Evidence Supporting the Formation of 2,3-Epoxy-3-methylindoline: A Reactive Intermediate of the Pneumotoxin 3-Methylindole

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The existence of a cytochrome P450-dependent 2,3-epoxide of the potent pneumotoxin 3-methylindole was indirectly confirmed using stable isotope techniques and mass spectrometry. Determination of hydride shift and incorporation of labeled oxygen in 3-methyloxindole and 3-hydroxy-3-methyloxindole, metabolites that may be in part dependent on the presence of the epoxide, were utilized as indicators of the epoxide's existence. One mechanism for the formation of 3-methyloxindole involves cytochrome P450-mediated epoxidation followed by ring opening requiring a hydride shift from C-2 to C-3. Through incubations of goat lung microsomes with [2-^{2}H]-3-methylindole, the retention of ^{2}H in 3-methyloxindole was found to be 81%, indicating a majority of the oxindole was produced by the mechanism described above. 3-Hydroxy-3-methylindolenine is an imine reactive intermediate that could be produced by ring opening of the 2,3-epoxide. The imine may be oxidized to 3-hydroxy-3-methyloxindole by the cytosolic enzyme aldehyde oxidase. Activities of this putative detoxification enzyme were determined in both hepatic and pulmonary tissues from goats, rats, mice, and rabbits, but the activities could not be correlated to the relative susceptibilities of the four species to 3-methylindole toxicity. The ^{18}O incorporation into either 3-methyloxindole or 3-hydroxy-3-methyloxindole from both $^{18}O_2$ and $H_2{}^{18}O$ was determined. The ^{18}O incorporation into 3-methyloxindole from $^{18}O_2$ was 91%, strongly implicating a mechanism requiring cytochrome P450-mediated oxygenation. Incorporation of ^{18}O into 3-hydroxy-3-methyloxindole indicated that the alcohol oxygen originated from molecular oxygen, also implicating an epoxide precursor. These studies demonstrate the existence of two new reactive intermediates of 3-methylindole and describe the mechanisms of their formation and fate.

Introduction

3-Methylindole, a degradation product of tryptophan, is a selective pulmonary toxicant that requires cytochrome P450 biotransformation to elicit its toxic effects (*1*). Most susceptible to 3-methylindole pneumotoxicity are goats and cattle, followed by mice, rats, and rabbits (*2*, *3*). Human susceptibility to 3-methylindole has not been thoroughly characterized to date, but the toxin is bioactivated by human microsomes and is a good substrate for human cytochrome P450 enzymes that are selectively expressed in lung tissue (*4*, *5*). Exposure to 3-methylindole occurs through ingestion of dietary tryptophan, followed by degradation to indole-3-acetic acid through decarboxylation and deamination. Indole-3-acetic acid is decarboxylated to 3-methylindole by a *Lactobaccillus sp*. bacterium in the gut (*6*). 3-Methylindole circulates systemically to the lung tissue where it is bioactivated to highly reactive intermediates (Scheme 1) that have the potential to alkylate proteins and other cellular macromolecules leading to toxicity (*7*).

One well-established reactive intermediate of 3-methylindole is 3-methyleneindolenine (*8*). This highly reactive methylene imine is produced by cytochrome P450-mediated dehydrogenation of 3-methylindole at the methyl position (*9*). The mechanism appears to proceed by hydrogen atom abstraction followed by a one-electron oxidation step to produce the reactive intermediate which alkylates proteins (*10*) through a Michael-like addition reaction.

Another highly reactive cytochrome P450-dependent intermediate that could participate in the toxicity of 3-methylindole is the 2,3-epoxide. The 2,3-epoxide of indole may be an intermediate in the formation of 3-hydroxyindole conjugates or oxindole from indole in rats (*11*). The 2,3-epoxide of *N*-acyl-3-methylindole is formed by chemical oxidation with dimethyldioxirane and rearranges to *N*-acyl-3-methyloxindole (*12*). Thus, indirect evidence exists for the formation of indole 2,3-epoxides, but we are unaware of studies that conclusively demonstrate the formation of indole epoxides by P450-mediated oxidation. 2,3-Epoxy-3-methylindole could have at least three potential chemical fates: (1) direct alkylation of cellular protein and/or nucleic acids, (2) ring opening, possibly accompanied by a "NIH shift" of the hydride at C-2, resulting in a stable oxindole, or (3) ring opening to produce an imine reactive intermediate,

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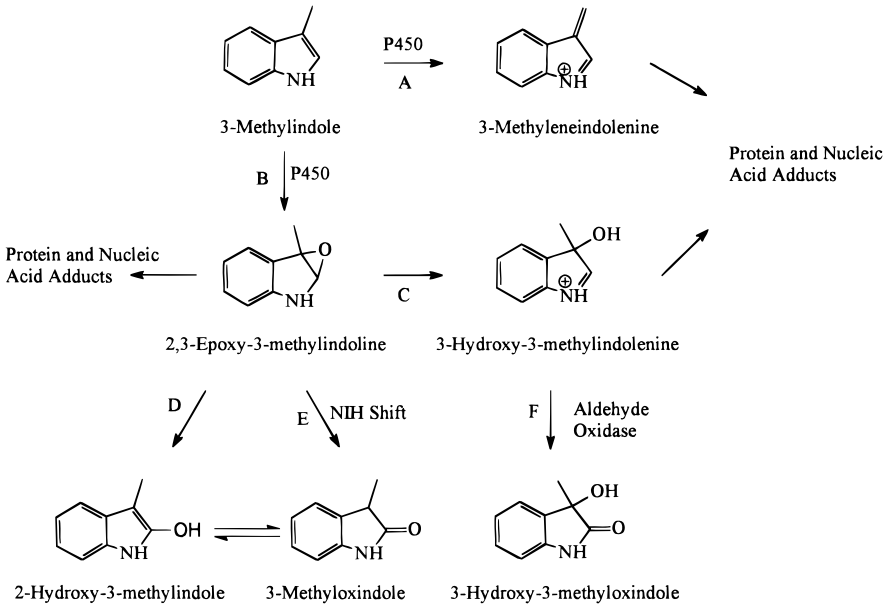
Scheme 1. Cytochrome P450-Mediated Bioactivation of 3-Methylindole[a]



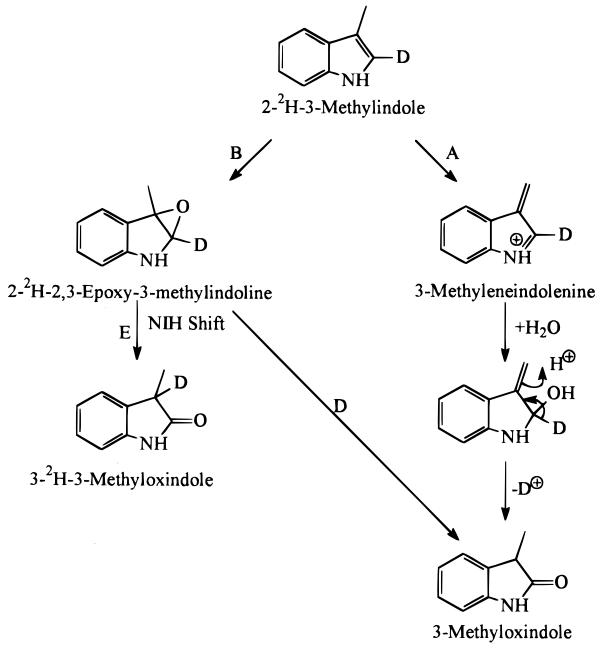
[a]Possible mechanisms include: dehydrogenation to 3-methyleneindolenine (pathway A); epoxidation (pathway B) followed by ring opening to C-2, resulting in the formation of 3-methyloxindole by both hydride shift (pathway E) and proton loss (pathway D) mechanisms; or epoxide ring opening to C-3 leading to 3-hydroxy-3-methylindolenine (pathway C) and its oxidation by aldehyde oxidase (pathway F). The possibility of reactive intermediate conjugation with proteins and/or nucleic acids is indicated. 3-Methyleneindolenine is depicted in the protonated form based on considerations of the basicity of indolenine nitrogen atoms (*22*). Alphabetical discriminators for each possible pathway are kept consistent in subsequent schemes.

3-hydroxy-3-methylindolenine, which can alkylate cellular macromolecules or be detoxified by further oxidation to 3-hydroxy-3-methyloxindole.

3-Methyloxindole that is formed from 2,3-epoxy-3-methylindoline through the NIH shift mechanism would retain the C-2 H-atom at C-3 (Scheme 2, B and E). However, another mechanism for the formation of 3-methyloxindole from the epoxide involves loss of the H atom from C-2 and the formation of an enol that tautomerizes to the keto form, 3-methyloxindole (Schemes 1 and 2, D). The mechanism of deuterium loss could also involve a "direct hydroxylation" mechanism wherein a formal epoxide is not formed (vide infra) during the oxidation of 3-methylindole to 3-methyloxindole. Finally, a mechanism for deuterium loss could occur through hydration of the dehydrogenated intermediate, 3-methyleneindolenine (Scheme 2, A). Therefore, characterization of the 3-methyloxindole formed from [2-^{2}H]-3-methylindole can be used to support the production of the epoxide and to assess the extent of the hydride shift mechanism versus other mechanisms in ring opening of the epoxide (Scheme 2).

It is possible that P450-mediated oxygenation of 3-methylindole by an addition/rearrangement mechanism occurs. This process would be similar to other aromatic hydroxylation reactions that have been proposed (*13*) to proceed through the addition of the triplet-like active oxygen species of reduced P450 to the π system of the aromatic ring, followed by rearrangement to form the enol directly, without formal formation of the epoxide. In fact, initial addition to the indole ring may be electronically more favorable than addition to a benzene-like aromatic system that does not contain nitrogen, because of the higher electron density of the indole ring. However, distinguishing between these two mechanisms is quite difficult using the methods employed in the current report.

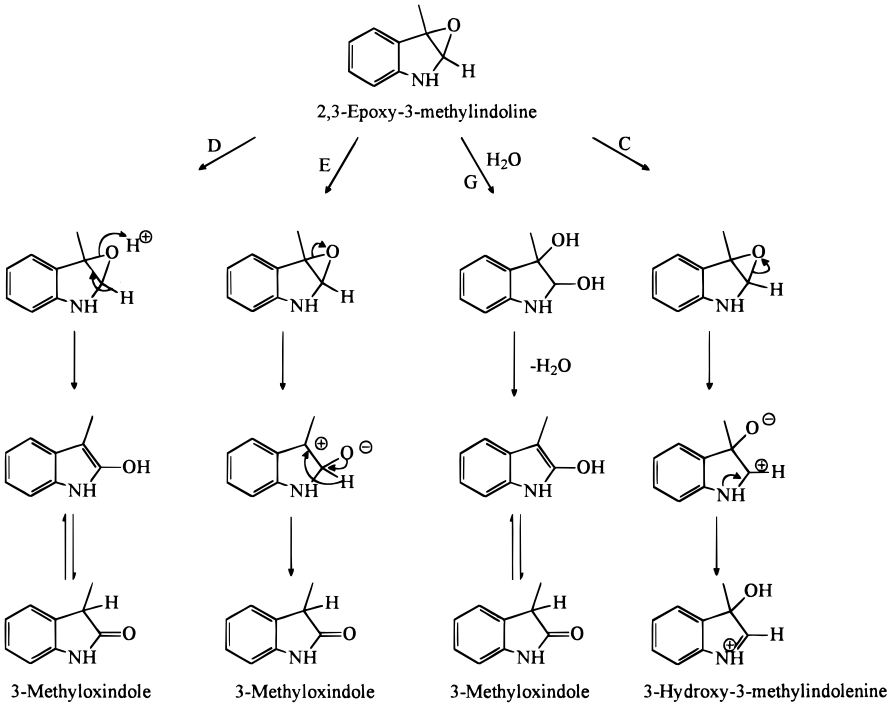
Scheme 2. Potential Fates of the Deuterium Label after Oxidation of [2-^{2}H]-3-Methylindole by P450 Enzymes to Produce 3-Methyloxindole[a]



[a] Mechanisms include: epoxidation (pathway B) followed by ring opening involving a hydride shift (pathway E) and ring opening not involving a hydride shift (pathway D) or dehydrogenation to 3-methyleneindolenine (pathway A) which is hydrated at C-2 and which subsequently aromatizes to the indole and undergoes enol/keto tautomerization to 3-methyloxindole.

The source of oxygen incorporated into 3-methyloxindole and also 3-hydroxy-3-methyloxindole is another indicator of the proposed epoxide intermediate. 3-Methyloxindole that is produced via a cytochrome P450-dependent epoxide should incorporate ^{18}O from molecular oxygen. Other mechanisms for the formation of 3-meth-

Scheme 3. Mechanisms of 2,3-Epoxy-3-methylindoline Ring Opening[a]



[a]Mechanisms include: proton loss and ring opening to C-2 (pathway D), followed by tautomerization to 3-methyloxindole; ring opening to C-2 (pathway E) involving a hydride shift from C-2 to C-3 forming 3-methyloxindole; hydration of the epoxide (pathway G) to the dihydrodiol that dehydrates and tautomerizes to 3-methyloxindole; and ring opening to C-3 (pathway C) forming 3-hydroxy-3-methylindolenine.

yloxindole include incorporation of oxygen from water through the formation of a diol and subsequent dehydration to 3-methyloxindole (Scheme 3, G). Therefore, an assessment of the incorporation of ^{18}O from either $^{18}O_2$ or $H_2{}^{18}O$ should provide an indication of the presence of the epoxide, the extent of ring opening versus the hydration/dehydration mechanism, or the possibility that hydration of the methylene imine is a predominant mechanism.

3-Hydroxy-3-methylindolenine is another putative reactive intermediate of 3-methylindole which could be produced by epoxide ring opening (Scheme 1, C). 3-Hydroxy-3-methylindolenine may be oxidized to 3-hydroxy-3-methyloxindole by the cytosolic enzyme aldehyde oxidase (EC 1.2.3.1) (*14*). Because 3-hydroxy-3-methylindolenine may be formed though the epoxide, the subsequent formation of 3-hydroxy-3-methyloxindole could be another indicator of the epoxide's existence. 3-Hydroxy-3-methyloxindole is in fact the major metabolite of 3-methylindole (*15*, *16*). Therefore, aldehyde oxidase may be a major contributor in the detoxification of 3-methylindole. Differing activities of this cytosolic enzyme for the hydroxyindolenine could contribute substantially to differences in species and organ susceptibility to 3-methylindole. By supplementing goat lung microsomal incubations with cytosolic proteins that contain aldehyde oxidase from different species, production of 3-hydroxy-3-methyloxindole can be monitored, simultaneously evaluating the presence of the epoxide and the role of aldehyde oxidase in species-selective 3-methylindole detoxification.

The present study investigated the possibility of the existence of an epoxide reactive intermediate of 3-methylindole. Through the use of the stable isotopes ^{18}O and ^{2}H, the formation and metabolic fate of the epoxide, 2,3-epoxy-3-methylindoline, was monitored, indirectly confirming its presence.

Experimental Procedures

Chemicals. 3-Methylindole, *N*-acetyl-L-cysteine, indole-3-carbinol, nicotinamide adenine dinucleotide phosphate (NADPH), ammonium acetate, THF, D_2O, and formic acid were purchased from Sigma Chemical Co. (St. Louis, MO). $^{18}O_2$ (50 atom %) was obtained from Cambridge Isotopes (Woburn, MA). Acetanilide, *p*-hydroxyacetanilide (acetaminophen), and $H_2{}^{18}O$ (95 atom %) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-Methyloxindole was a generous gift from Dr. James Carlson, Washington State University. 3-Hydroxy-3-methyloxindole and [2-^{2}H]-3-methylindole were synthesized as described below.

Synthesis. 3-Hydroxy-3-methyloxindole was prepared by adding CH_3MgBr to indole-2,3-dione in anhydrous THF according to a published method (*17*). The product was recrystallized from ether/CH_2Cl_2 and produced white crystals with a melting point of 160−161 °C, in 65% purified yield.

[2-^{2}H]-3-Methylindole was prepared by a modification of a published method (*18*). 3-Methylindole (15.2 mmol) was added to a flask which was evacuated and flushed with argon three times. THF (50 mL) was added to give a homogeneous mixture which was cooled to −70 °C. *n*-Butyllithium (6.1 mL of a 2.5 M hexane solution) was added, and a colorless precipitate formed. The solution was warmed to 25 °C over 10 min, and CO_2 was bubbled into the solution with vigorous stirring for 3 min to produce a colorless homogeneous solution. The solution was allowed to stand at 25 °C for 5 min at which point the solvent was removed under reduced pressure below 25 °C to give a colorless salt (lithium 3-methylindole-1-carboxylate) which was dried under a vacuum.

The flask was again evacuated and flushed with argon three times. THF was added to give a homogeneous pale-yellow solution which was cooled to −70 °C, and *tert*-butyllithium (10 mL of a 1.7 M pentane solution) was slowly added to give a pale-orange solution of lithium 2-lithio-3-methylindole-1-carboxylate.

This solution was warmed to −20 °C and kept at this temperature for 1 h. The solution was cooled to −70 °C, and D_2O (0.2 mL) was added to form [2-^{2}H]-3-methylindole-1-carboxylate. The solution was kept at −70 °C for 3 h and then slowly quenched with aqueous ammonium sulfate. The solution was warmed to 25 °C, and 2 N sulfuric acid was added to bring the solution to pH 4. The solution was extracted twice with diethyl ether, washed with water, dried over anhydrous $MgSO_4$, and filtered, and the solvent was evaporated under reduced pressure to give crude [2-^{2}H]-3-methylindole-1-carboxylic acid as a pale-yellow solid. The solid was then heated, yielding the decarboxylated product [2-^{2}H]-3-methylindole. The product was purified by column chromatography on silica gel using hexane as an eluent. The purified yield of [2-^{2}H]-3-methylindole (mp 96−97 °C) was 90%, and deuterium incorporation was 83%, measured in triplicate by LC/MS of the molecular ion cluster at 148/149 *m*/*z*.

Microsomal Preparation. Goat lungs were obtained from three male goats. The lungs were perfused in situ with ice-cold 0.05 M phosphate buffer containing 1 mM EDTA and 1.15% KCl and then frozen at −70 °C. Microsomes were prepared by standard centrifugal isolation procedures (*4*). Mouse livers were obtained from four male Swiss−Webster mice that were sacrificed by cervical dislocation. The livers were removed, and microsomes were prepared by standard centrifugal isolation procedures. For experiments requiring cytosolic protein, the cytosolic fraction from the microsomal preparation was used in each case. In all cases the cytosolic fraction was the supernatant from the first 105000*g* centrifugation step. This procedure was performed for the goat and mouse lung and liver tissues from animals described above. Cytosolic fractions were also obtained in an analogous manner from the lung and liver tissues from three male Sprague−Dawley rats and five male New Zealand white rabbits.

Incubations To Determine Extent of Hydride Shift Mechanism in the Formation of 3-Methyloxindole. Incubations contained 4 mM NADPH, goat lung microsomes containing 1.1 nmol of cytochrome P450/mL, and either 0.5 mM 3-methylindole or 0.5 mM [2-^{2}H]-3-methylindole. Incubations were performed in 0.01 M ammonium acetate, pH 7.4 in a final volume of 1 mL. Incubations were also performed in the presence of 4 mM *N*-actetyl-L-cysteine. The reaction was performed at 37 °C in a shaking water bath for 30 min and stopped by immersion in an ice bath and by the addition of an equivalent volume of ice-cold acetonitrile to both stop the reaction and precipitate protein. The precipitated protein was separated from the reaction mixture by centrifugation at 3100*g* in a Beckman GPR tabletop centrifuge. The supernatant was removed and concentrated to 200 μL in a Savant SpeedVac (model SVC100) for analysis by LC/MS.

^{18}O Incorporation into 3-Methyloxindole and 3-Hydroxy-3-methyloxindole from $^{18}O_2$ and $H_2{}^{18}O$. Incubations contained 1 mM 3-methylindole, goat lung microsomes containing 1.1 nmol of cytochrome P450/mL, 1 mM NADPH, and 0.05 M phosphate buffer in a final volume of 3 mL. Some incubations also contained 8 mM *N*-acetylcysteine. The incubations were performed in an air-tight four-flask glass manifold apparatus that was constructed so that it could be evacuated, be flushed with ultrapure N_2, and have $^{18}O_2$ (50 atom %) introduced from a lecture bottle. 3-Methylindole was incubated with goat lung microsomes in two of the flasks, and acetanilide was incubated with mouse liver microsomes in the other two. Hydroxylation of acetanilide to *p*-hydroxyacetanilide was utilized as a positive control for incorporation from molecular oxygen. ^{18}O Incorporation into *p*-hydroxyacetanilide was normalized to 100%. Incubations contained mouse liver microsomes because goat lung microsomes do not hydroxylate acetanilide to a large enough extent for reliable quantification. After adding all incubation components except substrate into the flasks, the system was cooled on ice and then evacuated and flushed with N_2 at least 10 times. The $^{18}O_2$ was introduced, substrate was added, and the system was heated to 37 °C for the 30-min incubation. The reactions were stopped by immersing the flasks into a dry ice/acetone bath, and the flasks were removed from the manifold. Three milliliters of ethyl acetate was added to the frozen incubation solutions, and as they thawed, they were mixed to denature protein and prevent further metabolism. The ethyl acetate layer was removed, and the aqueous solutions were extracted twice more with ethyl acetate. The organic extracts were pooled, evaporated to dryness under an N_2 stream at ambient temperature, and then derivatized in MSTFA/acetonitrile for analysis by GC/MS.

For incorporation from $H_2{}^{18}O$, incubations were performed in 0.05 M phosphate buffer that was prepared using water enriched with $H_2{}^{18}O$. The net isotopic enrichment in the incubation mixture was 44.7 atom %, and the final volume of the incubations was 0.5 mL. Incubations also contained goat lung microsomes (1.1 nmol/mL cytochrome P450) and 1 mM NADPH. For assessment of incorporation into 3-hydroxy-3-methyloxindole, mouse liver microsomes and cytosol were utilized because goat lung enzymes did not produce a significant amount of this metabolite. 3-Methylindole was added, and a 50-μL aliquot was removed from each incubation mixture and placed on ice. The incubation was then performed at 37 °C for 30 min and stopped by the addition of 500 μL of ice-cold ethyl acetate. The organic layers were removed, and the samples were extracted twice more with ethyl acetate. The extracts were pooled, evaporated to dryness, and derivatized with MSTFA for analysis by GC/MS. Five microliters of benzoyl chloride was added to the 50-μL aliquots that were removed just prior to incubation. The incorporation of ^{18}O into benzoic acid, in the conversion from benzoyl chloride in water, was normalized to 100%. The aliquots were then extracted three times with ethyl acetate. The extracts were pooled, evaporated to dryness, and derivatized with MSTFA for GC/MS analysis.

Aldehyde Oxidase Activity. Oxidation of *N*-methylnicotinamide, the model substrate for aldehyde oxidase, to *N*-methyl-2-pyridone-5-carboxamide (2-pyridone) and *N*-methyl-4-pyridone-3-carboxamide (4-pyridone) was assayed in lung and liver tissues of goat, mouse, and rabbit (*19*). The activity of aldehyde oxidase in rat tissues was not measured with this substrate. Product formation was monitored by UV analysis (300 nm) using a Gilford spectrophotometer. Potassium phosphate buffer (0.1 M, pH 7.8) (700 mL), and 0.005% EDTA were added to the cuvettes. Bovine serum albumin (50 μL of a 25 mg/mL solution) and catalase (50 μL of a 0.25 mg/mL solution) were added. The *N*-methylnicotinamide concentration was 0.5 M. An appropriate volume of water was added such that the cuvette volume was 1 mL at the time of the assay. The cuvette was then placed in a 25 °C water bath for 5 min; once the temperature of the solution was equilibrated, the cytosolic fractions were added. The cuvette was then placed in the spectrophotometer, and the absorbance was monitored at 300 nm. The change in absorbance was converted to μmol of product formed using an extinction coefficient of 4.23×10^3 mM^{-1} cm^{-1}.

3-Hydroxy-3-methyloxindole was quantified in incubations that generated 3-hydroxy-3-methylindolenine as a substrate for aldehyde oxidase. Incubations contained 0.5 mM 3-methylindole, 4 mM NADPH, goat lung microsomes containing 5 nmol of cytochrome P450/mL, and cytosolic protein, 1 mg/mL, from either liver or lung fractions from goat, mouse, rat, or rabbit. The higher P450 content was utilized because it was necessary to maximize the amount of 3-hydroxy-3-methylindolenine that was produced from 3-methylindole. Incubations also contained 20 μL of a 1 mM solution of 3-phenyloxindole for use as an internal standard (retention time of 15.2 min). Peak area ratios of 3-hydroxy-3-methyloxindole to 3-phenyloxindole were compared to a standard curve generated with synthetic 3-hydroxy-3-methyloxindole for quantification of 3-hydroxy-3-methyloxindole formation. Incubations were performed at 37 °C in a shaking water bath for 15 min and were stopped by immersion in an ice bath and by the addition of an equal volume of ice-cold acetonitrile. The precipitated protein was removed by centrifugation, and the supernatant was concentrated for analysis by HPLC.

Table 1. ^{18}O Incorporation into 3-Methyloxindole, 3-Hydroxy-3-methyloxindole, and APAP from $^{18}O_2$

metabolite	NAC	$^{18}O_0$	$^{18}O_1$	$^{18}O_2$	$^{18}O_2$ incorporation (%)	APAP correction (%)
APAP	−	0.52 ± 0.01	0.49 ± 0.01	N/A	98	
APAP	+	0.50 ± 0.01	0.50 ± 0.01	N/A	100	
3MOI	−	0.64 ± 0.01	0.36 ± 0.01	N/A	73	74
3MOI	+	0.55 ± 0.01	0.45 ± 0.01	N/A	90	91
APAP[a]	−	0.52 ± 0.01	0.47 ± 0.01	N/A	95	
3H3MOI[a]	−	0.49 ± 0.01	0.41 ± 0.01	0.02 ± 0.001	82, 4.2[b]	87, 4.4[b]

[a] These experiments were performed using mouse liver microsomes and cytosol. [b] Percentage of 3-hydroxy-3-methyloxindole that incorporated two atoms of oxygen from molecular oxygen. N/A, not applicable; APAP, *p*-hydroxyacetanilide; 3MOI, 3-methyloxindole; 3H3MOI, 3-hydroxy-3-methyloxindole. Incubations were performed in an air-tight system that was evacuated prior to $^{18}O_2$ introduction. Incorporation of ^{18}O into *p*-hydroxyacetanilide (APAP) was normalized to 100%, and metabolite incorporation is corrected for this value.

Liquid Chromatography for Analysis of 3-Hydroxy-3-methyloxindole. A Beckman HPLC with dual 114M pumps and a 421A pump controller was utilized. Metabolites were separated using a reversed-phase Ultramex C-18, 5-μm, 250- × 4.6-mm column (Phenomonex, Torrance, CA) by a gradient that began at 90% aqueous (0.01 M ammonium acetate, pH 6.0), 10% organic (acetonitrile). The gradient proceeded with linear increases to 35% organic after 5 min, 50% organic at 10 min, 55% organic at 15 min, 95% organic at 19 min, isocratic for 2 min, and back to 10% organic at 25 min. The column was reequilibrated for 7 min between injections. 3-Hydroxy-3-methyloxindole was detected using a Hewlett-Packard 1040A diode array detector and a Hewlett-Packard 9000 series, Pascal-based workstation. The compound's retention time was 7.5 min, and it was positively identified by its characteristic pattern of absorbance as compared to a synthetic standard.

GC/MS of ^{18}O-Labeled 3-Methyloxindole and *p*-Hydroxyacetanilide. The ratios of metabolically produced isotopomers were determined by mass spectrometry with a Finnigan MAT 4500 GC/MS. The GC was fitted with a 30-m DB-5 microbore column with 0.25-mm film thickness (J&W Scientific). The carrier gas was hydrogen at a flow rate of ca. 30 m s^{-1}. The GC oven temperature profile started at 100 °C for 2.0 min and was ramped at 10 °C min^{-1} for 13.0 min to 230 °C and then to 300 °C at 20 °C min^{-1} before returning to 100 °C. The injector, ionizer, and transfer line temperatures were 270, 120, and 280 °C, respectively. Ionization of the derivatized ^{18}O-labeled 3-methyloxindole (retention time 11.3 min) and *p*-hydroxyacetanilide (retention time 8.6 min) was accomplished by methane chemical ionization. The mass spectrometer was operated in the positive ion mode, and each sample was analyzed in triplicate.

Direct Probe Electron Impact MS of 3-[^{18}O]Hydroxy-3-methyloxindole. Electron impact mass spectra were recorded on a VG Micromass 7050E double focusing high-resolution mass spectrometer with VG Data System 2000. The mass spectrometer was operated in the direct probe electron impact mode, and ionization was achieved with a 70-eV electron beam.

LC/MS Analysis of 3-Methyloxindole Formed from [2-^{2}H]-3-Methylindole. The incubation mixtures from the NIH shift-tracking experiments were analyzed by LC/MS. The preconcentrated mixtures were injected using a Leap Technologies CTC-A200S autosampler into a Waters 626 liquid chromatograph. Metabolites were separated using a Phenomenex Ultramex 5 C18 IP (250-mm × 2.1-mm, 5-μm) column by the same instrument-controlled gradient described above for liquid chromatography. In this case, however, the organic phase was acetonitrile, containing 0.1% (v/v) formic acid, and the aqueous phase was H_2O, containing 0.1% (v/v) formic acid. Mass spectrometry was performed using a Finnigan model TSQ-7000 triple-quadrupole mass spectrometer with an atmospheric pressure ionization interface to the liquid chromatograph. A capillary temperature of 175 °C and a vaporizer temperature of 500 °C were used. A corona discharge voltage of 2.84 kV at 4.9 mA was used with the sheath gas pressure set at 70 psi N_2.

Results

NIH Shift-Tracking Experiments. The molecular ion for synthetic 3-methyloxindole had a *m*/*z* of 148 and a retention time of 12.5 min. After incubation of goat lung microsomes with [2-^{2}H]-3-methylindole, metabolically formed 3-methyloxindole with a *m*/*z* of 149 was produced by an NIH shift of the deuterium from position 2 to position 3, a process which is consistent with epoxide ring opening. Incubations were performed in the presence and absence of the thiol *N*-acetylcysteine in order to trap the component of 3-methyloxindole that may form via hydration of 3-methyleneindolenine, a mechanism not requiring hydride migration, as its thioether adduct. The deuterium retention in 3-methyloxindole was 71 ± 1.7% in the absence of *N*-acetylcysteine and 81 ± 2.5% in its presence. Percentages are the mean ± standard deviation of three incubations. An *N*-acetylcysteine adduct of the epoxide or the ring-opened 3-hydroxy-3-methylindolenine product was observed, but no products that appeared to be dihydrodiols were observed by LC/MS analysis. Analysis of the thiol adducts of these products is currently under investigation.

^{18}O Incorporation into 3-Methyloxindole and 3-Hydroxy-3-methyloxindole. The ratios of the isotopomers and amounts of ^{18}O incorporated into *p*-hydroxyacetanilide and 3-methyloxindole when 3-methylindole was incubated with and without *N*-acetylcysteine in an atmosphere that was 50 atom % $^{18}O_2$ are shown in Table 1. These results demonstrated that incorporation of oxygen in 3-methyloxindole was 74% and 91% from molecular oxygen in the absence and presence of *N*-acetylcysteine, respectively. Also shown are results of ^{18}O incorporation into 3-hydroxy-3-methyloxindole incubated as described above with the exception that mouse liver microsomes and cytosol, as opposed to goat lung microsomes alone, were used. Molecular oxygen incorporation into 3-hydroxy-3-methyloxindole was 87% for one atom and 4.4% for two atoms of oxygen. The incorporation results were corrected for the theoretical amount of ^{18}O which should be incorporated into *p*-hydroxyacetanilide (100% incorporation) and multiplied by 2 to correct for the fact that only 50% of the O_2 was labeled with ^{18}O. These corrections provided a measurement of the total amount of ^{18}O in 3-hydroxy-3-methyloxindole that originated from molecular oxygen.

The incorporation of ^{18}O from $H_2{}^{18}O$ is shown in Table 2. Incorporation results are shown for 3-methyloxindole and 3-hydroxy-3-methyloxindole. For these incubations the positive control for incorporation was benzoic acid which is rapidly formed from benzoyl chloride in water; it is assumed that ^{18}O incorporation is 100%. Again, because goat lung microsomes do not produce 3-hydroxy-

Table 2. ^{18}O Incorporation into 3-Methyloxindole, 3-Hydroxy-3-methyloxindole, and BA from $H_2{}^{18}O$

metabolite	$^{18}O_0$	$^{18}O_1$	$^{18}O_2$	BA correction (%)
BA	0.55 ± 0.03	0.44 ± 0.10	0	
3MOI	0.94 ± 0.04	0.03 ± 0.03	N/A	6.44
BA[a]	0.60 ± 0.02	0.37 ± 0.01	0	
3H3MOI[a]	0.57 ± 0.03	0.40 ± 0.02	0.02 ± 0.03	107

[a] These experiments were performed with goat lung microsomes and cytosol. N/A, not applicable; BA, benzoic acid produced from benzoyl chloride; 3MOI, 3-methyloxindole; 3H3MOI, 3-hydroxy-3-methyloxindole. Incubations were performed in buffer that had been prepared with $H_2{}^{18}O$ to produce a final isotopic enrichment of 44.7%. Incorporation of ^{18}O into benzoic acid (BA) was normalized to 100%, and metabolite incorporation is corrected for this value.

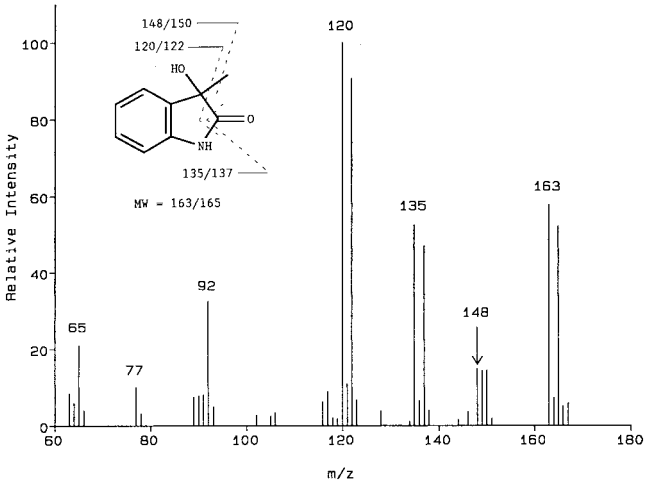


Figure 1. Electron impact, direct probe, high-resolution mass spectrum of 3-[^{18}O]hydroxy-3-methyloxindole, formed by cytochrome P450 oxidation in the presence of $^{18}O_2$ (50 atom %).

3-methyloxindole to an appreciable extent, mouse liver enzymes were used in this case. Only 6.44% of the oxygen in 3-methyloxindole was shown to be derived from water. Essentially all of one atom (107%) of oxygen in 3-hydroxy-3-methyloxindole came from water. The results were corrected for the amount of ^{18}O incorporated into benzoic acid from benzoyl chloride (100% theoretical incorporation) and also multiplied by 2.24 to correct for the fact that the enrichment of $H_2{}^{18}O$ in the incubation mixture was 44.7 atom %.

Electron Impact Mass Spectrum of ^{18}O-Labeled 3-Hydroxy-3-Methyloxindole. The direct probe electron impact mass spectrum of 3-hydroxy-3-methyloxindole that was formed by mouse liver microsomes under a 50 atom % ^{18}O atmosphere is shown in Figure 1. The major ions in the spectrum have the following *m/z*, assignments, and relative intensities, respectively: *m/z* 163/165/167, M^+ (58, 52, 6.0); 148/150, $[M - CH_3]^+$ (15, 14); 135/137, $[M - CO]^+$ (53, 47); 120/122, $[M - CH_3\text{-}CO]^+$ (100, 91).

Aldehyde Oxidase Experiments. Products of *N*-methylnicotinamide oxidation by aldehyde oxidase present in the cytosolic fractions of goat, mouse, and rabbit lung and liver tissues are shown in Figure 2. The enzyme present in goat pulmonary and hepatic tissues oxidized the model substrate to a much lesser extent than that in the rabbit tissues. The enzyme from mouse lung cytosol did not oxidize the substrate to any measurable level, but the mouse liver cytosol had an activity that was lower than that of the goat and higher than that of the rabbit.

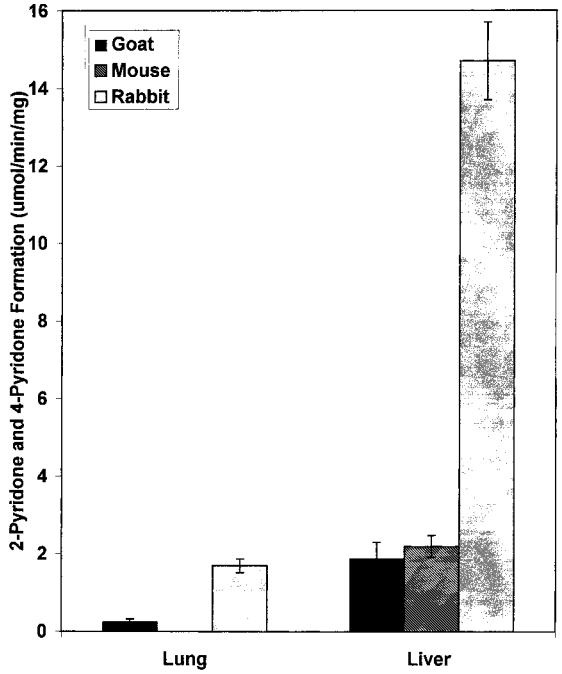


Figure 2. Aldehyde oxidase-catalyzed oxidation of *N*-methylnicotinamide to 2-pyridone and 4-pyridone. Error bars represent standard deviations of two or more replicates from at least three animals.

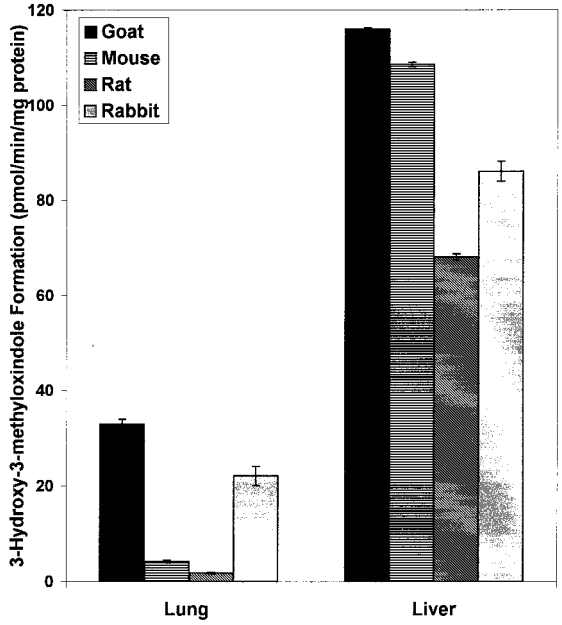


Figure 3. Aldehyde oxidase-catalyzed oxidation of 3-hydroxy-3-methylindolenine to 3-hydroxy-3-methyloxindole. Error bars represent standard deviations of two or more replicates from at least three animals.

For both liver and lung tissues, the rabbit exhibited the greatest activity. As expected (*14*), the activities in liver cytosols were much higher than in the lung tissues for all species.

Aldehyde oxidase-catalyzed conversion of 3-hydroxy-3-methylindolenine to 3-hydroxy-3-methyloxindole was monitored by HPLC. The results are reported in Figure 3. The amount of substrate for the aldehyde oxidase reaction, 3-hydroxy-3-methylindolenine, was normalized in that all incubations contained the same concentration of goat lung microsomal cytochrome P450. Therefore, the production of 3-hydroxy-3-methylindolenine from cyto-

chrome P450-mediated metabolism of 3-methylindole should have been relatively constant. The source of aldehyde oxidase was the cytosolic protein fractions from both liver and lung from goats, mice, rats, and rabbits. 3-Hydroxy-3-methyloxindole was not produced in incubations that contained microsomes alone or cytosolic protein alone (data not shown). Surprisingly, both liver and lung from the goat, the most susceptible species to 3-methylindole pneumotoxicity, exhibited the highest aldehyde oxidase activities for this substrate. The activity in mouse lung was extremely low, while the hepatic activity from this species was nearly as high as that of the goat. The rat exhibited the lowest activities for both lung and liver. The rabbit exhibited an activity nearly as high as the goat for lung, but the activity was lower than both goat and mouse activity for the liver. Expectedly, incubations containing liver cytosol had much higher aldehyde oxidase activities than lung cytosol.

Discussion

Cytochrome P450-mediated epoxidation reactions play a critical role in the biotransformation and subsequent toxicity of many xenobiotics. Perhaps one of the most notable is aflatoxin B_1. This fungal metabolite is biotransformed by cytochrome P450 CYP3A forms to its 8,9-epoxide (*20*) in human lung tissues. The carcinogenicity of 1,3-butadiene is also linked to mono- and diepoxides that are produced by cytochrome P450 enzymes (*21*). Therefore, given that cytochrome P450 enzymes have the potential to oxygenate 3-methylindole to its 2,3-epoxide, it is reasonable to hypothesize that this 2,3-epoxide could play some role in the pneumotoxicity of this compound. Data in this work provide evidence for the existence and the chemical and metabolic fate of this epoxide.

It is possible that oxidation of 3-methylindole to 3-methyloxindole or 3-hydroxy-3-methylindolenine by cytochrome P450 enzymes occurs without the intervention of a formal epoxide or that the epoxide is formed by ring closure of a tetrahedral intermediate, formed by addition (*13*) of a triplet-like FeO species to the 2- or 3-position of the indole moiety. As stated previously, the techniques employed in the current investigations do not permit us to conclude unequivocally that the 2,3-epoxide is released from the active site of the enzyme(s). Studies have shown (*12*), however, that 2,3-epoxides of 2- and 3-substituted *N*-acylindoles can be synthesized chemically, and some of these epoxides are stable overnight at 0 °C. When these indole 2,3-epoxides are warmed to room temperature, they form substituted oxindoles. Thus, these studies demonstrate that 2,3-epoxy-3-methylindoline may be a reasonably stable intermediate, and its formation and release by P450 enzyme(s) are not unreasonable mechanisms of P450-mediated oxygenation of 3-methylindole.

3-Methyloxindole is a stable metabolite of 3-methylindole that theoretically can be formed from the 2,3-epoxide (Scheme 3, D, E, G). As described above, one mechanism for 3-methyloxindole formation is through the epoxide that undergoes an NIH shift. Ring opening of the epoxide probably occurs by another mechanism as well, which does not require a hydride shift for formation of the oxindole; this mechanism invokes hydrogen loss from position 2 and ring opening to the enol with subsequent tautomerization to the keto form. Another mechanism for 3-methyloxindole formation likely involves the hydration of 3-methyleneindolenine at position 2 and a subsequent tautomerization. The studies presented here show that all of the possible mechanisms for 3-methyloxindole formation occur, at least to a small extent.

^{18}O Incorporation from molecular oxygen into 3-methyloxindole demonstrated that 74% of the metabolite contained the label. When this experiment was performed in the presence of *N*-acetylcysteine, 91% of the metabolite contained the label. This confirms that multiple mechanisms for this metabolite's formation are likely occurring (Scheme 2, A). When *N*-acetylcysteine is present in the incubation, one or more reactive intermediates, e.g., 3-methyleneindolenine, that lead to the oxindole by incorporation of oxygen from water are being trapped as the *N*-acetylcysteine thioether conjugate before the metabolite forms. Therefore, in the presence of *N*-acetylcysteine, 91% of the oxindole is probably produced from the epoxide. This leaves 9% whose mechanism of formation is unexplained but could be produced by hydration of the epoxide followed by a dehydration step in which the ^{18}O label is lost (Scheme 3, G). A worldwide shortage of ^{18}O at the time these experiments were performed necessitated the use of extremely small incubation volumes and did not permit experiments in both the absence and presence of *N*-acetylcysteine for studies of ^{18}O incorporation from $H_2{}^{18}O$. As shown in Table 2, the incorporation of ^{18}O into 3-methyloxindole from water was roughly 6%, a value much smaller than the expected 26% based on incorporation from air in the absence of *N*-acetylcysteine. An extremely small amount of 3-methyloxindole was produced in these incubations, probably due to the small incubation volumes. Therefore, reliable quantification of this metabolite was extremely difficult, and poor analytical precision led to a wide range in the incorporation results. Consequently, 20% of the oxygen incorporated into 3-methyloxindole in the absence of *N*-acetylcysteine was unidentified.

3-Methyloxindole's retention of ^2H, when [2-^{2}H]-3-methylindole was used as the substrate in the incubation, also indicates the presence of the epoxide and that at least one mechanism for ring opening involves an NIH shift. In the absence of *N*-acetylcysteine the ^2H retention by 3-methyloxindole was 71%, and in its presence the retention went up to 81%, an increase of 10%. This is somewhat inconsistent with the ^{18}O incorporation data, which displayed an increase in ^{18}O incorporation of 17% in the presence of *N*-acetylcysteine. In both cases *N*-acetylcysteine is presumably depleting the fraction of 3-methyloxindole that forms by hydration of another reactive intermediate (3-methyleneindolenine). If 3-methyleneinodolenine is the precursor of the oxindole, ^{18}O from molecular oxygen would not be incorporated and ^2H would not be retained. Therefore, it might be expected that changes in ^{18}O incorporation and ^2H retention, in the presence of *N*-acetylcysteine, would be of the same magnitude.

However, one might not necessarily expect the absolute percentage changes to be the same due to the possibility that multiple mechanisms of epoxide ring opening may be occurring that lead to the oxindole, perhaps not involving an NIH shift. Ring opening leaving oxygen at position 2, can lead to an enol that tautomerizes to the oxindole, resulting in the loss of the label to water. (Scheme 3, D). Another possibility involves cytochrome P450-mediated direct hydroxylation of 3-methylindole at

C-2 through an addition−rearrangement pathway (*13*) to form the enol which also tautomerizes to the keto form (3-methyloxindole). In both cases, the oxygen incorporated into the oxindole does, in fact, arise from molecular oxygen, but ^2H at position 2 would be lost.

3-Hydroxy-3-methylindolenine is another reactive intermediate of 3-methylindole that likely results from epoxide ring opening leaving the oxygen at position 3. This transient electrophilic intermediate appears to be oxidized to the stable metabolite, 3-hydroxy-3-methyloxindole, by the cytosolic enzyme aldehyde oxidase. Both the ^{18}O incorporation characteristics and the putative detoxification pathway were investigated in this work.

The incorporation of molecular oxygen into 3-hydroxy-3-methyloxindole was higher than that of 3-methyloxindole, 87% versus 74%. The ratios of the $^{18}O_0$, $^{18}O_1$, and $^{18}O_2$ isotopomers indicate that, except for a minor amount of incorporation of two oxygen atoms that originated from molecular oxygen, the vast majority of the compound was formed by incorporation of only one atom from molecular oxygen. There are several possible scenarios for oxygen incorporation into the metabolite, but the one that best fits the data is one in which one site incorporates oxygen from molecular oxygen and the other incorporates oxygen from water. In this case the expected ratios of the isotopomers $^{18}O_0$, $^{18}O_1$, and $^{18}O_2$ in 3-hydroxy-3-methyloxindole, from either molecular oxygen or water, would be 1:1:0. This scenario best fits the data as presented in Tables 1 and 2. The very small amount of the $^{18}O_2$ isotopomer was probably produced by an uncharacterized mechanism that involves incorporation of two oxygen atoms from molecular oxygen.

The mass spectral data (Figure 1) also support a mechanism for 3-hydroxy-3-methyloxindole formation that involves incorporation of only one oxygen from molecular oxygen and that the site of incorporation is the alcohol oxygen. This also supports a mechanism involving the epoxide that ring-opens to position 3. The only fragmentation mechanism resulting in the M − 28 ions at *m*/*z* 135/137 is loss of CO; thus, the retention of approximately an equal isotopomeric ratio (^{18}O atom % of H_2O was 50%) in the *m*/*z* 135/137 fragment cluster indicates that the site of incorporation must be the alcohol at C 3 (Figure 1).

Aldehyde oxidase appears to catalyze the oxidation of 3-hydroxy-3-methylindolenine to 3-hydroxy-3-methyloxindole; therefore, it is reasonable to hypothesize that the enzyme plays an important role in the detoxification of this 3-methylindole reactive intermediate. Presumably, animal species with higher pulmonary aldehyde oxidase activities for this substrate, e.g., rabbits (*14*), would be less susceptible to 3-methylindole-induced pneumotoxicity. When the model substrate (*N*-methylnicotinamide) for this enzyme was utilized, the pattern of enzyme activity for the species and tissue types appeared to confirm this hypothesis; i.e., rabbits had higher enzyme activities in both lung and liver than mice or goats. However, in incubations with 3-methylindole, goats, the most susceptible species, exhibited the highest aldehyde oxidase activities for this substrate in both liver and lung cytosol. Thus, *N*-methylnicotinamide does not appear to be a good surrogate substrate for 3-hydroxy-3-methylindolenine; the isozyme of aldehyde oxidase that catalyzes the oxidation of 3-hydroxy-3-methylindolenine may be different from the isozyme that catalyzes the oxidation of *N*-methylnicotinamide. If 3-hydroxy-3-methylindolenine plays a role in the toxicity of 3-methylindole, then aldehyde oxidase is probably involved in detoxification processes and may be protective of hepatic cells. However, the relative order of species sensitivities to the pneumotoxic effects of 3-methylindole does not correlate to a relative lack of aldehyde oxidase activity in pulmonary tissues of sensitive species.

Presented here is an indirect confirmation that a cytochrome P450-dependent 2,3-epoxide of 3-methylindole exists and can be characterized by monitoring its decomposition products. Additional work is required to elucidate the epoxide's role and the role of 3-hydroxy-3-methylindolenine in 3-methylindole-mediated pneumotoxicity. An analysis of the protein and/or nucleic acid alkylation sites for each of the proposed reactive intermediates is essential to an understanding of the cascade of events that likely lead to the observed toxicity. Work describing the nature of thiol conjugates of these reactive intermediates is underway.

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