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MECHANISM OF METABOLIC CLEAVAGE OF A FURAN RING

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ABSTRACT:

We studied the mechanism of metabolic cleavage of a furan ring, using a new hypolipidemic agent, ethyl 2-(4-chlorophenyl)-5-(2-furyl)oxazole-4-acetate (TA-1801), as a model compound. A TA-1801 analogue labeled with deuterium at the 5-position of its furan ring was administered orally to rats. The analysis of urinary metabolites by GC/MS revealed that the deuterium of the furan was retained in the ring-opened metabolite (M_3). Metabolic cleavage of furan has been generally considered to proceed by hydroxylation of the 5-position followed by tautomerism and hydrolysis of the resulting 5-hydroxyfuran derivative. However, if the cleavage proceeded by this pathway, the deuterium of the 5-position would be eliminated during hydroxylation. Therefore, we propose that the ring was cleaved directly to form an unsaturated aldehyde, considering the mechanism of oxidation by cytochrome P-450. Although this "intermediate" was not detected in the biological specimens, a synthetic unsaturated aldehyde was transformed to the actual urinary metabolites M_2 and M_3 (major ring-opened metabolites) in the isolated rat liver.

The metabolism of furan ring has not been studied well, whereas hydroxylation of the benzene ring has been known as a very common biotransformation process of the aromatic ring of drugs. The furan rings of many drugs (*e.g.* furosemide, prazosin) has been reported to be stable in their metabolic pathways (1–3), so the metabolism of furan is known for only a few compounds such as a food preservative AF-2[1] (4), a calcium antagonist dichlofurime (5), and a hypolipidemic agent TA-1801 (6). Biotransformation of the furan ring is of interest, because the metabolism observed for these three compounds differs from that of the benzene ring. No hydroxyfuran derivatives were isolated in their study, but the furan rings were cleaved to give carboxypropionyl derivatives. The mechanism of the ring cleavage of furan is unknown.

There is one more report for metabolic cleavage of the furan ring. In the study about the toxicity of 3-methylfuran, a naturally occurring potential atmospheric contaminant (7), Ravindranath *et al.* (8) trapped methylbutenedial formed by metabolic oxidation of 3-methylfuran, by use of semicarbazide in the hepatic microsomal system. However, since the formation of this unsaturated aldehyde was reported to be only in a small amount *in vitro*, the aldehyde has not been considered as an important intermediate in the major metabolic pathway of the drugs that produced ring-opened metabolites *in vivo*. On the other hand, Fur and Labaune (5) reported that the furan of diclofurime was biotransformed *via* an epoxide to a 5-hydroxyfuran derivative by cytochrome P-450 with a process analogous to the major mechanism for benzenoids (5). The resulting metabolite, 5-hydroxyfuran, was reported to be tautomerized to a lactone and hydrolyzed to give a ring-opened metabolite, a carboxypropionyl derivative. This proposed pathway has been generally accepted, although there is no direct evidence.

[1] Abbreviations used are: TA-1801, ethyl 2-(4-chlorophenyl)-5-(2-furyl)oxazole-4-acetate; AF-2, 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; AcOEt, ethyl acetate; TMS, trimethylsilyl.

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TA-1801 is hydrolyzed at the site of the ester bond by the first metabolic reaction. The resulting metabolite M_1 is further metabolized by either glucuronidation at the carboxyl group or cleavage of the furan ring to form the metabolites M_2 and M_3 (6). In the course of the study on TA-1801, we found that a carboxypropionyl derivative (M_2) was formed by oxidation of another metabolite, a hydroxybutyril derivative (M_3). This observation is in conflict with the metabolic pathway proposed for dichlofurime, because the carboxypropionyl derivative was reported to be the first metabolite after ring cleavage in the metabolic pathway of dichlofurime.

Therefore, we studied further the metabolic route of TA-1801 in order to clarify the mechanism of ring cleavage of furan compounds, using a TA-1801 analogue labeled with deuterium at the 5-position of the furan moiety, the "key" position of cleavage. Considering the result of this experiment and the mechanism of oxidation by cytochrome P-450, we propose an unsaturated aldehyde as the first intermediate in the metabolic route of ring cleavage. In this report, we also show that the metabolites, M_2 and M_3, are formed actually from a synthetic unsaturated aldehyde (T-0694), the *trans* form isomer of the proposed intermediate, in the isolated rat liver.

Materials and Methods

Chemicals. TA-1801, T-0694, and the authentic samples of metabolites M_1, M_2, and M_3 were synthesized in the Applied Biochemistry Research Laboratory, Tanabe Seiyaku Co., Ltd. Fig. 1 shows their chemical structures. ^{14}C-TA-1801 was synthesized in our laboratory from 2-^{14}C-bromoacetic acid (Amersham Corp.) as a starting labeled material. It had a specific radioactivity of 10.0 μCi/mg and a radiochemical purity of more than 99% as determined by TLC. Methanol-d_4 and sulfuric acid-d_2 (96–98%) were purchased from E. Merck (Darmstadt, FRG). Their isotope enrichments were more than 99%. Hexamethyldisilazane and BSTFA were obtained from Tokyo Kasei (Tokyo, Japan). Nikkol (HCO-60), a surface-active agent, was purchased from Nikko Chemical (Tokyo, Japan). Fluosol-43 was purchased from Green Cross Corp. (Osaka, Japan).

Deuterium Exchange of Furan Hydrogen of ^{14}C-TA-1801 (synthesis of ^{2}H, ^{14}C-M_1 Methyl Ester). To a stirred solution of 60 mg of ^{14}C-TA-1801 (0.18 μCi/mg) in 2.5 ml of methanol-d_4 was added 0.2 ml of sulfuric

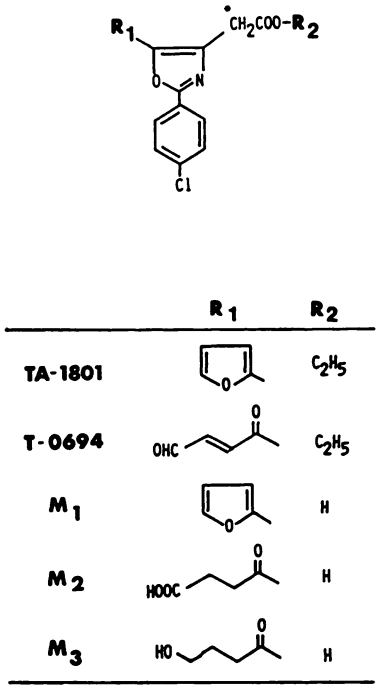

FIG. 1. *Chemical structures of TA-1801 and its related compounds.* The *asterisk* indicates the labeled position of ^{14}C-TA-1801.

acid-d_2 slowly, and the mixture was refluxed for 4 hr. After cooling, in order to avoid the acid-catalyzed re-exchanged of deuterium in the product, 600 mg of $NaHCO_3$ was added. Five drops of 4 M NaOH was added, and the product was extracted into benzene (6 ml × 2), which was separated from the methanol layer, washed with a small volume of saturated NaCl solution, then evaporated to dryness, giving a crude yield of 98.5% calculated by total radioactivity. Its radiochemical purity was 95.4% as determined by TLC on silica gel plates ($60F_{254}$, Merck) developed with benzene/AcOEt/acetic acid (5:4:0.2, v/v) (R_f M0.6). This crude product was supplied to animal experiments without any purification.

Isolation of Urinary Metabolites. ^{2}H, ^{14}C-M_1 methyl ester was administered orally (20 mg/rat) to two male Sprague-Dawley rats (310 g) as a suspension in a 0.1% aqueous Nikkol solution (2 ml/rat).

Urine collected up to 24 hr was adjusted to pH 2.0 and extracted twice with 4 volumes of AcOEt. AcOEt extract was evaporated to dryness, and the residue was subjected to TLC (silica gel plate developed with *n*-hexane/AcOEt/acetic acid = 20:80:4, v/v). Radioactive areas on the plate were detected by TLC scanner (Aloka, TRM-101) and scraped off, and the metabolites were eluted with AcOEt. Identification of metabolites was carried out by comparison of R_f values on TLC and GC/MS data with their authentic samples.

Measurement of Radioactivity. Radioactivity was measured by a Packard Tri-Carb 460 CD liquid scintillation counter after the samples of urine or organic solution were dissolved in Triton X-100-based scintillator (2,5-diphenyloxazole, 4 g; 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene, 0.1 g; toluene, 660 ml; and Triton X-100, 330 ml).

GC/MS and ^{1}H NMR. The GC/MS analysis was performed with a Hitachi M-80A gas chromatograph-mass spectrometer equipped with a 003B data processing system. A glass column (1 m × 3 mm i.d.) packed with 3% OV-1 on Gas-chrom Q (100–120 mesh) was used and heated from 200°C to 290°C at 5°C/min. The carrier gas was helium at 30 ml/min. The injection, separator, and ion source temperatures were 250, 270, and 180°C, respectively. The ionizing voltage was 20 eV. The samples were injected after methylation or trimethylsilylation. Methyl derivatives of metabolites were prepared by treatment of the samples with a large excess of diazomethane in a mixture of diethyl ether and methanol. TMS derivatives were obtained by treatment with sufficient amounts of hexamethyldisilazane or BSTFA at 60°C for 30 min.

^{1}H NMR were recorded on a JEOL FX-100 spectrometer in $CDCl_3$ solution, with tetramethylsilane as an internal standard.

Liver Perfusion. Male Sprague-Dawley rats were anesthetized by diethyl ether. The livers were surgically isolated after cannulation of the bile duct, portal vein, and thoracic inferior vena cava, followed by perfusion with a Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 1.1 g of glucose per liter by the method of Miller (9).

The isolated liver was placed on the waiting platform of the perfusion apparatus which was modified slightly from that described by Miller (9) and perfused with 100 ml of a 20% Fluosol-43 suspension in Krebs-Henseleit bicarbonate buffer (pH 7.4, containing 1.1 g of glucose per liter) at a rate of 9-15 ml/min under 20 cm hydrostatic pressure in the portal vein. In our system, the medium was equilibrated with mixed gas, O_2/CO_2 (95:5), at a perfusate reservoir by bubbling. Drug (0.5 mg) was dissolved in 0.3 ml of dimethyl sulfoxide and added to perfusate.

After addition of the drug, bile was collected from the cannula for 2 hr and analyzed.

HPLC. The HPLC system comprised a Waters Model 660 Solvent Programmer, two Model 6000A pumps, and a Hitachi Model 638-41 uv absorbance detector (310 nm). Separation was effected with a Nucleosil C-18 column (150 × 4.6 mm i.d.) using a linear gradient of 30-60% acetonitrile in 0.01 M phosphate buffer (pH 3.0) over 10 min, at a flow rate of 1.5 ml/min. Final conditions were maintained for 10 min at the end of the program.

Bile was diluted with water and subjected to a filter unit with a pore size of 0.45 μm (Nihon Millipore Co., Ltd., Tokyo, Japan) before injected to HPLC. Biliary metabolites were quantified by comparison of peak heights with those of known amounts of the authentic samples added to control bile.

Results

Deuterium Exchange of Furan Hydrogen of ^{14}C-TA-1801 (Synthesis of ^{2}H, ^{14}C-M_1 Methyl Ester). Fig. 2 shows the mass spectra of authentic M_1 methyl ester (*upper chart*) and the product obtained by treatment of ^{14}C-TA-1801 with methanol-d_4 and sulfuric acid-d_2 (*lower chart*). The molecular ion peaks (cluster) of the product appeared at m/z 320–323, whereas those of M_1 methyl ester appeared at m/z 317–319. This indicates that the ethyl ester moiety in TA-1801 was converted to the methyl-d_3 ester completely. In addition, this suggests that one hydrogen atom somewhere other than the ester was exchanged partly by deuterium. Therefore, it can be seen that ^{14}C-TA-1801 was converted to M_1-d_1 methyl-d_3 ester (d_4-compound) by treatment with methanol-d_4 and sulfuric acid-d_2. The content of d_4-compound was estimated to be 50–60% of the product by comparison

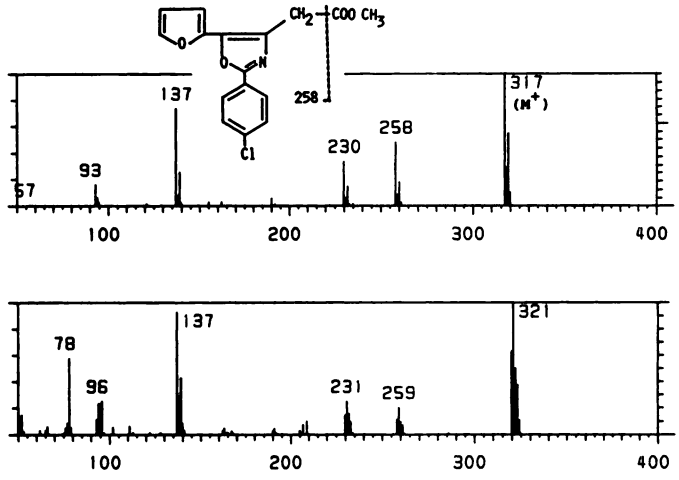

FIG. 2. *Mass spectra of M_1 methyl ester; nonlabeled (upper) and deuterium-labeled (lower).*

of the intensities of the isotope peaks of the molecular ion (M^+) with those of no-deuterium compound.

Fig. 3 shows the aromatic region of a ^1H NMR spectrum of this product. Some changes were seen only in the signals of the furan protons, whereas the other protons showed no change compared with the no-deuterium compound. When the integrated area of each proton signal was compared with the others, only the intensity of the H-5 proton of furan (δ: 7.53 ppm) was decreased to 45% of the other proton. On the other hand, the unusual shape of the H-4 signal (δ: 6.51 ppm) indicates the presence of deuterium at the 5-position of the furan, since the signal is considered to be the mixture of a double doublet ($J_{4,5}$ = 1.7 Hz, $J_{4,3}$ = 3.6 Hz) attributable to the no-deuterium compound and a simple doublet ($J_{4,3}$ = 3.6 Hz) attributed to the deuterated compound. The latter doublet signal was shifted by 0.007 ppm to upfield by the isotope effect. The H-3 signal (δ: 6.69 ppm) did not show the long range coupling with H-5, although the H-5 signal showed the coupling ($J_{5,3}$ = 0.7 Hz) with H-3. This is only explained by the presence of deuterium at the 5-position, since the single doublet ($J_{3,4}$ = 3.6 Hz) of the deuterated compound emerges just over the double doublet of the nondeuterated compound and hides the split due to the long range coupling.

Therefore, it was concluded that the deuterium-labeled position other than the methyl residue of the product was located specifically at the 5-position of furan ring, and its content was 50–60%.

It was difficult to elevate the deuterium content at the 5-position, since the severer or longer reflux exchanged the other hydrogens with deuterium.

Administration of ^2H, ^{14}C-M_1 Methyl Ester and GC/MS Analysis of Its Metabolites. ^2H, ^{14}C-M_1 methyl ester described above was administered orally to rats. This compound was metabolized by the same pathway as TA-1801 (ethyl ester of M_1); *i.e.* hydrolysis of ester gave M_1 and ring cleavage of the furan gave M_2 and M_3 (6). After oral administration, metabolites M_1, M_2, and M_3 were isolated from 24-hr urine. The crude yields of them were 1.1, 1.5, and 2.4 mg, respectively. These metabolites were analyzed with GC/MS after methylation or trimethylsilylation and identified with the authentic samples. Fig. 4 shows their mass spectra. In the spectrum of M_1, the cluster pattern of the molecular ion (*m/z* 375–378) or some fragment ions (*m/z* 360–363, 330–333) differed from that of nondeuterated M_1 which showed only the cluster peaks of chlorine. It was the same as the cluster pattern observed in the spectrum of the administered compound, deuterated M_1 methyl ester (fig. 2). Therefore, the deuterium at the furan ring of administered compound was retained in M_1.

In the mass spectrum of M_3 (fig. 4, *lower*), the molecular ion (*m/z* 467–470) or some fragment ions (*m/z* 452–455, 411–414) also indicated the presence of deuterium. The content of deuterium was considered to be the same as that of the administered compound (fig. 2) or metabolite M_1 (fig. 4, *upper*), because their cluster patterns were the same.

On the other hand, the mass spectrum of M_2 (fig. 4, *middle*) showed the absence of deuterium. The cluster patterns of the molecular ion (*m/z* 365–367) and other fragment ions were the same as the nondeuterated compound. They showed only the presence of chlorine.

Metabolism by Isolated Liver. Isolated livers were perfused with synthesized metabolites M_1, M_2, and M_3. Metabolites excreted in bile after perfusion for 2 hr were quantified by HPLC. The quantities of resulting metabolites are shown in table 1. After perfusion of a liver with M_1, biliary excretion of M_1, M_2, and M_3 was 15.0%, 2.9%, and 1.8% of the dose, respectively, up to 2 hr. After perfusion with M_2, only M_2 was excreted (43.2% of the dose). A liver perfused with M_3, however, excreted not only M_3 (14.4%) but also M_2 (45.6% of the dose).

The experiment of liver perfusion was run by one liver for one compound. So the data of table 1 can not be presented with standard deviations. However, this does not matter, because the purpose of this experiment was to know whether one metabolite might be a precursor of the other or not. In quantification of each metabolite by HPLC, the sensitivity and reproducibility were quite good.

An isolated liver was also perfused with a synthetic unsaturated aldehyde (T-0694) which was an isomer of the proposed intermediate of metabolic cleavage of the furan ring. Fig. 5 shows the HPLC elution profile of bile excreted up to 2 hr from the T-

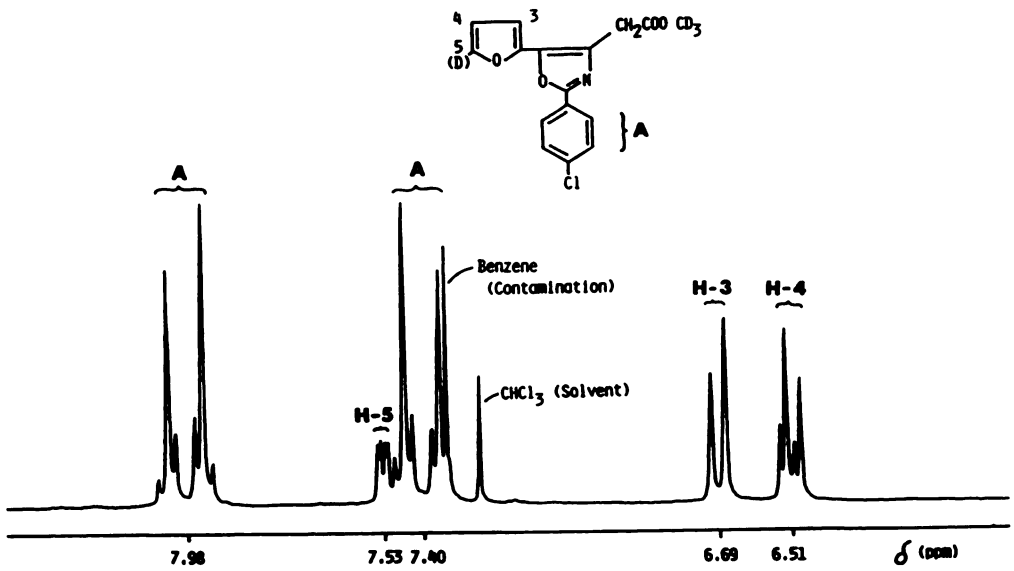

FIG. 3. *^1H NMR spectrum of ^2H-M_1 methyl ester.*

This is an expanded area of interest (δ:6.0–8.5 ppm) taken from the whole spectrum measured in $CDCl_3$ at 100 MHz.

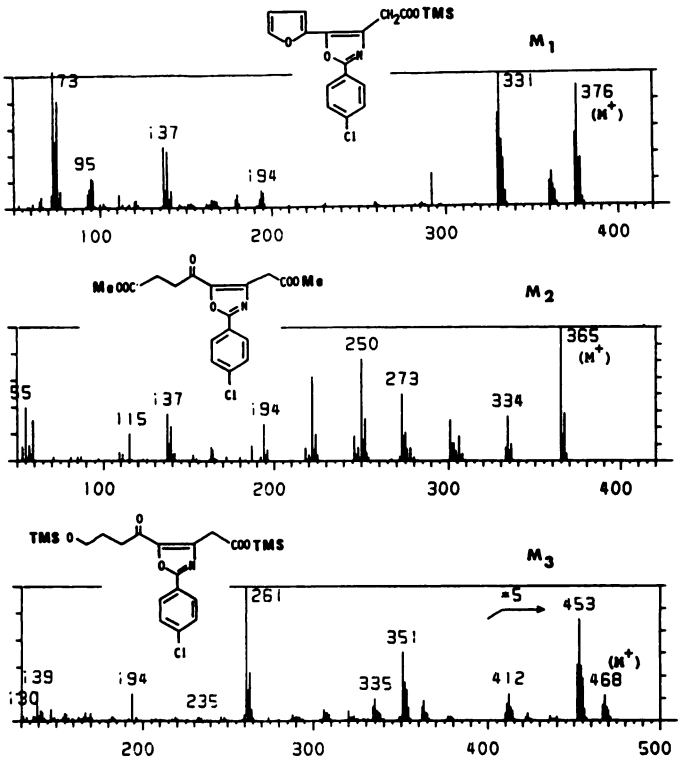

FIG. 4. *Mass spectra of the metabolites M_1, M_2, and M_3 obtained by oral administration of ^2H-M_1 methyl ester to rats.*

The metabolites were isolated from 24-hr urine, and their spectra were measured as the TMS or methyl derivatives.

TABLE 1

Biliary excretion of metabolites M_1, M_2, and M_3 from isolated rat livers after perfusion of M_1, M_2, M_3, and T-0694

Bile was collected for 2 hr after the isolated liver was perfused with the compounds at the dose of 0.5 mg.

Perfused Compounds	Excretion (% of the Dose)		
	M_1	M_2	M_3
M_1	15.0	2.9	1.8
M_2		43.2	
M_3		45.6	14.4
T-0694		3.4	1.6

0694-perfused liver (*B*), and that from a control liver without drug perfusion (*A*). The elution profile indicated the formation of two metabolites which had the same retention times as the authentic samples of M_2 and M_3. The mass spectra of these metabolites were identical with those of synthetic M_2 and M_3. The amounts of M_2 and M_3 excreted in bile were determined by the peak heights in HPLC and shown in table 1. M_2 and M_3 were excreted at the rate of 3.4% and 1.6% of the dose, respectively, up to 2 hr after perfusion of the liver with T-0694.

Discussion

Our experiments with TA-1801 showed that: 1) the deuterium at the 5-position of furan was retained in the hydroxybutyril derivative M_3 after cleavage of the ring, but not in the carboxypropionyl derivative M_2, and 2) in isolated livers, M_2 was formed from M_3, but M_3 was not formed from M_2. Therefore, the metabolic pathway for TA-1801 is possibly $M_1 \rightarrow M_3 \rightarrow M_2$, and the deuterium labeled in M_1 seems to be eliminated during the

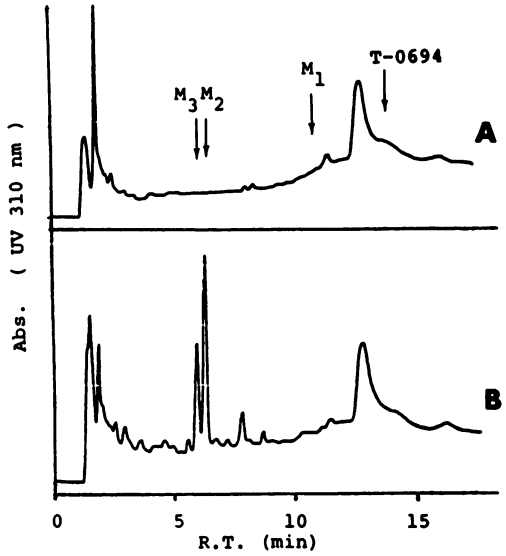

FIG. 5. *HPLC profiles of bile excreted from a no drug-perfused liver (A) and from a T-0694-perfused liver (B).*

HPLC conditions were: Nucleosil C-18 column, 4.6 mm × 15 cm, 1.5 ml/min, 0.01 M phosphate buffer (pH 3.0) with a linear acetonitrile gradient from 30% to 60% in 10 min. In this condition, the authentic samples of drugs and metabolites were eluted with the retention times indicated by the *arrows* over the chromatogram *A*.

oxidative reaction of M_3 to M_2. By these results, we are going to discuss the mechanism of metabolic cleavage of the furan ring.

The furan ring is stable like other aromatic rings, but cytochrome P-450 will be able to attack the ring directly. Although we did not show experimentally the involvement of this enzyme, we obtained a reasonable pathway for ring cleavage by assuming the action of cytochrome P-450.

For several substrates such as vinyl halides (10) and cyclopropylamines (11), cytochrome P-450-catalyzed oxidations were studied and have been postulated to proceed by discrete, one-electron steps. The first step of cytochrome P-450-catalyzed oxidation of aromatic rings is thought to be removal of an electron of a substrate by a perferrylcytochrome [Fe(V)═O] (12). the resulting radical cation of the substrate could undergo radical-radical reaction, or could react with nucleophiles at the cationic center. In many cases, it would react immediately with the nearby [Fe(IV)—O] species of cytochrome to form a substrate-O-Fe(IV)-enzyme complex (fig. 6). After the formation of this complex, there are two possible pathways of the opening of furan ring. Route A is a pathway *via* epoxide formation proposed by Fur and Labaune (5) on the analogy of the well-known metabolic reaction for benzenoids. Route B is a pathway *via* the formation of unsaturated aldehyde which Ravindranath *et al.* (8) reported in the study of 3-methylfuran. Route B proceeds by direct cleavage of the C—O bond of the furan ring when the oxygen is transferred to the substrate from cytochrome P-450. This type of cleavage does not occur for the benzenoid because the C—C bond of the benzene ring is stable enough. Metabolic oxidation of the benzene ring will proceed by only route A, the formation of the epoxide.

If cleavage occurred by route A, the deuterium at the 5-position of the furan of TA-1801 would be eliminated in the course of the formation of the 5-hydroxyfuran from the epoxide, (migration of deuterium like the NIH shift (13) would be denied by the absence of deuterium in M_2), and the first ring-opened product

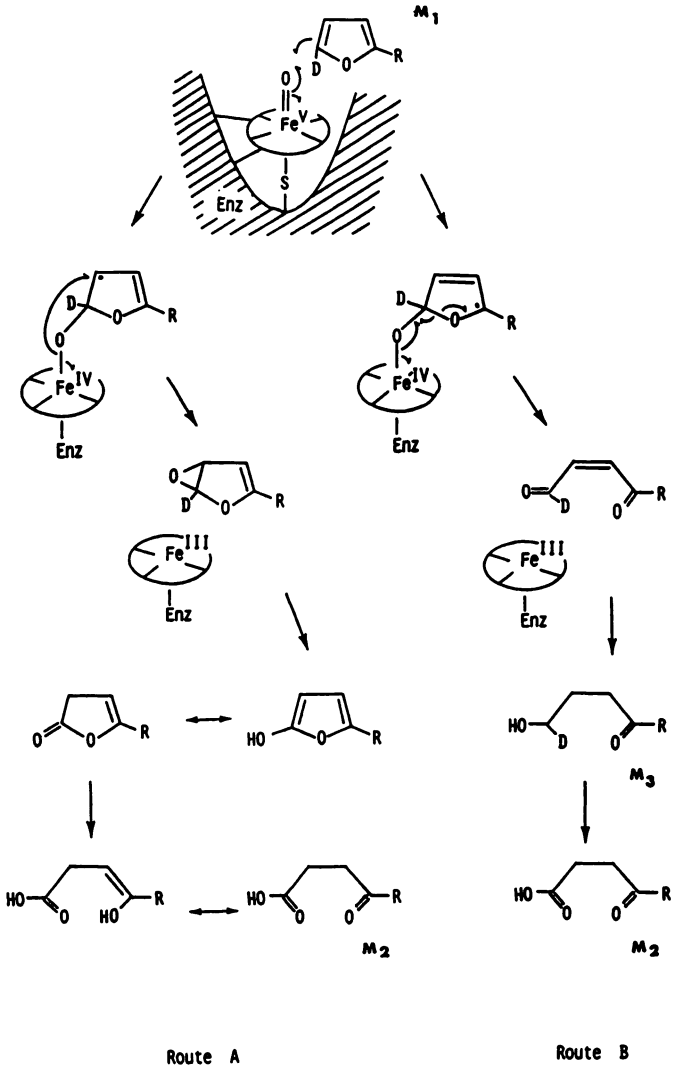

FIG. 6. *Possible metabolic routes for cleavage of the furan ring.*

D, deuterium labeled at the furan ring of TA-1801.

would be M_2. These do not coincide with the results described above.

If cleavage underwent by route B, *i.e.* if the furan ring of TA-1801 was metabolized into unsaturated aldehyde, followed by its metabolic reduction to a saturated alcohol, the metabolite M_3 would be formed with retention of deuterium. Therefore, route B is reasonable for the results of our experiments, *i.e.* the retention of deuterium in M_3 and the formation of M_3 prior to M_2.

In order to confirm our speculation that this unsaturated aldehyde might be a "real" intermediate, we investigated the formation of M_3 and M_2 from this "intermediate" by the experiment of liver perfusion. Since the synthesized sample of the postulated intermediate (*cis* form) was very labile and isomerized spontaneously to the *trans* form, this *trans* isomer (ethyl ester, T-0694) was used for our experiment. Although T-0694 are not transformed spontaneously to M_2 or M_3 in buffer solution (pH 7.4, 37°C), it was reduced to the saturated alcohol (M_3) and then oxidized to the carboxylic acid (M_2) in the isolated liver.

Therefore, it is concluded that TA-1801 is metabolized *via* route B. Other furans such as AF-2 and diclorfurime might be metabolized also *via* route B.

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