11.5-kb Sst I–Eco RI fragment and the sequence encoding the carboxy terminus lies within the 5-kb Eco RI–Sst I fragment (Fig. 3B, dashed line).

Studies of the molecular genetics of toxin formation will not only provide valuable tools for the study of toxin structure and function but may also lead to the development of nontoxic cross-reacting proteins and synthetic vaccine candidates.

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7. A 0.1-ml sample of a culture grown overnight in brain-heart infusion broth (BHI) at 34°C was used to inoculate a Columbia blood agar plate (England Laboratories, 100 by 15 mm) containing 5 percent sheep blood and 2 percent agar. The cultures were incubated anaerobically (Gas Pak System, Baltimore Biological Laboratories) at 34°C for 20 to 24 hours. The cells were then removed with cotton swabs and resuspended in 50 m*M* glucose, 25 m*M* tris (*p*H 8.0), and 25 m*M* EDTA (solution 1), with 1 ml of buffer for each plate. Plasmid DNA was isolated from 1 ml of this suspension as described by D. Ish-Horowicz and J. F. Burke [*Nucleic Acids Res.* **9**, 2989 (1981)].
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Reactive Metabolites from the Bioactivation of Toxic Methylfurans

Abstract. *An important mechanism of toxicity of furans involves the cytochrome P-450 monooxygenase–catalyzed bioactivation of the compound in situ directly within the target tissues to highly reactive electrophilic products. The unsaturated aldehydes acetylacrolein and methylbutenedial have been identified as the principal reactive intermediates of 2- and 3-methylfuran, respectively, that are produced and bound covalently to tissue macromolecules in hepatic and pulmonary microsomal systems in vitro.*

Reduced nicotinamide adenine dinucleotide phosphate–dependent (NADPH) and oxygen-dependent activation of toxic furan compounds catalyzed by cytochrome P-450–dependent microsomal monooxidases results in the formation of highly reactive metabolites that bind covalently to tissue macromolecules (*1–4*). Epoxidation of the furan ring has been thought to be involved in the activation, a process analogous to the major mechanism of metabolism for benzenoid compounds. In addition, aflatoxin B_1 is almost certainly activated by epoxidation of the dihydrofuran ring in its structure (*5–7*).

Evidence that a furan epoxide is the intermediate in mixed-function oxidase metabolism of simple furans is lacking:

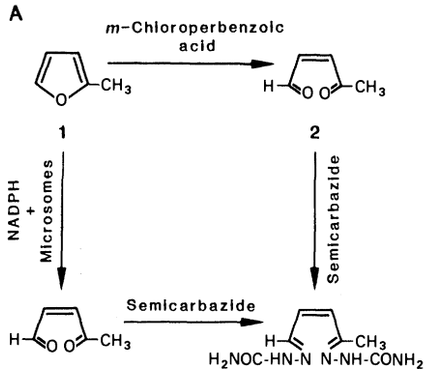

Fig. 1. Scheme of the chemical syntheses of (A) AA and (B) MBD and their formation from in vitro microsomal incubations containing 2-MF and 3-MF, respectively (see *17*).

no furan epoxides have been isolated and no metabolites resulting from attack of nucleophiles (for example, water to form dihydrodiols) have been identified. Although evidence exists for formation of glutathione adducts of 4-ipomeanol (*8*), the structures of the adducts remain to be determined.

Indirect evidence for epoxidation of the furan-containing drug furosemide has been reported. Addition of 1,2-epoxy-3,3,3-trichloropropane (TCPO) to mouse liver microsomal incubation mixtures has been found to result in a nearly twofold increase in covalent binding (*9*). The increase was attributed to the inhibition of epoxide hydrolase by TCPO, resulting in higher concentration of the active metabolite in the microsomes. Addition of TCPO has scant effect on the covalent binding of either 4-ipomeanol (10) or 2-(*N*-ethylcarbamoylhydroxymethyl)furan (*11*). Either an epoxide is not formed in the latter two cases, or an epoxide is formed but is not a substrate for the hydrolase, or an epoxide is formed which is so reactive that it undergoes alternate reactions before reaching the hydrolase enzyme.

Other evidence indicates that cytochrome P-450–catalyzed oxidations may proceed by discrete, one-electron steps. Oxidation by removal of a hydrogen atom or an electron as an initial step in cytochrome P-450–catalyzed metabolism has been postulated for several substrates, such as vinyl halides (*12*), cyclopropyl amines (*13*), organosulfides (*14*), and norbornane (*15*). The result of such a one-electron oxidation of a furan by the cytochrome P-450 perferryl intermediate [Fe(V)O] is the formation of a radical cation in which the radical or cationic site or both can be stabilized by the furan oxygen. Such a species may be stable enough to leave the site of formation but too reactive to migrate far. Cationic radicals could undergo radical-radical reactions or hydrogen atom abstraction or could react with nucleophiles at the cationic center. A radical-radical reaction between the furan radical cation and the Fe(IV)O species (which has radical character) would be facile, and the cationic

intermediate obtained could close to give an epoxide. A lower energy pathway, however, might be a ring-opening reaction to form the unsaturated dialdehyde without the epoxide intermediate. Unsaturated aldehydes have reactive functionality and could react with tissue constituents by either Michael addition to the double bond or nucleophilic addition to the aldehyde (*16*). The following experiments were therefore designed to determine whether such intermediates are formed from 3-methylfuran (3-MF) and 2-methylfuran (2-MF) and to assess to what extent they could be involved in the covalent binding and toxicity of these methylfurans.

We first attempted to use nucleophilic sulfhydryl trapping reagents to obtain stable conjugates of the unsaturated aldehydes for structural studies. Even though such adducts were apparently formed rapidly with glutathione, cysteine, and *N*-acetylcysteine, they were not stable enough to allow isolation. The use of semicarbazide was therefore explored as an alternate trapping agent suitable for the suspected dialdehyde intermediates.

The disemicarbazones of the suspected unsaturated aldehyde products from 2-MF and 3-MF (Fig. 1)—acetylacrolein (AA) and methylbutenedial (MBD), respectively—were first prepared by chemical syntheses (*17*). Rat hepatic microsomal incubations were then performed in the presence and absence of NADPH, semicarbazide, and 2-MF or 3-MF. High-performance liquid chromatography (HPLC) analyses (Fig. 2) of extracts from incubations with 3-MF showed peaks corresponding to the synthetic disemicarbazone of MBD. When NADPH or semicarbazide or both were deleted, no such peaks were observed. A similar experiment (*18*) performed with rat lung microsomes and 3-MF also resulted in a peak corresponding to MBD disemicarbazone and again only from incubations containing both semicarbazide and NADPH. A small amount of MBD disemicarbazone was detectable in the absence of NADPH, probably due to air oxidation of 3-MF. Similar results were observed with AA formation from 2-MF. Subsequent preparative HPLC, purification by gel permeation chromatography, and further comparisons by mass spectroscopy with the AA and MBD disemicarbazone standards confirmed the identities of the AA and MBD disemicarbazones produced in the microsomal incubations (Fig. 3).

Another experiment was designed to determine whether these aldehydes were responsible for the covalent binding of 3-MF observed in vitro. When a compari-

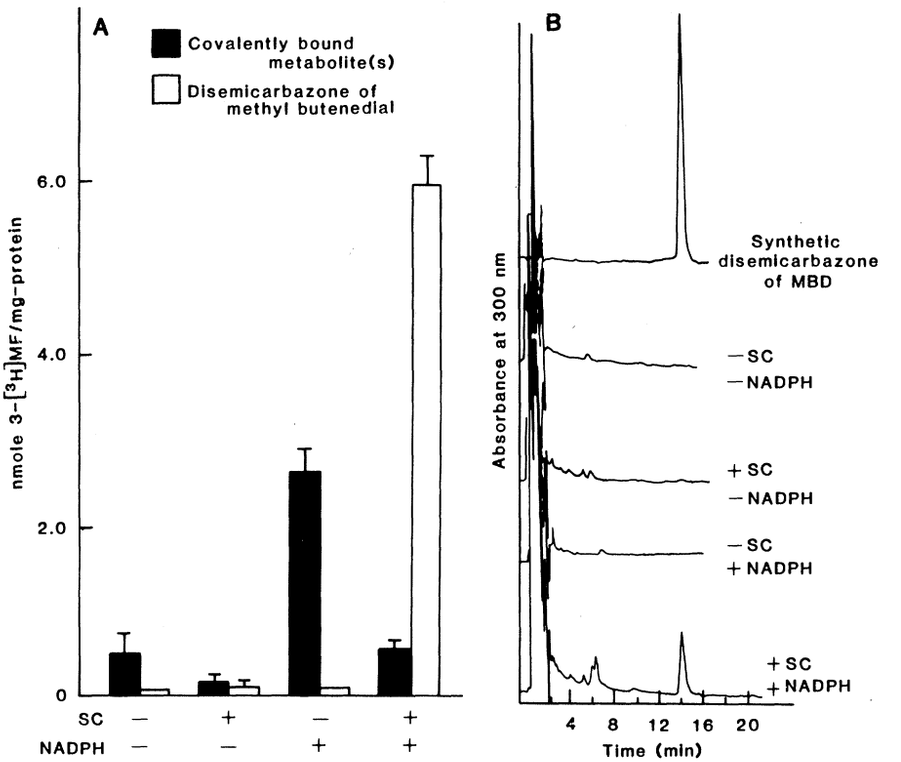

Fig. 2. (A) Effect of semicarbazide on covalent binding of 3-[^{3}H]MF to pulmonary microsomal protein and (B) the HPLC analyses of the metabolite formed (see *18*).

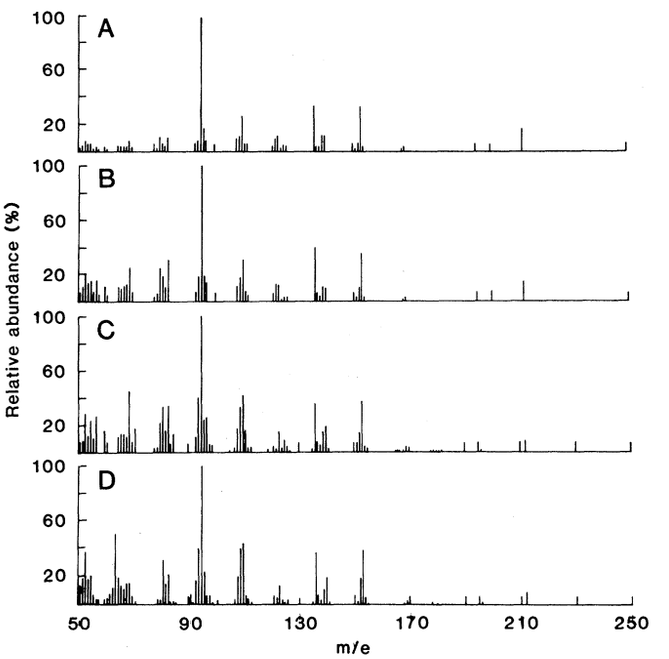

Fig. 3. Mass spectra of (A) synthetic disemicarbazone of AA, (B) metabolite from microsomal incubation containing 2-MF and semicarbazide (SC), (C) synthetic disemicarbazone of MBD, and (D) metabolite from incubation containing 3-MF and SC (M^+ = 212 in all cases). Spectra were obtained from LKB model 9000 mass spectrometer in the electron impact mode with a block temperature of 290°C and an electron voltage of 70 eV.

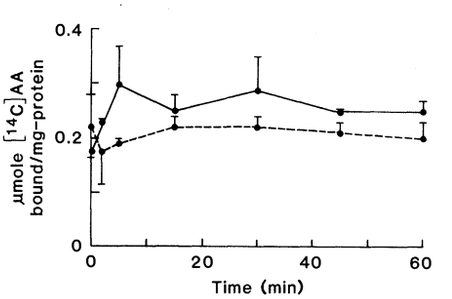

Fig. 4. Covalent binding of [^{14}C]AA to rat liver microsomes in the presence (dashed lines) and absence (solid lines) of NADPH (see *19*).

son was made of the covalent binding and the amounts of MBD disemicarbazone produced in pulmonary microsomal incubations in the presence and absence of NADPH and semicarbazide (Fig. 2), there was an inverse relation between the two measures. Semicarbazide inhibited the NADPH-dependent covalent binding of 3-MF, presumably by trapping the reactive aldehyde intermediate (MBD) before it could react and bind irreversibly to the microsomal macromolecules. Enzyme-catalyzed binding was not observed in the absence of NADPH. Although the presence of semicarbazide prevented covalent binding of 3-MF metabolite to microsomal protein, a twofold increase was observed in the amount of 3-MF metabolized.

Finally, we investigated the possibility that the unsaturated aldehyde might require further activation (for example, by oxidation of the olefinic linkage) before it was bound. However, synthetic AA was shown to be bound quickly in microsomal incubations (*19*), and there was no further enhancement by the presence of NADPH (Fig. 4). Thus, it appears that the aldehyde is the principal binding species.

These results provide new insight into the metabolic activation of toxic furans. At least in the case of the alkylfurans, the unsaturated aldehyde intermediates are the products of microsomal metabolism. Therefore, these reactive compounds may be the ultimate toxic metabolites responsible for target tissue alkylation and for toxicity produced by the parent furans in vivo. Epoxides, even if formed transiently, appear not to play a major role in the covalent binding nor in the toxicity of these simple furans.

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17. (A) Acetylacrolein **2** (AA), synthesized by *m*-chloroperbenzoic acid oxidation of 2-MF **1** [N. Clauson-Kaas and J. Fakstorp, *Acta Chem. Scand.* **1**, 415 (1947)], was derivatized to the disemicarbazone **3** with semicarbazide hydrochloride. (B) 3-MF, **4**, on reaction with bromine and methanol, yielded dihydrodimethoxy 3-MF **5** [J. A. Hirsch and A. J. Szur, *J. Heterocycl. Chem.* **9**, 523 (1972)] which was cleaved to methylbutenedial **6** (MBD) in situ with semicarbazide hydrochloride to give the disemicarbazone of MBD **7**. The identities of all the products were confirmed by mass spectral analyses (Fig. 4) and by nuclear magnetic resonance [solvent, D_6-dimethylformamide; internal standard, tetramethylsilane; disemicarbazone of AA, δ (ppm) 10.15 (1H, s), 9.42 (1H, s), 6.52 (3H, m), 2.02 (3H, s); disemicarbazone of MBD, δ (ppm) 10.22 (1H, s), 10.15 (1H, s), 6.34 (1H, s), 6.5 (2H, m), 2.04 (3H, d)].
18. 3-[^{3}H]MF was synthesized as described by R. B. Franklin *et al.* [*J. Labelled Compd. Radiopharm.* **15**, 569 (1978)]. Lung microsomes were prepared from male Sprague-Dawley rats (*10*). Incubations were performed in the presence (+) or absence (−) of 60 m*M* semicarbazide (SC) or 4 m*M* NADPH or both, as indicated, in triplicate at 37°C for 30 minutes with a total volume per incubation of 2 milliliters of phosphate buffer containing 2 mg microsomal protein and 20 m*M* 3-[^{3}H]MF (specific activity, 1 mCi/mmole). Incubations were run in an air atmosphere and terminated by the addition of 2 ml of ice-cold methanol. After centrifugation, the supernatant was analyzed by HPLC for the metabolites and the precipitated proteins were assayed for covalently bound radioactivity (*10*). The HPLC analysis of the supernatants and the synthetic standard MBD disemicarbazone were performed on a Whatman Partisil 5-ODS3 column with 7 percent acetonitrile-water as mobile phase. Similar HPLC conditions were used to separate MBD disemicarbazone from rat hepatic microsomal incubations containing 3-MF and to identify AA-disemicarbazone from rat pulmonary and hepatic microsomal preparations containing only 2-MF in the presence of both SC and NADPH. The HPLC retention volumes for the disemicarbazones of MBD and AA were 14.4 and 9.14 minutes, respectively.
19. [^{14}C]Acetylacrolein was obtained by oxidation of 2-[^{14}C]MF, which was synthesized by reaction of 2-furyl lithium with [^{14}C]methyl iodide and purified by preparative gas chromatography, and the chemical identity was established by mass spectral measurement. Oxidation of 2-[^{14}C]MF (1.2 mmole; specific activity, 0.0017 mCi/mmole) by *m*-chloroperbenzoic acid gave [^{14}C]AA, which was purified by flash chromatography on silica gel (specific activity, 0.0017 mCi/mmole; radiochemical purity > 97.5 percent), and chemical identity was established by mass spectral measurement ($M^+ = 98$) and NMR [solvent, $CDCl_3$; internal standard, tetramethylsilane; δ (ppm) 2.38 (3H, s), 6.15 (1H, dd, J = 12 and 7 Hz), 6.96 (1H, d, J = 12 Hz), 10.2 (1H, d, J = 7 Hz)]. Liver microsomes were prepared from Sprague-Dawley rats (*10*). Microsomal incubations were run in triplicate at 37°C and in a total volume of 5 milliliters of phosphate buffer containing 50 m*M* [^{14}C]AA, 5 mg of microsomal protein, and, where appropriate, 10 m*M* NADPH. Equal portions (0.5 ml) were removed at specified time intervals and transferred into equal volumes of ice-cold methanol, and the microsomal protein was assayed for covalently bound radioactivity (*10*).

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Synthesis of Compounds with Properties of Leukotrienes C_4 and D_4 in Gerbil Brains After Ischemia and Reperfusion

Abstract. *6-Sulfidopeptide–containing leukotriene-like immunoreactivity was synthesized in gerbil forebrains after bilateral common carotid occlusion and reperfusion. At 5, 10, or 15 minutes of ischemia, concentrations increased significantly and became more marked on reperfusion. Immunoreactivity was highest in forebrain gray matter and was below the detection limit of the assay in brain regions remote from the zone of ischemia. In vitro experiments with vascular cells and organ cultures of cerebral arteries indicate that the cerebral blood vessel wall is not a major source of biosynthetic activity in the brain. These experiments demonstrate leukotriene biosynthesis by the brain. Because synthesis occurs during ischemia and reperfusion and because leukotrienes are potent vasoconstrictors and promoters of tissue edema, they may play a role in the pathophysiology of cerebral ischemia.*

The 6-sulfidopeptide–containing leukotrienes [slow reactive substances (SRS's)] are a family of pharmacologically active peptidolipids that are synthesized from arachidonic acid by way of the lipoxygenase pathway (*1*). These molecules are more potent than other derivatives of arachidonic acid (such as the prostaglandins) in their ability to promote plasma leakage and constrict blood vessels (*2*). During cerebral ischemia, deacylation of membrane phospholipids and release of arachidonic acid occurs (*3*). During reperfusion, arachidonic acid is converted enzymatically to products of the cyclooxygenase pathway (prostaglandins) (*4*). It has been suggested that prostaglandin accumulation after stroke may contribute to decreases in blood flow and cerebral edema formation in the affected brain region (*5*). This is partially confirmed by studies showing that blood flow increases in the brain's microcirculation after treatment with a combination of indomethacin, heparin, and prostacyclin (*6*). However, in other experiments brain edema did not abate despite adequate inhibition of prostaglandin synthesis by indomethacin (*4*). Since arachidonic acid is a precursor for SRS's, and since SRS's, in relatively small amounts, produce vasoconstric-