

Slow Oxidation of Acetoxime and Methylethyl Ketoxime to the Corresponding Nitronates and Hydroxy Nitronates by Liver Microsomes from Rats, Mice, and Humans

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Acetoxime and methylethyl ketoxime (MEKO) are tumorigenic in rodents, inducing liver tumors in male animals. The mechanisms of tumorigenicity for these compounds are not well defined. Oxidation of the oximes to nitronates of secondary-nitroalkanes, which are mutagenic and tumorigenic in rodents, has been postulated to play a role in the bioactivation of ketoximes. In these experiments, we have compared the oxidation of acetoxime and methylethyl ketoxime to corresponding nitronates in liver microsomes from different species. The oximes were incubated with liver microsomes from mice, rats, and several human liver samples. After tautomeric equilibration and extraction with n-hexane, 2-nitropropane and 2-nitrobutane were quantitated by GC/MS-NCI (limit of detection of 250 fmol/injection volume). In liver microsomes, nitronate formation from MEKO and acetoxime was dependent on time, enzymatically active proteins, and the presence of NADPH. Nitronate formation was increased in liver microsomes of rats pretreated with inducers of cytochrome P450 and reduced in the presence of inhibitors (n-octylamine and diethyldithiocarbamate). Rates of oxidation of MEKO (v_{\max}) were 1.1 nmol/min/mg (mice), 0.5 nmol/min/mg (humans), and 0.1 nmol/min/mg (rats). In addition to nitronates, several minor metabolites were also enzymatically formed (two diastereoisomers of 3-nitro-2-butanol, 2-hydroxy-3-butanone oxime and 2-nitro-1-butanol). Acetoxime was also metabolized to the corresponding nitronate at rates approximately 50% of those observed with MEKO oxidation in the three species examined. 2-Nitro-1-propanol was identified as a minor product formed from acetoxime. No sex differences in the capacity to oxidize acetoxime and MEKO were observed in the species examined. The observed results show that formation of sec-nitronates from ketoximes occurs slowly, but is not the only pathway involved in the oxidative biotransformation of these compounds. Due to the lack of sex-specific oxidative metabolism, other metabolic pathways or mechanisms of tumorigenicity not involving bioactivation may be involved in the sex-specific tumorigenicity of ketoximes in rodents.

Key Words: sex-specific tumorigenicity; oximes; nitronates; mouse; rat; human.

Ketoximes are chemicals widely used in technical products, some ketoximes such as methylethyl ketoxime (MEKO) are also present in consumer goods such as paints and sealants. MEKO and several other oximes show a relatively low acute toxicity, but MEKO induces liver tumors in male mice and male rats at the highest exposure concentration, but not in female mice and rats after inhalation (15, 75, and 375 ppm, 6 h per day, 5 days per week, for 18 months in mice and 26 months in rats) exposure (IHF, 1993a,b). Carcinomas were statistically increased in male mice and rats at the 375-ppm exposure level. Sex-specific tumor induction in male rats was also observed with acetoxime after administration in drinking water (Mirvish *et al.*, 1982). The mechanisms of sex-specific tumorigenicity of MEKO and related oximes are not well defined. MEKO is not mutagenic in *Salmonella typhimurium* and does not induce DNA-damage in primary hepatocytes obtained from male rats (IHF, 1995). However, some other oximes are mutagenic in *Salmonella typhimurium* (Rogers-Back *et al.*, 1988).

Since the observed sex-differences in tumorigenicity of MEKO and acetoxime are possibly based on different biotransformation reactions in male and female rodents, the mechanisms of biotransformation for oximes need to be elucidated. Acetoxime and MEKO may be oxidized by cytochromes P450 or FMO to give alkylnitronates. Evidence for an oxidation of acetoxime to propane 2-nitronate has been presented (Kohl *et al.*, 1992). Sec-alkylnitronates are potent mutagens in bacteria and induce DNA-damage (Goggelmann *et al.*, 1988), they are tautomers of the anion of sec-nitroalkanes and their formation has been implicated in the mechanisms of tumorigenicity of several sec-nitroalkanes (Conaway *et al.*, 1991; Fiala *et al.*, 1987, 1989; Kohl *et al.*, 1994).

In this study, we comparatively quantified the oxidation of MEKO and acetoxime to the respective nitronates in liver microsomes from both sexes of three species, in order to elucidate possible sex differences which could be responsible for the gender-difference tumors in response to ketoximes in rodents. The results obtained did not show sex differences in the capacity of liver microsomes to oxidize MEKO and acetoxime. Moreover, a 10-fold difference in the capacity for

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ketoimine oxidation between mice and rats does not support a decisive role for ketoimine oxidation in tumorigenicity.

MATERIAL AND METHODS

Chemicals. Standard solutions of propane 2-nitronate and butane 2-nitronate were generated from 2-nitropropane and 2-nitrobutane by dissolving in 1 N sodium hydroxide at room temperature and storing the solution in the dark for 0.5 hours. 2-Nitrobutane was synthesized by the method of Kornblum *et al.*, (1956), with 2-bromobutane as starting material. The obtained product had a purity of >98% as checked by GC/MS and was characterized by ¹H-NMR, ¹³C-NMR, and mass spectrometry.

¹H-NMR (250 MHz, CDCl₃): δ (ppm) = 1.6 [d, 3H, C-1], 4.5 [m, 1H]; 1.9 [m, 2H]; 0.9 [t, 3H]. ¹³C-NMR (63 MHz, [DEPT], CDCl₃): δ (ppm) = 18.8 [s, C-1], 84.9 [s, C-2], 28.4 [s, C-3], 10.1 [s, C-4]. Mass spectrum (electron impact, 70 eV): m/z (relative intensity) = 41 (100%) [M⁺-CH₃-NO₂-H], 57 (88%) [M⁺-NO₂], 104 (2%) [MH⁺]. Mass spectrum (chemical ionization, negative-ion detection): m/z = 46 [NO₂⁻], 102 [M⁻-H], 86 [M⁻-OH].

MEKO was supplied by AlliedSignal (Morristown, NJ) with a purity of 99.5% as determined by GC/MS. Acetoxime, 2-nitropropane, 2-nitro-1-propanol, 3-nitro-2-butanol, and all other chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) in the highest purity available.

Animals and treatment. Adult female and male B6C3F1 mice and female and male Wistar rats used for all studies were obtained from Harlan-Winkelmann (Borchen, Germany). Cytochrome P450 induction experiments were performed according to literature protocols with phenobarbital (Benoit *et al.*, 1992), 3-methylcholanthrene (Benoit *et al.*, 1992), and pyridine (Dekant *et al.*, 1995). Twenty-four hours after the last administration of the inducers, animals were killed by cervical dislocation.

Microsome preparation. Human liver specimens were obtained from the Keystone Skin Bank (Exton, PA) and the liver bank in Kiel, Germany. Human liver samples, medically unsuited for liver transplantation were also acquired, under the auspices of the Washington Regional Transplant Consortium (Washington, DC). For more details on donors see (Harris *et al.*, 1994; Urban *et al.*, 1994). All procedures for microsome preparation were performed at 4°C. To minced livers (rats) and tissue specimens (human), a three-fold volume (w/v) of 0.154 M KCl (pH 7.2–pH 7.4) was added and tissues were homogenized. The homogenate was then centrifuged at 12,500 g for 10 min and the resulting supernatant subjected to a centrifugation at 160,000 g at 4°C for 60 min to sediment microsomes. The microsomal pellet was resuspended in 0.01 M phosphate buffer (pH 7.4) and centrifuged again for 60 min at 160,000 g at 4°C. The resulting pellet was suspended in 0.01 M phosphate buffer (pH 7.4) and aliquots of the microsomal preparations were quickly frozen in liquid nitrogen and stored at -70°C. Protein concentrations were determined with a Bio Rad protein assay kit with bovine serum globuline as standard (Bradford, 1976).

Incubation conditions. MEKO (0.1–10 mM) and acetoxime (5 mM) were incubated with liver microsomal protein (3 mg/mL) in 0.1 M phosphate buffer (pH 7.4) containing 5 mM NADPH in a total volume of 500 μL in all experiments. The reaction mixture was preincubated for 5 min at 37°C in the absence of MEKO or acetoxime and started by the addition of MEKO or acetoxime. For discrimination between flavin-containing monooxygenases and cytochrome P450 enzymes, the chemical inhibitors n-octylamine (5 mM), diethylthiocarbamate (0.1 mM and 0.3 mM), or coumarin (0.05 mM) were preincubated in the presence of NADPH for 5 min at 37°C before addition of MEKO. After 20 min (0, 10, 20, or 30 min in the case of time-dependent incubations) at 37°C, the reactions were stopped by putting the vials on ice. Microsomal protein was removed by centrifugation at 160,000 g for 15 min at 4°C. After centrifugation, the supernatant was analyzed as described below.

Quantitation of nitronate formation in subcellular fractions. To an aliquot (450 μL) of the supernatant of microsomal incubations, 10 nmol of the internal standard (propane 2-nitronate in incubations with MEKO and butane 2-nitronate in incubations with acetoxime) and 1 mL n-hexane were added, and

samples were vigorously stirred for 20 h at room temperature. The organic layer was then separated and dried on MgSO₄, 1-nitropentane (10 nmol) was added as a second internal standard and aliquots (2 μL) of the sample were analyzed by GC/MS with splitless injection (valve time 1.0 min).

GC/MS analyses. GC/MS analyses were performed on a Fisons Trio 2000 mass spectrometer coupled to a Carlo Erba 8000 series GC and equipped with an AS 800 autosampler (Fisons Instruments, Mainz, Germany). For separations of 2-nitropropane and 2-nitrobutane, a DB WAX (J and W Scientific, Folsom, CA) fused-silica capillary column (30 m, 0.25 mm I.D., 0.25 μm film thickness) with helium as carrier gas (33 cm/sec) were used under the following conditions: oven temperature at 40°C for 4 min followed by a linear program from 40°C to 150°C with a heating rate of 15°C and held at 150°C for 2 min. Injector temperature was 180°C and transfer line temperature was 220°C. The ion source temperature was adjusted to 110°C and methane was used as reactant gas. Quantitation of 2-nitropropane, 2-nitrobutane, and 1-nitropentane was performed with chemical ionization and negative ion detection in the single ion monitoring mode with a detection limit of 250 fmol/2μL injection volume. Characteristic fragments [m/z = 102 (M⁻-H) and m/z = 46 (NO₂⁻)] were monitored for the quantitation of 2-nitrobutane in relation to the internal standards of 2-nitropropane and 1-nitropentane. Peak areas were referenced to calibration curves with authentic material. Calibration curves were obtained from defined concentrations of the nitronates added to the incubation buffer, followed by sample preparation procedures identical to those with microsomal incubations.

For separation and quantitation of 3-nitro-2-butanol, 2-nitro-1-propanol, and the other minor metabolites, the following GC-conditions were used: oven temperature of 45°C for 1 min followed by a linear program from 45°C to 230°C with a heating rate of 15°C and hold at 230°C for 3 min. No other parameters were changed. The metabolites were quantified by selected ion monitoring (SIM) after chemical ionization and negative-ion detection. During the gas chromatographic separation, the following ions were monitored to quantitate the minor metabolites: m/z = 119 (M⁻) and 46 (NO₂⁻), 3-nitro-2-butanol; m/z = 105 (M⁻) and 46 (NO₂⁻), 2-nitro-1-propanol and m/z = 119 (M⁻) and 46 (NO₂⁻), 2-nitro-1-butanol or 3-nitro-1-butanol.

To confirm the presence of 2-nitro-1-propanol, 3-nitro-2-butanol and the other metabolites, another type of fused-silica capillary column, DB FFAP (30 m, 0.25 mm ID, 0.25 μm film thickness) (J & W Scientific, Folsom, CA) was used, with the following separation conditions: injector temperature of 220°C, oven temperature of 45°C followed by a linear program from 45°C to 200°C with a heating rate of 10°C holding the 200°C for 2 min. All other parameters were used as described above.

RESULTS

Metabolite Identification

When acetoxime and MEKO were incubated with microsomal fractions from rodent liver in the presence of oxygen and NADPH, GC/MS analysis of the extracts of these incubations showed the presence of new peaks. Under the conditions of the sample workup, the nitronates formed by oxidation of the oximes tautomerize to the corresponding nitroalkanes (Nielsen, 1969) which are extracted into n-hexane and may be analyzed by GC/MS. Figure 1 shows the gas chromatographic separation of the hexane layer from an incubation of MEKO with liver microsomes from male mice. A large peak (R_T = 8.3 min) was observed that had not been present in control incubations without MEKO or NADPH or in heat-treated microsomes. The mass spectrum (Fig. 1, top) of the peak was identical to that of 2-nitrobutane. Moreover, the retention time of the reference compound and the compound formed by microsomal oxidation

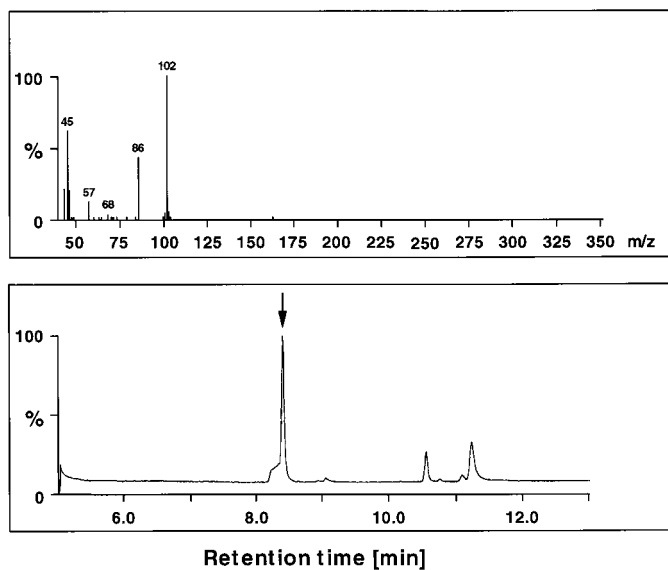


FIG. 1. GC/MS-analysis of an incubation of MEKO with mouse liver microsomes. The chromatogram shows the signal of 2-nitrobutane at 8.38 min after n-hexane extraction of a typical incubation of MEKO with liver microsomes. The electron capture mass spectrum represents the full-scan spectrum of 2-nitrobutane.

were identical. 2-Nitrobutane formation from MEKO was also confirmed by recording electron impact mass spectra (SIR-mode) of the metabolite (data not shown). Using other separation conditions, four additional new peaks (besides 2-nitrobutane), not present in controls, were observed by separation of the organic phase obtained from MEKO incubations. Figure 2 shows the mass spectra of the obtained products. The spectra of the compounds eluting at 11.0 min and 11.2 min were identical to those obtained from the diastereomers of 2-nitro-3-butanol

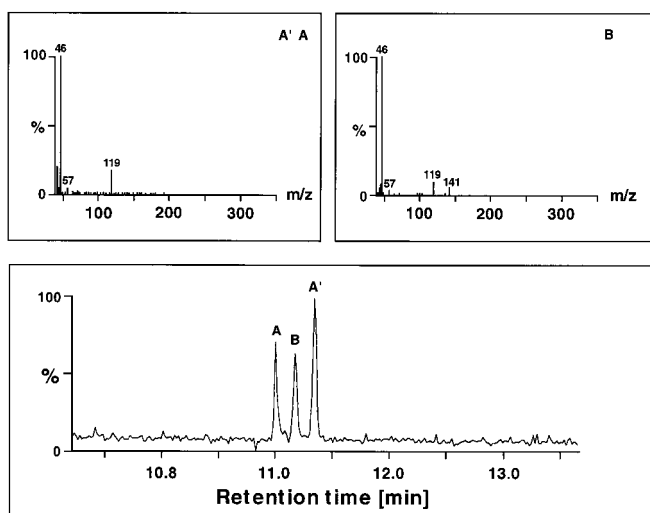


FIG. 2. Three new metabolites of MEKO formed after P450 oxidation; A and A' represent the diastereomers of 3-nitro-2-butanol and B likely another isomer of 3-nitrobutanol.

and co-eluted with the reference compounds. The third peak showed a fragmentation very similar to that seen with 2-nitro-3-butanol; therefore, the metabolite likely represents 2-nitro-1-butanol or 3-nitro-1-butanol. The fourth new peak eluted earlier from the column and showed an electron capture mass spectrum with few fragments (Fig. 3). The putative molecular ion $(M-H)^-$ indicates that this metabolite may represent a hydroxylation product of MEKO such as 2-hydroxy-3-butanone oxime. Incubation of the nitronate of 2-nitrobutane with liver microsomes also gave both diastereomers of 3-nitro-2-butanol with higher rates of formation.

In incubation of acetoxime in the presence of enzymatically competent microsomes, a major and a minor product were observed. The mass spectrum (Fig. 4) of the major metabolites was identical to those of 2-nitropropane, the minor metabolite had an identical spectrum (not shown) as synthetic 2-nitro-1-propanol. Both compounds also co-eluted with the synthetic reference compound, under different chromatographic conditions.

Metabolite Quantitation

In general, electron capture mass spectrometry is more sensitive than electron impact mass spectrometry for organic chemicals with electronegative substituents (Giese, 1997). Moreover, electron capture mass spectra often show little fragmentation providing increased sensitivity in selected ion monitoring. With sec-nitroalkanes, proton abstraction at the α -carbon in electron capture mass spectrometry gives the $(M-H)^-$ fragment as a major ion. In the EI-spectra of sec-nitroalkanes, the M^+ fragment is only a minor ion and therefore not useful for quantitation. In the case of 2-nitrobutane, the absolute detection limit in electron impact ionisation was 1 pmol as compared with 250 fmol in NCI. Quantitation of nitroalkanes was performed in the NCI-mode with internal standards and calibration curves with authentic materials. The matrix standards were linear in the range of concentrations of nitroalkanes present in samples (up to 0.1 mM in the incubation) and calibration standards were analyzed within every sample series. A relative standard deviation of the response factors across the standard range of less than 10% was observed.

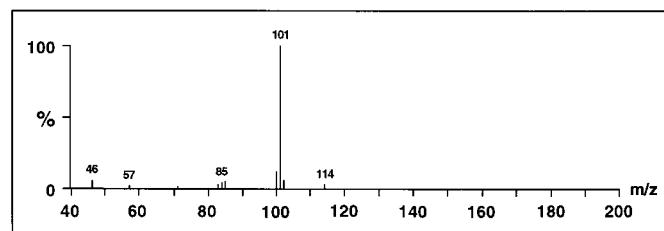


FIG. 3. Mass spectrum of the putative metabolite 2-hydroxybutanone-3-oxime.

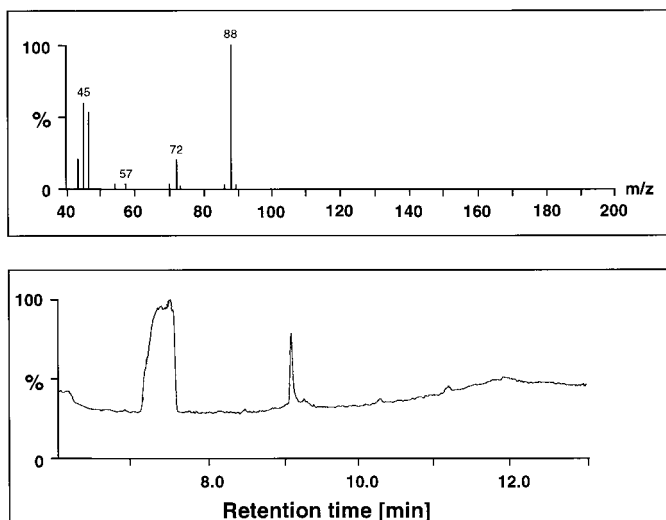


FIG. 4. GC/MS analysis of an incubation of acetoxime with mouse liver microsomes. The peak at 7.50 represents 2-nitropropane (mass spectrum above) after n-hexane extraction.

Sex and Species Differences in Ketoxime Oxidation

When acetoxime and MEKO were incubated with liver microsomal fractions from male and female rats and mice and liver microsomes obtained from human donors (4 males, 4 females) no significant sex differences were observed in the extent of nitronate formation. The oxidation of MEKO to the nitronate by microsomal fractions was generally more efficient (more than twofold) as compared to that of acetoxime (Table 1). Liver microsomes from both sexes of mice exhibited the highest activities for the oxidation of MEKO followed by human liver (Table 2) and rat liver microsomes. From MEKO, both diastereomers of 2-nitro-3-butanol were formed with rates of <20 pmol/mg/min. Due to the lack of reference compounds, the other products could not be quantified. However, the obtained peaks were small. Assuming similar response factors, the rates of formation of the other isomer of 2-nitrobutanol and also of 2-hydroxy-3-butanone oximes has to be assumed as <20 pmol/min/mg protein. In the case of these minor metabolites, only small species differences were observed.

The rates of acetoxime oxidation to propane 2-nitronate were lower than those determined for MEKO oxidation; however, similar differences in the capacity to oxidize acetoxime were seen in the species used. The rates of formation of 2-nitro-1-propanol from acetoxime were very low and in the range of 20 pmol/min/mg protein.

Effects of P450 Inducers and Inhibitors on Ketoxime Oxidation

In incubations with microsomes from methylcholanthrene (inducer of cytochrome P450 1A1) pretreated rats a twofold increase in the rates of nitronate formation from MEKO, with microsomes from phenobarbital (inducer of cytochrome P450

TABLE 1
Oxidation of MEKO to Butane 2-Nitronate and of Acetoxime to Propane 2-Nitronate in Liver Microsomes from Male and Female Rats and Mice

Liver microsomes (species)	Formation of butane 2-nitronate from MEKO (pmol/min/mg)	Formation of propane 2-nitronate from acetoxime (pmol/min/mg)
Wistar rats, male	108.22 \pm 22	66.2 \pm 2.4
Wistar rats, female	163.8 \pm 41	85.8 \pm 1.1
B6C3F1 mice, male	1341.9 \pm 354	288.7 \pm 44
B6C3F1 mice, female	1439.2 \pm 134	479.2 \pm 124

Note. K_m for the oxidation of MEKO was determined as 0.9 mM in mouse liver microsomes and 3.1 mM in rat liver microsomes. Results are mean \pm SD from two incubations. Each sample was analyzed twice.

2B)-treated rats a threefold higher rate and with microsomes of pyridine (inducer of cytochrome P450 2E1)-treated rats a six-fold higher rate was observed, as compared to microsomes from uninduced rats. In comparison to MEKO, the oxidation of acetoxime with induced microsomes resulted in a larger increase in the rates of nitronate formation between threefold for phenobarbital, four-fold for methylcholanthrene and six-fold for pyridine (Table 3).

To discriminate between FMO and cytochrome P450 as catalysts of MEKO, oxidation heat inactivation experiments were performed. As shown in Table 4 the preincubation of microsomes for 3 min at 45°C in the absence of NADPH resulted in a decrease of activity in comparison to untreated controls of only 20%. The small decrease indicates a major

TABLE 2
Oxidation of MEKO and Acetoxime to Butane 2-Nitronate resp. Propane 2-Nitronate in Human Liver Microsomes

Liver microsomes (different donors)	Formation of butane 2-nitronate [pmol/min/mg]	Formation of propane 2-nitronate [pmol/min/mg]
Human (13), male, 56 years	507.8	200.8
Human (15), male, 24 years	426.7 \pm 51	160.6
Human (11), female, 31 years	265.8 \pm 47	106.8
Human (14), female, 48 years	459.1 \pm 173	175.3
Human (Hum3), male, 44 years	681.1 \pm 69	nd
Human (L4), female, 69 years	892.9 \pm 151	nd
Human (17), male, 42 years	174.5 \pm 26	nd
Human (19), female, 31 years	325.9 \pm 9	nd

Note. K_M was determined as 0.91 mM for MEKO. Results are mean \pm SD from two incubations for MEKO and from one incubation with acetoxime; nd = not determined. Each sample was analyzed twice.

TABLE 3
Oxidation of MEKO and Acetoxime to Corresponding Nitronates in Liver Microsomes from Male Rats Pretreated with Cytochrome P450 Inducers

Microsomal preparation	Formation of butane 2-nitronate from MEKO [pmol/min/mg protein]	Formation of propane 2-nitronate from acetoxime [pmol/min/mg protein]
No pretreatment	108.2 ± 22	66.2 ± 2.4
Methylcholantrene pretreated rats	267.0 ± 26	292.7 ± 22
Phenobarbital pretreated rats	384.3 ± 11	204.2 ± 12
Pyridine pretreated rats	685.5 ± 21	845.7 ± 385

Note. Results are mean ± SD from two incubations. Each sample was analyzed twice.

participation of cytochromes P450 in MEKO oxidation (Ziegler, 1988). In incubations with coumarin (0.05 mM), a specific inhibitor for cytochrome P450 2A, no effect (Hong *et al.*, 1997) was observed on nitronate formation from MEKO (Table 4). With diethyldithiocarbamate, a specific inhibitor for cytochrome P450 2E1 (Guengerich, 1991) in concentrations of 0.3 mM, a decrease in nitronate formation of approximately 70% was observed. The inhibition of microsomal oxidation of MEKO in the presence of n-octylamine (5 mM) was more efficient and nitronate formation was decreased to <10% of untreated controls (Table 4). The rates of formation of the other MEKO metabolites were also decreased in the presence of n-octylamine and in the presence of diethyldithiocarbamate. In summary, these data suggest participation of several cytochromes P450 in the oxidation of MEKO and acetoxime to the corresponding nitronates.

DISCUSSION

Exposure to high concentrations of MEKO causes liver tumors only in male rats and male mice (IHF, 1995). In the case of acetoxime, its hepatocarcinogenicity has been postulated to be due to the oxidation of acetoxime to propane 2-nitronate, a genotoxicant and carcinogen (Kohl *et al.*, 1992).

The aim of this study was to characterize the oxidative biotransformation of ketoximes and compare the capacity of different species to catalyze these reactions. Correlations of the results with sex differences in the responses to ketoximes and the different potencies of the two compounds may help to elucidate the relevant mechanisms of bioactivation for ketoximes. One major problem of this work was the elaboration of a sensitive method for quantitation of *in vitro* formed nitronates. A method based on GC/MS had to be developed to quantify the sec-nitroalkane tautomers of the corresponding nitronates. At a pH between 5 and 8, the tautomeric equilib-

rium of butane 2-nitronate and 2-nitrobutane is adjusted within 24 h. The supernatants of incubates were mixed with n-hexane to efficiently extract the nitroalkane tautomer. Since the sec-nitroalkane does not distribute back to the aqueous layer, a quantitative transformation of the nitronate to the nitroalkane tautomer is obtained. Quantitation of the nitroalkanes, using highly sensitive electron capture mass spectrometry, permitted exact determination of the low amounts of nitronate formed by metabolic oxidation of MEKO and acetoxime and the identification of further ketoxime metabolites formed in low concentrations.

The structures of metabolites elucidated show that the oxidation of MEKO (and also of acetoxime) is more complicated than previously proposed (Fig. 5). Both MEKO and acetoxime are slowly oxidized, likely by cytochromes P450, to the corresponding nitronates. In addition to nitronate formation, cytochrome P450 catalyzes the oxidation of MEKO and butane 2-nitronate to nitroalcohols. This reaction results, in the case of MEKO, in the formation of several isomers. Acetoxime may only be oxidized to 2-nitro-1-propanol. Both direct oxidation of the oximes to the alcohol followed by oxidation of the oxime moiety to the nitronate, or vice versa, may occur at low rates.

The results presented here show that liver microsomes from these three different species are capable of slowly oxidizing MEKO and acetoxime to the corresponding nitronates. The rates of nitronate formation both from MEKO and from acetoxime are different in the three species examined. Mice generally exhibited the highest capacities for oxidation and human liver samples showed an intermediate capacity. The capacity of rat liver microsomes to catalyze ketoxime oxidation was 10-fold lower than that of mice microsomes.

Experiments with microsomes from animals pretreated with inducers of cytochromes P450 confirmed that nitronate formation is significantly increased by cytochrome P450 induction as described for acetoxime (Kohl *et al.*, 1992). The low specificity of the inducers suggest that different cytochrome P450 enzymes may catalyze the oxidation of MEKO. This conclusion is also supported by the results of the experiments with P450-specific inhibitors like diethyldithiocarbamate and coumarin.

TABLE 4
Inhibition of MEKO Oxidation in Liver Microsomes from Male Rats in the Presence of Enzyme Inhibitors

Pretreatment	Formation of butane 2-nitronate from MEKO (% of control)
45°C, 3 min	82
n-Octylamine (5 mM)	7
Diethyldithiocarbamate (0.3 mM)	32
Coumarin (0.05 mM)	120
Without NADPH	3
100°C, 5 min	3

Note. Rates in control were 108.2 ± 22.2 pmol/min/mg protein.

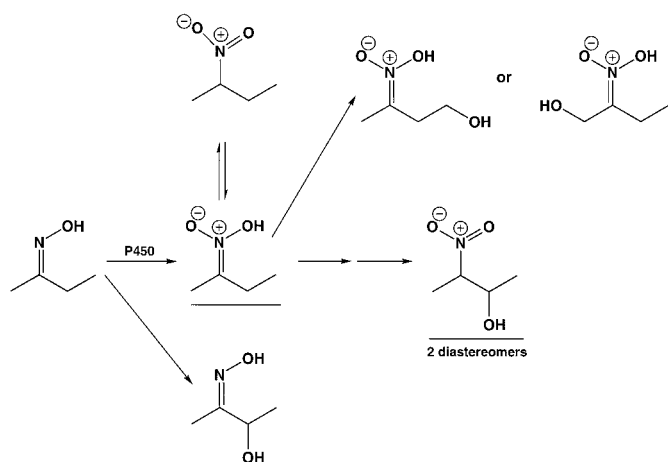


FIG. 5. Biotransformation of methylethyl ketoxime (MEKO) by rodent liver microsomes. Conclusively identified products are underlined.

The role of metabolic oxidation in acetoxime and MEKO tumorigenicity in the livers of male rodents is still unclear. The determined capacities for MEKO and acetoxime oxidation do not correlate with sex and species differences in the toxic responses to MEKO and acetoxime (Mirvish *et al.*, 1982). A sex difference should be observed in the rates of oxidation if the reaction would result in the ultimate toxic metabolite. Therefore, nitronate formation alone is very unlikely to account for the sex-specific tumor response to MEKO and acetoxime. However, other products formed downstream from the nitronate may be involved.

Also, the results of these studies indicate that the rates of MEKO-oxidation to butane 2-nitronate between rats and mice, although small, differ by 10-fold. This also does not support an involvement of nitronate formation in tumorigenicity of MEKO, as the tumor incidences in male rats and mice exposed to identical levels of MEKO (IHF, 1993a) were relatively similar despite the 10-fold higher capacity of mouse liver compared to rat liver to oxidize MEKO to butane 2-nitronate.

Moreover, the similar rates observed in rodent liver microsomes to oxidize MEKO and acetoxime also do not explain the large differences in liver toxicity observed between MEKO and acetoxime. Based on results of comparative 90-day oral toxicity studies on both acetoxime and MEKO performed at similar dose levels, acetoxime appears to be much more potent than MEKO in its effects on the liver (AlliedSignal, 1977, 1991). With acetoxime, liver lesions consisting of bile duct proliferation, cytoplasmic vacuolization, and foci of cellular alteration were present in male livers at dose levels of 50 mg/kg and 250 mg/kg, beginning as early as 45 days at the higher dose. No evidence of liver toxicity was evident with MEKO at dose levels as high as 225 mg/kg after 90 days of exposure. The higher potency of acetoxime is not consistent with its lower

rate of oxidation reactions, further questioning the significance of the metabolic oxidation in toxicity.

In conclusion, the obtained data suggest that nitronate- or hydroxy nitronate-formation alone is not sufficient to explain the tumorigenicity of ketoximes. A mechanism for DNA-damage by ketoximes has been proposed based on the observation of DNA-amination by 2-nitropropane and apparent identical DNA-damage after administration of large oral doses of acetoxime (Hussain *et al.*, 1990; Sodum *et al.*, 1994). For 2-nitropropane, the mechanism involves sulfate conjugation which supposedly forms acetoxime O-sulfate which is hydrolyzed to hydroxylamine O-sulfate which aminates DNA (Sodum *et al.*, 1993). With ketoximes, this mechanism requires oxidation to the nitronate, since acetoxime itself seems not to be a substrate for sulfotransferases (Kreis *et al.*, 1998). Since sex-specific expression of sulfotransferases in rodents has been described (Falany and Wilborn, 1994), this mechanism may explain the sex-specific tumor response. However, it is questionable if the low rates of oxidation of MEKO and acetoxime observed in the present studies could produce sufficient concentrations of nitronates to cause liver tumors. Therefore, other mechanisms of tumorigenicity not related to nitronate mutagenicity and DNA-damage may thus be operative.

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