AFG_2
concentration in feed (μg/kg)	219 ± 7 (213−226)[a]	306 ± 4 (302−310)	321 ± 6 (314−326)	270 ± 3 (266−272)
concentration in muscle (ng/kg)	150 ± 20 (130−170)	150 ± 10 (130−160)	120 ± 20 (90−140)	180 ± 20 (150−200)
UF × 10^3	0.70 ± 0.08 (0.59−0.78)	0.48 ± 0.04 (0.42−0.52)	0.39 ± 0.08 (0.28−0.44)	0.67 ± 0.08 (0.56−0.74)

[a] Range of concentration.

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between accumulation and elimination. If accumulation continues during an elimination experiment, a large $t_{1/2}$ will be determined. Absorption and accumulation of AFB_1 are influenced by experimental conditions, such as the amount and route of administration (oral ingestion or ip injection). Shelton et al.[8] administered AFB_1 by ip injection, and it is considered that the difference in $t_{1/2}$ values depends on the difference in exposure method.

AFL was not detected in the muscle or liver of the control fish (expt A). Changes of AFL concentrations in muscle in expt C are shown in Figure 2A. AFL concentrations were approximately half of AFB_1. However, changes in the concentration during the experiment were similar to those of AFB_1. AFL concentrations in the muscle ranged between 60 and 140 ng/kg during the uptake experiments and did not change significantly during the uptake experiment. AFL concentrations did not change significantly until 24 h after the final feeding on day 21 of the exposure experiment. As shown in Figure 2A, the concentrations decreased rapidly with time after initiating feeding with the control feed. Sampling 42 h after final feeding found no AFL in one sample among three muscle samples. The elimination rate constant (α) was determined by applying the data during the elimination experiment to the eq 2, and $t_{1/2}$ was calculated by using eq 3, based on α. The α and $t_{1/2}$ of AFL in the muscle were 2.02 d^{-1} and 0.34 day (8.16 h), respectively.

The AFL concentration was higher in liver than in muscle (Figure 1B). Changes with time in the liver were the same as those recognized in muscle. Furthermore, the changes were the same as the results recognized in the experiments with AFB_1. For the liver, α and $t_{1/2}$ were again calculated, on the basis of the changes in AFL concentration in the elimination experiment. The α and $t_{1/2}$ of AFL were 2.41 day^{-1} and 0.29 day (6.96 h), respectively. It was clear from the results of the present research that AFL, a metabolite of AFB_1, was also eliminated more rapidly from muscle and liver than AFB_1.

It was considered from the remarkably large α values that AFB_1 and AFL were not accumulated in high concentration in fish species such as rainbow trout. As a conclusion from the research, the AFB_1 concentration in muscle of aquaculture fish fed AFB_1-containing feed (ca. 500 μg/kg) decreased below the detection limit of the most sensitive analytical method (20 ng/kg) at 1.54 days (36.96 h) after rearing of fish fed uncontaminated feed.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81-42-398-7364. Fax: +81-42-368-7364. E-mail: yamadah0703@yahoo.co.jp.

Notes

Safety. AFs are hazardous due to potential hepatotoxicity and carcinogenicity, and aflatoxin epoxides are potent mutagenic agents. Gloves and masks are essential for the manipulation of AFs to prevent intake. Thus, experiments using large amounts of AFs, as in the preparation of contaminated feed and chemical analysis, and the rearing experiments, were conducted in a laboratory equipped with ventilation facilities. Rearing experiments were conducted in semistatic systems. Discharged rearing water and the waste contaminated with AFs were incinerated by a waste disposal company. AFs adhered on the glassware were destroyed using sodium hypochlorite solution.

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