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Formation Route of Sulfur-Containing Metabolites of Afloqualone, a New Centrally Acting Muscle Relaxant, in Rat[1)]

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The formation route of the sulfur-containing metabolites of afloqualone [6-amino-2-fluoromethyl-3-(*o*-tolyl)-4(3*H*)-quinazolinone, AFQ], a centrally acting muscle relaxant, was studied in rats. When ^{14}C-*N*-acetyl AFQ 2-mercapturate (AFQM) methyl ester was administered orally to normal rats, it was excreted in the bile as AFQM, one of the major biliary metabolites of AFQ, and in the urine as sulfur-containing metabolites of AFQ such as methylsulfinyl and methylsulfonyl metabolites of AFQ. This indicated that AFQM was a precursor of these methylsulfinyl and -sulfonyl metabolites. Pretreatment of rats with antibiotics significantly reduced the urinary excretion of ^{14}C-AFQM and its metabolites as compared with normal rats. Oral administration of ^{14}C-AFQ to antibiotics-treated rats also significantly reduced the amounts of the sulfur-containing metabolites excreted into the urine, but did not affect the amounts of other non-sulfur-containing metabolites. In addition, oral administration of ^{14}C-AFQ to bile-duct-ligated rats gave results similar to those in the antibiotics-treated rats. These results indicate that in normal rats microfloral metabolism is necessary for the formation of the methylsulfinyl and -sulfonyl metabolites from AFQM and other unidentified mercapturate pathway conjugates excreted into the bile. The metabolites of AFQ produced by the microflora are reabsorbed into the systemic circulation, processed by further metabolism in the liver, and in turn excreted in the urine.

Keywords——afloqualone; centrally acting muscle relaxant; methylsulfinyl metabolite; methylsulfonyl metabolite; formation route; intestinal microflora; antibiotics-treated; bile-duct-ligated; enterohepatic-renal disposition; rat

Afloqualone [6-amino-2-fluoromethyl-3-(*o*-tolyl)-4(3*H*)-quinazolinone, AFQ] is a new centrally acting muscle relaxant.[2,3)] In previous studies we have shown that the rat, dog and monkey, when given AFQ orally, excrete in the urine sulfur-containing metabolites possessing either a methylthio (only found in the dog), methylsulfinyl or methylsulfonyl group.[4,5)] The introduction of these sulfur-containing groups into drug metabolites is a recently discovered metabolic pathway.[6,7)] Although sulfur-containing metabolites derived from about fifteen compounds have been reported so far, many of them possess the sulfur-containing groups directly linked to an aromatic ring. A few papers have described metabolic modifications on the side chain of an aromatic ring,[6)] but none of them reports the displacement of a fluorine atom by a sulfur-containing group. Although several mechanisms of sulfur incorporation have been proposed, none is unequivocally established.

The present study deals with the elucidation of the formation route of the sulfur-containing metabolites of AFQ in rats.

Experimental

Compounds——^{14}C-AFQ was synthesized in our laboratory by the method described previously.[2,4)] The product has a specific radioactivity of 9.0 μCi/mg and a radiochemical purity of more than 98% as determined by thin-layer chromatography (TLC). ^{14}C-AFQ was diluted with an appropriate amount of non-radioactive AFQ before use. Since AFQ is light sensitive, all procedures were carried out in an area protected from light as far as possible.

^{14}C-*N*-Acetyl AFQ 2-mercapturate, *N*-acetyl-*S*-[6-acetamido-3-(*o*-tolyl)-4(3*H*)-quinazolinone-2-yl] methyl-L-cysteine (AFQM), and its methyl ester were synthesized from ^{14}C-AFQ according to the method described previously.[8] The specific radioactivity of ^{14}C-AFQM methyl ester was 0.3 μCi/mg, and the radiochemical purity was 98% as determined by TLC. Other chemicals and solvents were of the best grade commercially available.

Animal Experiments——Male Wistar rats weighing about 200 g were used. Unless otherwise noted, animals were permitted free access to water but not to food from 16 h before and until 4 h after the administration of labelled compounds.

A catheter was introduced into the common bile duct of rats anesthetized with urethane (1 g/kg, *i.p.*), and 1 h after the operation the rats were given orally ^{14}C-AFQ at a dose of 20 mg/kg in a 0.5% carboxymethylcellulose suspension or ^{14}C-AFQM methyl ester at a dose of 20 mg/kg in a solution of ethanol–water (1 : 1, v/v). The bile was collected up to 24 h after the administration.

Under ether anesthesia, the common bile duct of rats was ligated surgically and the rats were given orally ^{14}C-AFQ (20 mg/kg). The animals were housed individually in stainless steel metabolism cages. The urine was collected 24 h after the administration.

Antibiotics-treated rats received twice a day an oral dose of 25 mg/kg each of bekanamycin sulfate (Meiji Seika) and lincomycin hydrochloride (Japan Upjohn) for 5 d. On the 6th day, the rats were given the last dose of the antibiotics followed by an oral dose of ^{14}C-AFQ (20 mg/kg) or ^{14}C-AFQM methyl ester (20 mg/kg) 1 h after the administration of the antibiotics. The urine and feces were collected 72 h after the administration.

All the urine and bile samples collected were stored at −20 °C until analysis.

Measurement of Radioactivity——Radioactivity was measured with a liquid scintillation spectrometer (Packard, Tri-Carb 460CD). The urine and bile samples were suitably diluted with water and 0.5 to 1 ml portions were added to 12 ml of a Triton X-100 based scintillator (PPO 4 g, dimethyl POPOP 0.1 g, toluene 660 ml and Triton X-100 330 ml). The feces were homogenized with 5 to 10 volumes of water and aliquots of the homogenate were combusted in an automatic sample oxidizer (Aloka, ASC-113) to measure the radioactivity.[9] Radioactivity of TLC bands was measured using the same scintillator after scraping off each radioactive area of silica gel into a counting vial containing 2 ml of methanol.

Isolation of Urinary and Biliary Metabolites——The urine and bile samples were adjusted to pH 9 with 2 N NH_4OH and extracted three times with two volumes of ethyl acetate (AcOEt). The extract was designated as MA fraction. The residual aqueous solution was adjusted to pH 2 with 2 N HCl and extracted with AcOEt. The extract and the residual aqueous solution were designated as MB and MC fractions, respectively.

In some experiments, the MC fraction of the bile was neutralized with 2 N NH_4OH and diluted with two volumes of 0.2 M acetate buffer (pH 5.0). The solution was incubated with β-glucuronidase (Tokyo Zoki Chemicals, bovine liver) and sulfatase (Sigma, H-2) at 37 °C for 16 h. After the incubation, the mixture was extracted with AcOEt as described above.

Each AcOEt extract was concentrated to a small volume and subjected to TLC. TLC was carried out on silica gel $60F_{254}$ plates (Merck, precoated) employing the following solvent systems: I = benzene–THF (1 : 1), II = *n*-BuOH–AcOH–H_2O (4 : 1 : 1) and III = THF–$CHCl_3$–acetone–conc. NH_4OH (15 : 10 : 10 : 1) (all ratios by volume). Radioactive areas on the plate were detected by autoradiography on X-ray films (Kodak, NS-5T) and ultraviolet (UV)-absorbing areas under a UV lamp. After detection, the radioactive areas on the plate were scraped off into micro test tubes, extracted with tetrahydrofuran (THF) or methanol and subjected to radioactivity measurement and mass spectrometric analysis.

Derivatization——Trimethylsilyl (TMS) derivatives were prepared by treating the samples with sufficient amounts of *N,O*-bis(trimethylsilyl)acetamide (BSA, Gasukuro Kogyo) at 60 °C for 30 min. Methyl derivatives were prepared by treating the samples with a large excess of CH_2N_2 in a mixture of ether and ethanol.

Mass Spectrometry——All mass spectra (MS) were taken at an ionization voltage of 30 eV on a Hitachi RMU-6MG gas chromatograph-mass spectrometer equipped with a Hitachi 002B data processing system. Samples were introduced into either the gas chromatography (GC) or direct probe inlet system. GC was performed as follows. A 1-m × 4-mm glass column packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh, Applied Science) was used and heated from 200 to 280 °C at 3 °C/min. The carrier gas was helium at 40 ml/min. The source, separator and injection port temperatures were 180, 280 and 250 °C, respectively.

Results

Biliary Metabolites of AFQ

When ^{14}C-AFQ was orally administered to bile-duct-cannulated rats, about 63% of the administered radioactivity was excreted in the 24-h bile (Table I). When this radioactivity was fractionated by solvent extraction, the radioactivities in the MA (basic), MB (acidic) and MC (polar) fractions were 2.0, 18.9 and 41.6%, respectively (Table I). TLC of the MB fraction showed the presence of two major radioactive spots (Fig. 1) and their radioactivities were 10.1

Table I. Radioactivity Excreted in the Bile of Rats after Oral Administration of ^{14}C-AFQ and ^{14}C-AFQM Methyl Ester

		Percent of dose	
		^{14}C-AFQ	^{14}C-AFQM methyl ester
Biliary excretion	4 (h)	20.4	8.4
	8	39.8	13.1
	24	62.5	25.4
Extracted fraction[a]	MA	2.0	0.6
	MB	18.9	23.5
	MC	41.6	1.3
TLC spots from MB[b]	MC_1 (AFQM)	10.1	21.1
	MC_2	3.8	—

a) The 24-h bile samples were fractionated by solvent extraction. See the text.
b) The metabolites in the MB fraction were quantified by TLC.

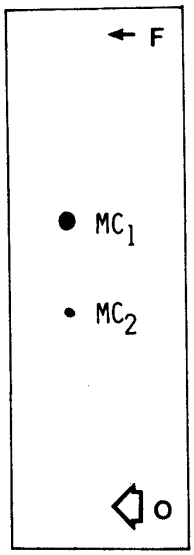

Fig. 1. TLC Autoradiogram of the Acidic Metabolite Fraction in the Bile of Rats after Oral Administration of ^{14}C-AFQ

The solvent system employed was *n*-BuOH–AcOH–H_2O (4:1:1, v/v).

and 3.8% of the dose, respectively. These two spots were positive to the $K_2Cr_2O_7$–$AgNO_3$ reagent,[10] suggesting the presence of mercapturic acid conjugates. The mass spectrum (MS) of the larger spot MC_1 after methylation with CH_2N_2 was identical with that of a synthetic sample of AFQM methyl ester. The chemical structure of the smaller spot MC_2 has not been determined so far.

When the MC fraction was hydrolyzed with β-glucuronidase and sulfatase and extracted with AcOEt, only a very small amount of the radioactivity in the MC fraction was transferred into the AcOEt layer, suggesting the absence of glucuronic and sulfuric acid conjugates. In view of the presence of the mercapturate (AFQM) in the MB fraction, the MC fraction may contain other mercapturic acid pathway metabolites such as glutathione and cysteine conjugates.

Biliary and Urinary Metabolites of AFQM Methyl Ester

Since ^{14}C-AFQM methyl ester could be prepared in a more satisfactory purity than ^{14}C-AFQM and the methyl ester was expected to yield the free acid form readily by the action of various esterases *in vivo*, further metabolism of AFQM was studied by administering ^{14}C-AFQM methyl ester to rats. When ^{14}C-AFQM methyl ester was orally administered to bile-

Table II. Cumulative Excretion of Radioactivity in the Urine and Feces of Rats after Oral Administration of ^{14}C-AFQ and ^{14}C-AFQM Methyl Ester

Compound	Animal treatment	Time after dosage (h)	Percent of dose	
			Urine	Feces
^{14}C-AFQ (20 mg/kg)	Untreated[a] (7 rats)	3	12.6	—
		6	19.3	—
		24	48.3	39.5
		48	49.2	44.0
		72	49.5	44.6
	Antibiotics-treated (2 rats)	4	18.9	—
		8	26.5	—
		24	30.3	38.9
		48	31.5	65.1
		72	31.7	69.3
	Bile-duct ligated (3 rats)	4	47.2	—
		8	70.8	—
		24	84.6	—
^{14}C-AFQM methyl ester (20 mg/kg)	Untreated (3 rats)	4	0.7	—
		8	1.4	—
		24	12.0	73.9
		48	13.6	85.3
		72	13.7	85.7
	Antibiotics-treated (3 rats)	4	0.7	—
		8	1.5	—
		24	2.5	69.2
		48	2.9	96.2
		72	3.0	97.3

a) These data are taken from the previous paper.[4]

duct-cannulated rats, about 25% of the administered radioactivity was excreted into the 24-h bile (Table I). About 90% of the biliary radioactivity was transferred to the MB fraction. TLC and mass spectrometric analyses of this fraction revealed the presence of AFQM as a major metabolite. The amount of this metabolite accounted for about 83% (21% of the dose) of the biliary radioactivity (Table I). Thus, the administration of ^{14}C-AFQM methyl ester instead of ^{14}C-AFQM was justified at least for qualitative purposes.

When ^{14}C-AFQM methyl ester was orally administered to normal rats, about 14% of the administered radioactivity was excreted into the urine within 72 h as shown in Table II. The radioactivity excreted into the 8-h urine was only 1.4% of the dose and most of the urinary radioactivity was excreted from 8 to 24 h after the administration. In the previous study, when ^{14}C-AFQ was orally administered to normal rats, about 50% of the dose was excreted in the 72-h urine, and of this, nearly two-fifths (19.3% of dose) was excreted in 6 h[4] (Table II). These results indicate that there is a lag time for the urinary excretion of the metabolites from AFQM.

When the MA fraction of the 8—24-h urine from the ^{14}C-AFQM methyl ester-treated rats was subjected to two-dimensional TLC, at least five radioactive spots were observed as shown in Fig. 2B. By comparing the *Rf* values with those of the metabolites obtained after the administration of ^{3}H-AFQ (Fig. 2A) and those of synthetic samples, these five compounds

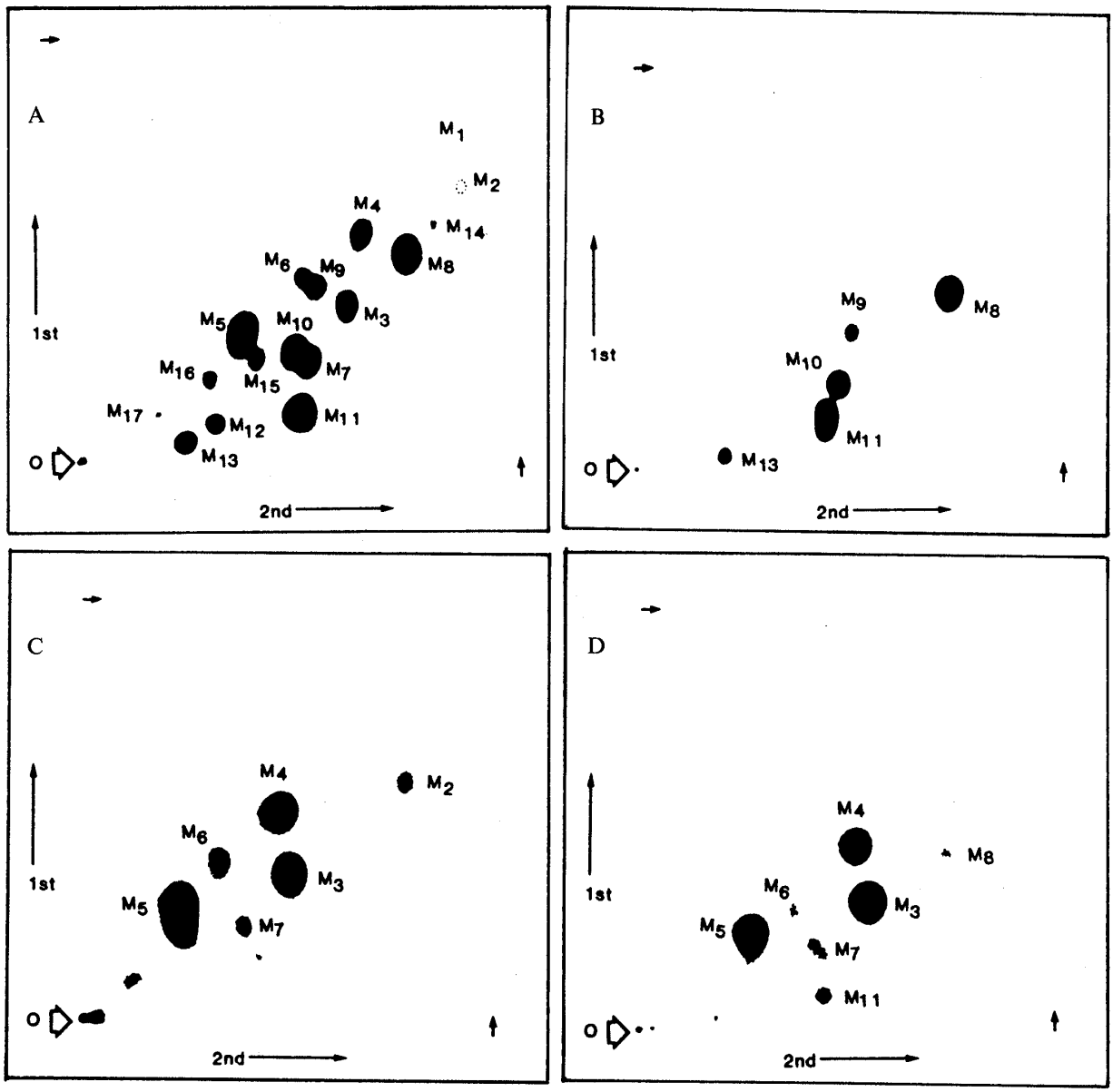

Fig. 2. Two-Dimensional TLC Autoradiograms of the Neutral and Basic Metabolite Fraction in the Urine

A: ^{3}H-AFQ to normal rats.[4)]
B: ^{14}C-AFQM methyl ester to normal rats.
C: ^{14}C-AFQ to antibiotics-treated rats.
D: ^{14}C-AFQ to bile-duct-ligated rats.
Solvent system employed: first dimension (ascending) benzene–THF (1:1, v/v) and second dimension (from left to right) THF–$CHCl_3$–acetone–conc.NH_4OH (15:10:10:1, v/v).

were suggested to be the methylsulfinyl and -sulfonyl metabolites described in the previous paper.[4)] The structures of the major metabolites M_8, M_{10} and M_{11} were confirmed by analysis of their mass spectra (Fig. 3). Table III shows the amounts of these metabolites in the 8—24-h urine; M_8 accounted for about 23% (2.7% of the dose) of the urinary radioactivity. The methylthio metabolites postulated as precursors of the methylsulfinyl metabolites were not observed (Fig. 2B).

In order to examine the possibility of involvement of intestinal microflora, ^{14}C-AFQM methyl ester was orally administered to antibiotics-treated rats. Although the excretion of radioactivity in the 0—8-h urine was similar to that obtained from normal rats, the

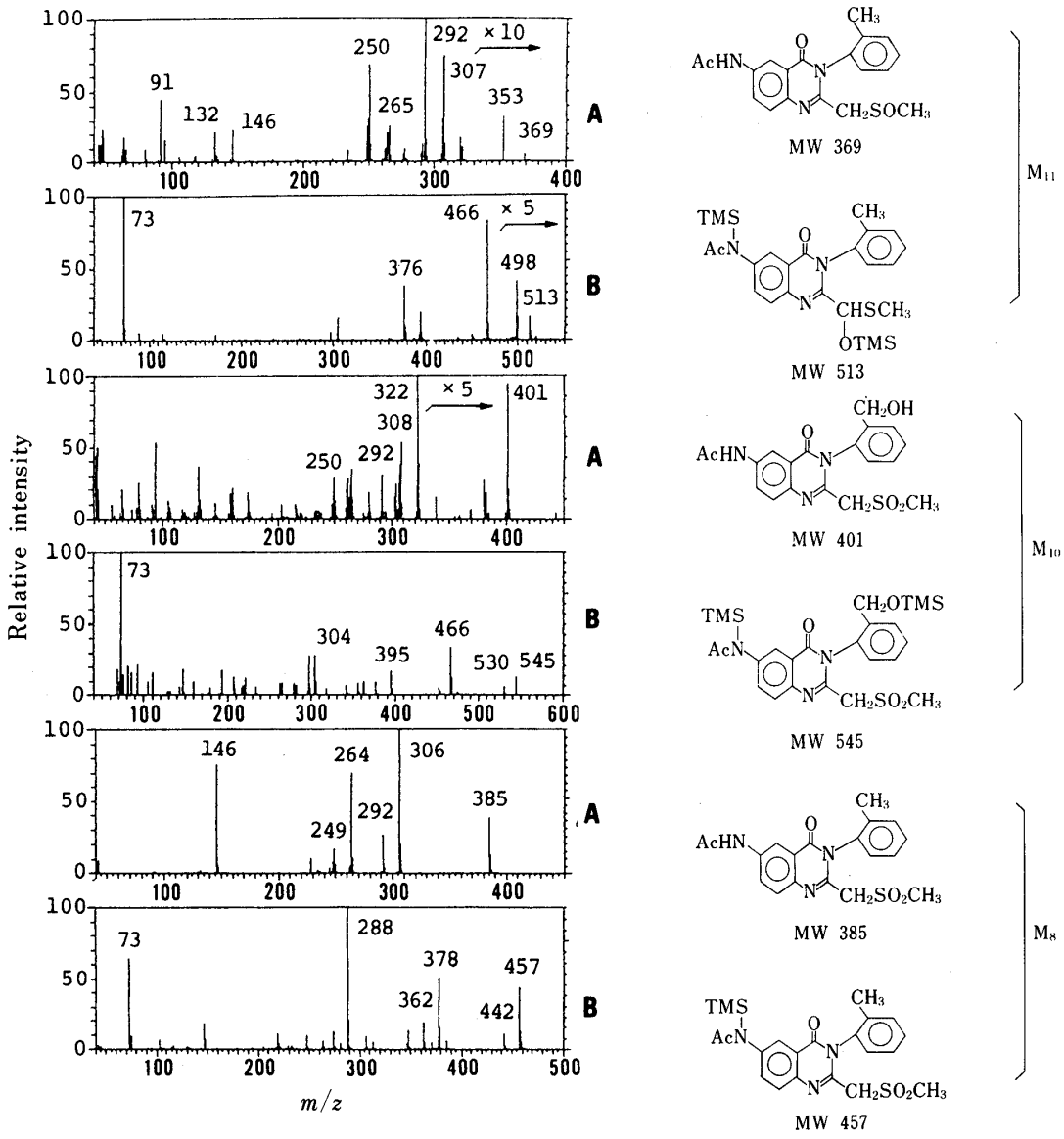

Fig. 3. Mass Spectra of M_8, M_{10} and M_{11}

A: direct-inlet probe introduction.
B: GC introduction as TMS derivatives.

radioactivity excreted from 8 to 24 h was significantly reduced and the fecal radioactivity in that period was correspondingly increased (Table II). Thus, the intestinal microflora seemed to be necessary for the conversion of AFQM to the late-appearing methylsulfinyl and -sulfonyl metabolites.

Urinary Metabolites of AFQ in Antibiotics-Treated and Bile-Duct-Ligated Rats

When ^{14}C-AFQ was administered orally to antibiotics-treated rats, the amount of radioactivity excreted into the 8-h urine was similar to or greater than that obtained from normal rats (Table II). However, the amount excreted from 8 to 24 h, the time period when the metabolites of AFQM methyl ester were excreted, was again reduced significantly while the corresponding fecal radioactivity was increased in the antibiotics-treated rats. This again suggests that the intestinal microflora was responsible for the conversion of the poorly absorbable biliary metabolites of AFQ to more absorbable metabolites, which upon entering the systemic circulation were metabolized to the late-appearing urinary metabolites. The two-

TABLE III. Excretion of Metabolites of ^{14}C-AFQ and ^{14}C-AFQM Methyl Ester in the 24-h Urine of Rats

Metabolite	Formula			Percent of dose											
				^{14}C-AFQ									^{14}C-AFQM methyl ester		
				No treatment[a]			Antibiotics treatment ($N=2$)			Bile-duct ligated ($N=3$)			No treatment ($N=3$)[b]		
				MA[c]	MB	MC	MA	MB	MC	MA	MB	MC	MA	MB	MC
	R_1	R_2	R_3	33.0	5.7	9.6	11.4	10.6	8.3	13.2	47.2	24.2	9.5	1.2	1.3
M_2	$COCH_3$	F	H	0.1			0.1			0.1					
M_3	$COCH_3$	F	OH	1.0			1.9			4.1					
M_4	$COCH_2OH$	F	H	1.7			1.8			1.7					
M_5	$COCH_2OH$	F	OH	5.8			5.7			4.6					
M_6	$COCH_2OH$	OH	H	0.6			0.3			0.2					
M_7	$COCH_3$	H	OH	1.3			0.1			0.2					
M_{14}	$COCH_3$	OH	H	0.2			0.03			ND					
MC_1	$COCH_3$	mer[d]	H		2.6			6.6			24.4				
M_8	$COCH_3$	SO_2CH_3	H	4.1			0.02			0.1			2.7		
M_9	$COCH_2OH$	SO_2CH_3	H	2.2			ND			ND			0.4		
M_{10}	$COCH_3$	SO_2CH_3	OH	3.0			ND			0.1			1.3		
M_{11}	$COCH_3$	$SOCH_3$	H	7.0			0.05			0.2			3.4		
M_{12}	$COCH_2OH$	$SOCH_3$	H	0.9			ND			ND			0.3		
M_{13}	$COCH_3$	$SOCH_3$	OH	2.0			ND			0.1			0.4		

a) These data are taken from the previous paper.[4]
b) 8—24-h urine.
c) MA, neutral and basic metabolite fraction; MB, acidic metabolite fraction; MC, polar metabolite fraction.
d) *N*-Acetylcysteinyl. ND, not detected.

dimensional TLC pattern of the MA fraction of the 24-h urine (Fig. 2C) was different from that obtained with normal rats (Fig. 2A) in that the radioactive spots corresponding to the sulfur-containing metabolites were missing or barely visible. Mass spectral analyses of the main spots M_2—M_7 revealed that they were non-sulfur-containing metabolites in various quantities as shown in Table III. There were no marked differences in the amounts of these non-sulfur-containing metabolites obtained from the antibiotics-treated rats and normal rats, indicating that the pretreatment with the antibiotics did not influence the formation of the non-sulfur-containing metabolites.

^{14}C-AFQ was then orally administered to bile-duct-ligated rats to examine the effect of prevention of enterohepatic circulation on the metabolism of AFQ. A much greater amount (about 85%) of the administered radioactivity (compared to 50% in the intact rats) was excreted in the 24-h urine (Table II). When the MA fraction (about 13% of the dose) of the 24-h urine was subjected to two-dimensional TLC, the autoradiogram pattern (Fig. 2D) was similar to that obtained from the antibiotics-treated rats, which lacked the sulfur-containing metabolites. Mass spectral analyses of the main spots M_3—M_5 showed that they were all non-sulfur-containing metabolites, but there were a few slight, but significant, spots (M_8, M_{11}) corresponding to the sulfur-containing metabolites (Fig. 2D, Table III). About 50% of the radioactivity in the MB fraction was the mercapturate (AFQM) (Table III).

Discussion

In the previous studies many metabolites were isolated from the urine of rats, monkeys and dogs after oral administration of AFQ.[4,5] Figure 4 shows the probable routes of formation of the sulfur-containing metabolites of AFQ encountered in rats. The mercapturate of AFQ (MC_1, AFQM) appears to be formed from the glutathione conjugate *via* the mercapturic acid pathway metabolites.[11,12]

The sources of sulfur in these methylsulfinyl and methylsulfonyl metabolites have been reported by several authors: methionine or *N*-acetylmethionine by Miller *et al.*[13] and Mio *et al.*,[14] mercapturic acid derivatives by Chatfield and Hunter,[15] cysteine conjugates by Tateishi *et al.*,[16] and methylmercaptan by Stillwell *et al.*[17] The present studies showed that the mercapturate (AFQM) can be a source of the sulfur-containing metabolites of AFQ as reported by Chatfield and Hunter.[15] Although mercapturates have generally been considered to be the final metabolites derived from glutathione conjugates *via* a series of metabolic steps, the present results indicate that the mercapturate can be further converted to the methylsulfinyl or -sulfonyl metabolites.

The findings that there was a lag time in the excretion of radioactivity in normal rats after oral administration of ^{14}C-AFQM methyl ester, that the sulfur-containing metabolites were not found in the plasma of normal rats 1 h after oral administration of ^{14}C-AFQ[4] and that AFQM was excreted in the bile of rats orally given AFQ, suggested that the metabolism of AFQM to these sulfur-containing metabolites required enterohepatic circulation. This assumption was confirmed by the present result that after the oral administration of ^{14}C-AFQ to the bile-duct-ligated rats the amounts of the sulfur-containing metabolites excreted into the urine were drastically reduced (Fig. 2D). Namely, AFQM (and its precursors) excreted into the intestinal tract *via* the bile seem to be converted to the sulfur-containing metabolites during enterohepatic circulation.

Intestinal metabolism of foreign compounds is caused by the intestinal microflora and enzymes in the intestinal walls. In order to determine whether the intestinal microflora is involved in the formation of the sulfur-containing metabolites of AFQ, we examined the urinary metabolites of AFQ in antibiotics-treated rats. Pretreatment of rats with the antibiotics significantly reduced the amounts of the sulfur-containing metabolites excreted in

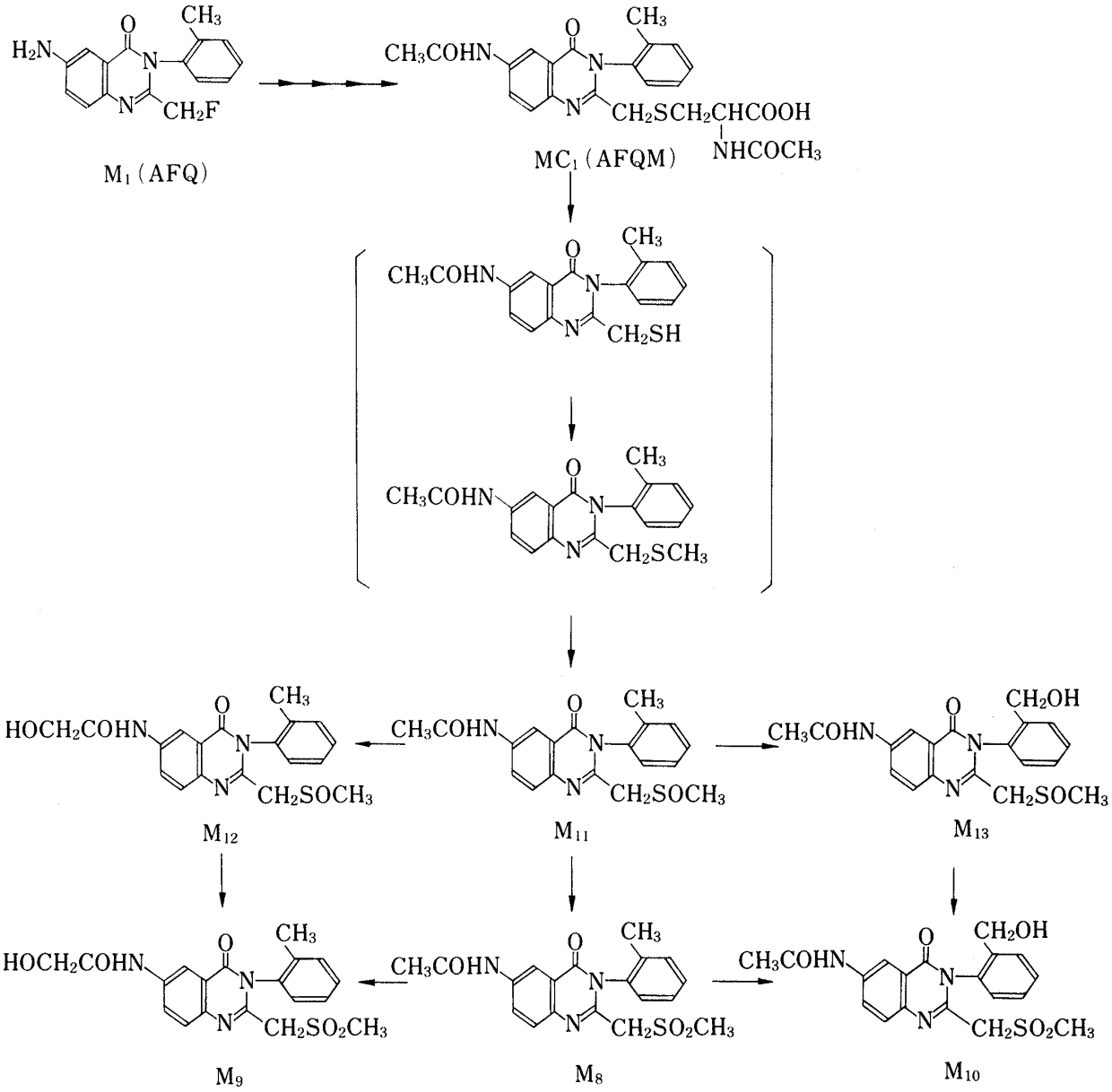

Fig. 4. Sulfur-Containing Metabolites of AFQ Found in the Urine of Normal Rats

the urine, but did not affect the amounts of non-sulfur-containing metabolites (Fig. 2C). These results indicate that the formation of the sulfur-containing metabolites in normal rats largely depends on the intestinal microfloral metabolism of biliary metabolites of AFQ.

The formation route of methylthio metabolites, which are considered to be precursor metabolites of methylsulfinyl derivatives, has been studied by some investigators. Tateishi *et al.*[16] reported that the cysteine conjugates of aromatic compounds such as bromazepam or bromobenzene were cleaved by cysteine conjugate β-lyase (C–S lyase) from rat liver and that the resultant thiols were methylated by *S*-adenosylmethionine to form the methylthio derivatives. An alternative pathway has been proposed by Bakke *et al.*,[18] in which the cysteine or mercapturic acid conjugates of propachlor and 2-acetamido-4-chloromethylthiazole excreted in the bile were cleaved by a C–S lyase of the intestinal microflora. The present results are similar to those obtained by Bakke *et al.* In these cases the contribution of the liver to the cleavage of the C–S bond appeared to be much smaller than that of the intestinal microflora. Tateishi *et al.* have also shown that the substrate specificity of their cys-

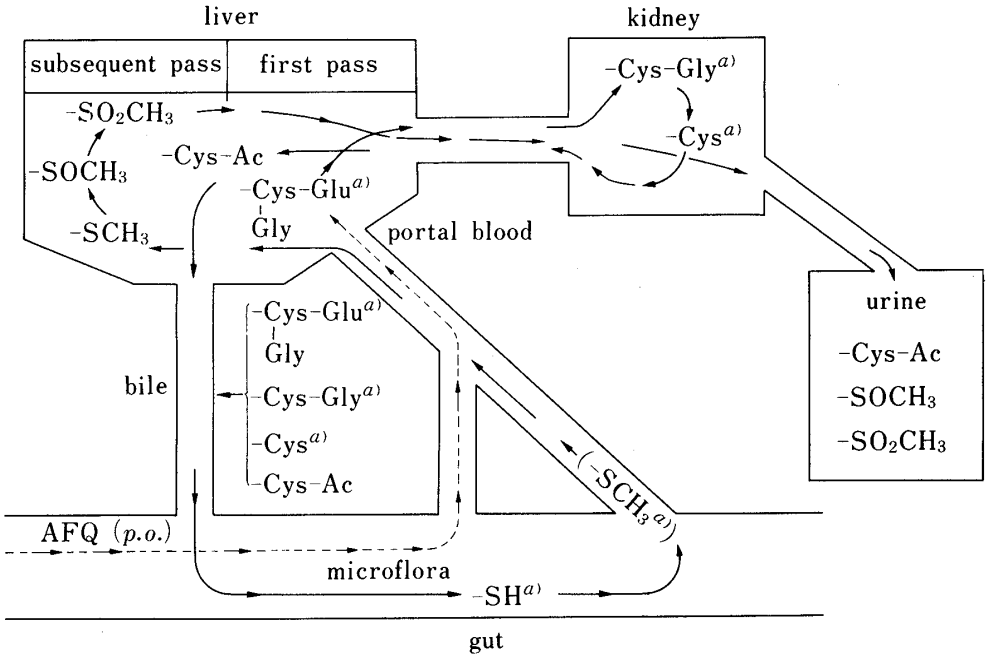

Fig. 5. Schematic Representation of the Formation of the Sulfur-Containing Metabolites of AFQ (Modified from a Figure in Huckel *et al.*, *Drug Metab. Dispo.*, **9**, 360 (1981))

a) Unidentified metabolites.

teine conjugate β-lyase is such that the enzyme is active towards certain cysteine conjugates, but not towards glutathione and mercapturic acid conjugates, and it efficiently cleaves cysteine conjugates of aromatic compounds, but not *S*-alkyl derivatives of cysteine. This is consistent with our finding that AFQM, an *S*-alkyl mercapturic acid conjugate, was not cleaved by the liver, but only after enterohepatic circulation.

It is uncertain so far whether methylation of the thiols produced by the intestinal microflora occurred in the intestinal wall[19] or in the liver.[16] It has been reported that methylthio metabolites undergo further *S*-oxidation by mono-oxygenase in the liver microsomes to form methylsulfinyl and methylsulfonyl derivatives.[20]

Although the mercapturate (AFQM) was certainly a precursor of the sulfur-containing metabolites, the amounts of the sulfur-containing metabolites in the urine after oral administration of AFQM methyl ester were smaller than those after oral administration of AFQ (Table III). This suggests that precursors other than AFQM were present. Bakke *et al.*[18] have reported that the cysteine conjugate, which is a precursor metabolite of the mercapturate, serves as a substrate for the C–S lyase of the intestinal microflora. Additionally Bray *et al.*[21] showed that the glutathione conjugate of *p*-chlorobenzyl chloride was readily hydrolyzed by the extracts of rat small intestine to the corresponding cysteine conjugate. Since the presence of the cysteine and glutathione conjugates of AFQ was suspected because of the resistance of the polar MC fraction to hydrolysis by β-glucuronidase and sulfatase in the bile of rats, a contribution of these conjugates to the formation of the sulfur-containing metabolites is likely.

By taking into consideration all the findings described above, the formation of the sulfur-containing metabolites found in the urine of normal rats can be explained in the following way. AFQ is absorbed from the gastrointestinal tract and first conjugated with glutathione. The glutathione conjugate is subjected to a series of metabolic reactions by the liver and kidney[12] to form the mercapturic acid pathway metabolites including AFQM and other conjugates, and then excreted into the intestinal tract *via* the bile. The biliary metabolites are

split by the intestinal microflora located in the lower gastrointestinal tract to form the thiols. The thiols are reabsorbed, methylated and *S*-oxygenated by either the intestinal wall or liver enzymes, and finally excreted in the urine. The probable routes of formation of the sulfur-containing metabolites are summarized in Fig. 5.

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References and Notes

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