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Oxidation of Methyl and Ethyl Nitrosamines by Cytochrome P450 2E1 and 2B1

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Supporting Information

ABSTRACT: Cytochrome P450 (P450) 2E1 is the major enzyme that oxidizes *N*-nitrosodimethylamine [*N*,*N*-dimethylnitrosamine (DMN)], a carcinogen and also a representative of some nitrosamines formed endogenously. Oxidation of DMN by rat or human P450 2E1 to HCHO showed a high apparent intrinsic kinetic deuterium isotope effect (KIE), ≥8. The KIE was not attenuated in noncompetitive intermolecular experiments with rat liver microsomes {^{D}V = 12.5; $^{D}(V/K)$ = 10.9 [nomenclature of Northrop, D. B. (1982) *Methods Enzymol. 87*, 607−625]} but was with purified human P450 2E1 [^{D}V = 3.3; $^{D}(V/K)$ = 3.7], indicating that C−H bond breaking is partially rate-limiting with human P450 2E1. With *N*-nitrosodiethylamine [*N*,*N*-diethylnitrosamine (DEN)], the intrinsic KIE was slightly lower and was not expressed [e.g., $^{D}(V/K)$ = 1.2] in noncompetitive intermolecular experiments. The same general pattern of KIEs was also seen in the $^{D}(V/K)$ results with DMN and DEN for the minor products resulting from the denitrosation reactions (CH_3NH_2, $CH_3CH_2NH_2$, and NO_2^-). Experiments with deuterated *N*-nitroso-*N*-methyl-*N*-ethylamine demonstrated that the lower KIEs associated with ethyl versus methyl oxidation could be distinguished within a single molecule. P450 2E1 oxidized DMN and DEN to aldehydes and then to the carboxylic acids. No kinetic lags were observed in acid formation; pulse−chase experiments with carrier aldehydes showed only limited equilibration with P450 2E1-bound aldehydes, indicative of processive reactions, as reported for P450 2A6 [Chowdhury, G., et al. (2010) *J. Biol. Chem. 285*, 8031−8044]. These same features (no lag phase for HCO_2H formation and a lack of equilibration in pulse−chase assays) were also seen with (rat) P450 2B1, which has a lower catalytic efficiency for DMN oxidation and a larger active site. Thus, the processivity of dialkyl nitrosamine oxidation appears to be shared by a number of P450s.

P450 enzymes are found throughout nature and function primarily as oxygenases.[1] In mammals, they oxidize sterols, fat-soluble vitamins, fatty acids, and various xenobiotics, including drugs, pesticides, and carcinogens. With regard to these xenobiotics, a subset of the P450s function with relatively slow rates of catalysis but with the advantage of broad substrate specificity.[2,3]

One of the reasons to study mammalian P450s is their role in chemical carcinogenesis.[4−6] Many carcinogens are inert until activated to electrophilic products that can bind to DNA.[7,8] One prominent group of carcinogens is the nitrosamines,[9] including many derived from tobacco.[10] Even simple alkyl nitrosamines, e.g., DMN and DEN, are activated by hydroxylation (Scheme 1). The point should be made that alkyl nitrosamines are of interest not only in the context of their presence in the artificial environment (e.g., industrial exposure and tobacco smoking) but also because they are formed endogenously from the reaction of secondary amines (e.g., dietary) with nitrite (formed by the endogenous reduction of nitrate) in the acidic milieu of the stomach.[9]

The two most prominent human P450s involved in nitrosamine metabolism are P450 2E1 and 2A6.[11−14] The kinetics of catalysis of oxidation of DMN and DEN by P450 2A6 have been extensively studied.[15] P450 2A6 oxidizes DMN with high kinetic deuterium isotope effects. An interesting feature (of the P450 2A6 oxidation of both DMN and DEN to aldehydes and then carboxylic acids) is the degree of processivity (i.e., lack of release of the first product prior to the second reaction) of the oxidation reactions (to carboxylic acids), as judged by the lack of a lag phase for carboxylic acid formation and by the results of pulse−chase experiments.[15]

P450 2E1 is the major enzyme involved in the N-demethylation of DMN in rats[16,17] and humans.[11] Studies with transgenic mice have shown that P450 2E1 is a major contributor to liver cancer caused by DEN.[18] Keefer et al.[19] showed that (per)deuteration of DMN greatly attenuated its ability to cause liver cancer in rats. Subsequent in vitro studies with rat liver microsomes reported a kinetic deuterium isotope effect on the K_m for DMN oxidation but not V_{max}.[20] This pattern (isotope effect on K_m but not k_{cat}) was observed in the oxidation of ethanol by recombinant human P450 2E1 and was interpreted in terms of a rate-limiting step following product formation.[21] However, this model could not be applied to P450 2A6 oxidation of DMN and DEN.[15]

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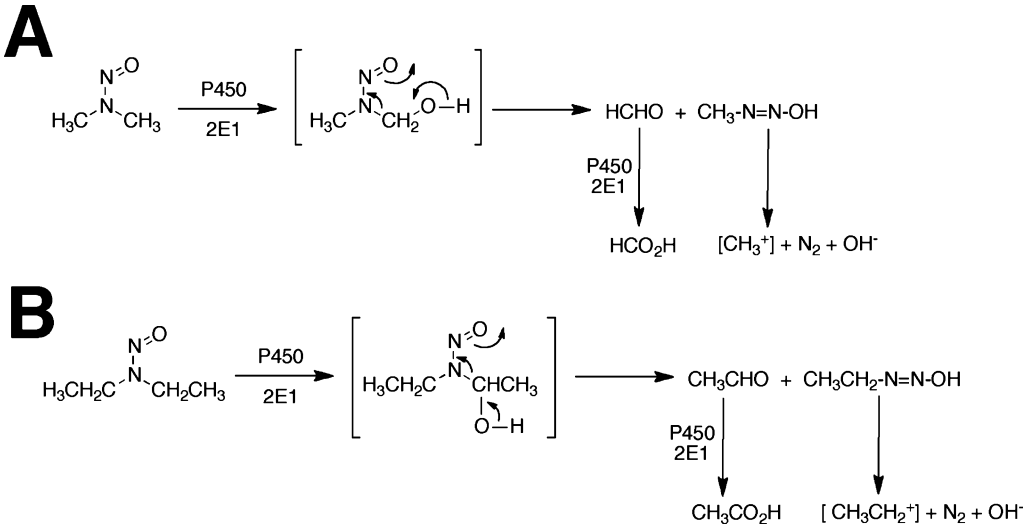
Scheme 1. Oxidations of *N,N*-Dialkyl Nitrosamines to Aldehydes and Acids, (A) DMN and (B) DEN



P450 2A6 and 2E1 have overlapping substrate specificities,[3,12] and the basis for this has been established in the crystal structures, both of which are now available.[22−24] We have also considered (rat) P450 2B1, an enzyme known to be able to catalyze DMN oxidation,[25,26] in the context that the active sites of members of P450 subfamily 2B are known to be larger[27] and might influence the processivity of sequential reactions.

In this work, we considered the oxidation of DMN and DEN with rat and human P450 2E1 and extended the work to MEN, which has elements of both DMN and DEN (i.e., both methyl and ethyl groups). We also found strongly expressed kinetic deuterium isotope effects for the oxidation of DMN and MEN (but not DEN) and processivity in the oxidations of DMN and DEN to carboxylic acids. The processivity of DMN oxidation was also observed with rat P450 2B1.

EXPERIMENTAL PROCEDURES

Caution! *These nitrosamines and nitrosamides are mutagens and carcinogens! All syntheses should be conducted in a fume hood with adequate ventilation, and appropriate skin protection should be used.*

Chemicals. DMN and DEN (d_0d_0, i.e., no deuterium on either alkyl group) were purchased from Sigma-Aldrich (St. Louis, MO) and purified by vacuum distillation before being used (~12 Torr). [^{14}C]DMN was purchased from Moravek Radiochemicals (Brea, CA). Before use in assays for conversion to formic acid, the aqueous material was purified by being passed through a Bakerbond quarternary amine SPE column to remove any carboxylic acids. All deuterated DMN and DEN preparations were synthesized as previously described.[15] MEN (d_0d_0) was purchased from SigmaAldrich and purified by vacuum distillation (57−58 °C, 12 Torr) (note that MEN is no longer available from Sigma-Aldrich, but a recent SciFinder search identified 11 other suppliers). β-Hydroxy DEN (*N*-nitroso-*N*-ethyl-2-hydroxyethylamine) was synthesized as described previously.[15] Specifically deuterated forms of MEN were prepared using the methods described in Figure S1 of the Supporting Information [the NMR spectra of nitrosamines are complex due to the *E*/*Z* character produced by the nitroso group (e.g., ref 15)], and the ^{1}H NMR spectra of deuterated MEN substrates are shown in Figure S1B of the Supporting Information for reference.

Enzymes. Liver microsomes were prepared by differential centrifugation[28] from male Sprague-Dawley rats (~150 g) that had been treated with ethanol (in the drinking water) to induce P450 2E1.[29] Recombinant human P450 2E1 was expressed in *Escherichia coli* and purified as described elsewhere,[30] except that a His_6 tag was added at the C-terminus and Ni^{2+}-nitriloacetic acid affinity chromatography was used in purification. Rat P450 2B1 was expressed from a plasmid obtained from P. F. Hollenberg (University of Michigan, Ann Arbor, MI) and purified by Ni^{2+}-nitriloacetic acid affinity chromatography.[31] P450 concentrations were determined spectrophotometrically using ferrous−CO versus ferrous difference spectra by the method of Omura and Sato.[32] Recombinant rat NADPH-P450 reductase[31] and human cytochrome b_5[33] were expressed in *E. coli* and purified as described elsewhere. P450 2E1, NADPH-P450 reductase, and cytochrome b_5 were mixed in a 1:2:1 ratio in the presence of 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine to reconstitute catalytic activity.[21,30] The same ratios were used for P450 2B1, but cytochrome b_5 was omitted.

Synthesis of *N*-Nitroso-*N*-ethylacetamide.[34] $BiCl_3$ (4.73 g, 15 mmol) and $NaNO_2$ (1.10 g, 16 mmol) were stirred in 10 mL of dry CH_2Cl_2, and *N*-ethylacetamide (1.31 g, 1.41 mL, 15 mmol) was added dropwise. The mixture was capped and stirred vigorously for 2 h at 23 °C, during which time a yellow color appeared. The solids were removed by filtration through paper, and the CH_2Cl_2 was removed carefully using a rotary evaporator. The product was further purified by silicic acid chromatography (CH_2Cl_2), yielding *N*-nitroso-*N*-ethylacetamide as a yellow oil, which chromatographed as a single spot on TLC (silica gel G, CH_2Cl_2, R_f = 0.77): HRMS MH^+ 117.0664 (calcd for $C_4H_9N_2O_2$, 117.0664); ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3H, J = 7.1 Hz, -CH_2CH_3), 2.76 (s, 3H, -$COCH_3$), 3.80 (q, 2H, -CH_2-, J = 7.1 Hz); UV λ_{max} 240, 420 nm.

Synthesis of *N*-Nitroso-*N*-methylformamide.[34] The same procedure utilized for *N*-nitroso-*N*-ethylacetanilide (vide supra) was used in the case of *N*-nitroso-*N*-methylformamide, except that the starting material was *N*-methylformamide: R_f = 0.78 (TLC, silica gel G, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 3.1 (s, 3H, -NCH_3), 10.03 (s, 1H, -CHO); UV (CH_3OH/H_2O) λ_{max} 240, 420 nm.

Determination of $t_{1/2}$ for *N*-Nitroso-*N*-methylformamide and *N*-Nitroso-*N*-ethylacetamide. *N*-Nitroso-*N*-methylacetamide was added to a quartz cuvette containing 1.0 mL of 100 mM potassium phosphate buffer (pH 7.4), and repetitive spectra were collected using an OLIS/Hewlett-Packard 8450A diode array spectrophotometer (Online Instrument Systems, Bogart, GA) (23 °C). The change in A_{240} was fit to a first-order exponential plot.

A solution of *N*-nitroso-*N*-ethylacetamide was prepared in 50 mM potassium phosphate buffer (pH 7.4) and immediately placed in the autosampler chamber of an Acquity UPLC system (Waters, Milford, MA). The sample tray was maintained at 37 °C. *N*-Nitroso-*N*-ethylacetamide was analyzed using a Waters Acquity UPLC system connected to a Waters photodiode array detector, utilizing an Acquity UPLC BEH C18 octadecylsilane column (1.7 μm, 2.1 mm × 100 mm). UPLC conditions were as follows. Buffer A consisted of 10 mM $NH_4CH_3CO_2$ and 2% CH_3CN (v/v), and buffer B consisted of 10 mM $NH_4CH_3CO_2$ and 95% (v/v) CH_3CN. The following gradient program was used, with a flow rate of 250 μL/min: from 0 to 5.5 min, linear gradient from 0 to 50% B (v/v); from 5.5 to 6.0 min, linear gradient to 100% B; from 6 to 8 min, held at 100% B; from 8 to 9.5 min linear gradient to 100% A; from 9.5 to 59 min, held at 100% A. The temperature of the column was maintained at 25 °C. Samples (20 μL) were infused with an autosampler every 60 min. A_{240} was monitored, and the peaks (t_R = 4.6 min) were integrated and fit to a single-exponential plot.

Assays of Catalytic Activity. *General.* As noted in our earlier work with P450 2A6,[15] assays of trace levels of HCHO, CH_3CHO, HCO_2H, and CH_3CO_2H are all highly problematic because of the ubiquitous presence of these compounds in many laboratory reagents. We found simple colorimetric methods unsatisfactory (e.g., Nash assay[35]) for sensitive assays because of the high blank levels, which are notorious.[28] In some cases, HPLC−UV methods could be used to measure hydrazone derivatives of aldehydes, if precautions were used to eliminate sources of contamination. In some critical situations, we utilized [^{14}C]DMN as the substrate. LC−-MS proved to yield low backgrounds if heavy isotopes of aldehydes were measured, particularly if enriched by ≥2 amu. HCO_2H is difficult to derivatize quantitatively[36] and was analyzed (as a radioactive product) using ion-exchange columns.[21] During the course of this work, [^{14}C]DMN became no longer commercially available, and our own synthetic strategies[15] could not be adapted to produce high specific radioactivity. Some of the studies of the rates of oxidation to formaldehyde were conducted with LC−MS assays with d_4- or [^{13}C]DMN, as noted, i.e., microsomal kinetic deuterium isotope studies and rates of oxidation by P450 2B1.

Nitrosamine N-Dealkylation Assays. Typical steady-state N-dealkylation reaction mixtures included 400 pmol of P450 2E1, 800 pmol of NADPH-P450 reductase, 400 pmol of cytochrome b_5, 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and varying concentrations of the nitrosamine substrate in 0.34 mL of 50 mM potassium phosphate buffer (pH 7.4). Reaction vials (clear glass, 1-dram, 4.0 mL) were sealed with Teflon-lined rubber septa because of the volatility of the substrates. Reconstituted enzyme solutions (P450 2E1, NADPH-P450 reductase, and cytochrome b_5) were dialyzed against glycerol-free 50 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM EDTA and 0.1 mM dithiothreitol (two changes over 12 h at 4 °C), before the addition of the phospholipid, to minimize complications arising from residual aldehydes known to be present in glycerol, as well as the inhibitory nature of glycerol on P450 2E1.[37,38] A 60 μL aliquot of an NADPH-generating system was used to start reactions (final concentrations of 10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 1 IU/mL yeast glucose-6-phosphate dehydrogenase).[28] Incubations were generally conducted for 15 min in a shaking water bath at 37 °C, terminated by the sequential addition of 100 μL of 10% (w/v) $ZnSO_4 \cdot 7H_2O$ and 100 μL of saturated aqueous $Ba(OH)_2 \cdot 8H_2O$, and centrifuged ($2 \times 10^3 \times g$). 2,4-Dinitrophenylhydrazine derivatization and analysis were as described previously.[15] 2,4-Dinitrophenylhydrazine was recrystallized twice from a CH_3OH/H_2O mixture (3:1), dried in vacuo, dissolved in 6 M HCl (0.1%, w/v), and washed multiple times with a hexane/CH_2Cl_2 mixture (7:3, v/v) before being used as a derivatization reagent, as described previously,[15] to minimize interference resulting from residual hydrazone contamination. Hexanes and CH_3CN were heated with and distilled from 2,4-dinitrophenylhydrazine to remove residual aldehydes.

Assays involving competitive kinetic deuterium isotope effects were conducted via LC−MS analysis (atmospheric-pressure chemical ionization, negative ion) of derivatized formaldehyde or acetaldehyde [source temperature of 550 °C, heated capillary voltage of 20 V, heated capillary temperature of 180 °C, ionization current of 5 μA, sheath gas (N_2) pressure of 70 psi, auxiliary gas (N_2) pressure of 10 psi]. Representative LC−MS data are shown in Figure S2 of the Supporting Information.

In some cases, aldehydes were derivatized as dansyl hydrazones and analyzed by LC−MS, as described in detail previously.[15]

The results of steady-state kinetic experiments (v vs S) were fit to hyperbolic plots using GraphPad Prism (GraphPad, San Diego, CA), and parameters and standard errors were obtained with this program using nonlinear regression.

Denitrosation Assays. Nitrite formation, an indirect measure of nitric oxide ($NO^\bullet$) formation, was measured colorimetrically by a previously described method.[39]

CH_3NH_2 and $CH_3CH_2NH_2$ were analyzed by derivatization with dansyl chloride[40] and LC−MS, using external standards. Incubations were generally conducted for 30 min in a shaking water bath at 37 °C and terminated by the addition of 100 μL of cold CH_3CN. Propylamine (10 μL, 1 nmol) was added as an internal standard, and the samples were centrifuged ($2 \times 10^3 \times g$). The supernatant (150 μL) was transferred into amber vials, and 10 μL of 1 M NaOH, 40 μL of 0.5 M Na_2CO_3, and 100 μL of dansyl chloride (1 mg/mL in CH_3CN) were added, in that order. The derivatization reaction mixture was heated at 40 °C for 15 min and dried under a gentle stream of N_2 at room temperature, and the residue was reconstituted in 100 μL of a H_2O/CH_3CN mixture (3:1, v/v). Dansylated products were analyzed by LC−MS on a Waters Acquity UPLC system using an Aquity UPLC BEH C18 octadecylsilane column (1.7 μm, 2.1 mm × 100 mm) connected to a ThermoFisher TSQ quantum mass spectrometer. LC conditions were as follows. Buffer A consisted of 0.1% HCO_2H and 5% CH_3CN (v/v), and buffer B consisted of 0.1% HCO_2H and 95% CH_3CN (v/v). The following gradient program was used, with a flow rate of 300 μL/min: from 0 to 4.0 min, linear gradient from 100 to 40% A; from 4.0 to 4.5 min, linear gradient to 100% B; from 4.5 to 6.5 min, held at 100% B; from 6.5 to 7.0 min, linear gradient to 100% A; from 7.0 to 9.0 min, held at 100% A. The temperature of the column was maintained at 40 °C. Samples

(15 μL) were infused with an autosampler. Dansylated products of the alkylamines were detected in the selected ion monitoring (SIM) mode using respective m/z values. The mass spectrometer was tuned using an authentic dansylated CH_3NH_2 standard.

DEN β-Hydroxylation Assay.[15] Typical reaction mixtures included 400 pmol of 2E1, 800 pmol of NADPH-P450 reductase, 400 pmol of cytochrome b_5, 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and 1 mM d_0- or d_4-DEN in 100 μL of 50 mM potassium phosphate buffer (pH 7.4). An NADPH-generating system was used to initiate reactions. Incubations were conducted for 30 min in a shaking water bath at 37 °C and terminated by the addition of 100 μL of cold CH_3CN. The supernatant was transferred, and CH_3CN was removed under a stream of nitrogen. The volume of the reaction mixture was increased to 100 μL with H_2O. β-Hydroxy DEN was analyzed by LC−MS on a Waters Acquity UPLC system using an Aquity UPLC BEH C18 octadecylsilane column (1.7 μm, 2.1 mm × 100 mm) connected to a ThermoFisher TSQ quantum mass spectrometer. LC conditions were as follows. Buffer A consisted of 0.1% HCO_2H and 5% CH_3CN (v/v), and buffer B consisted of 0.1% HCO_2H and 95% CH_3CN (v/v). The following gradient program was used, with a flow rate of 300 μL/min: from 0 to 2.0 min, 100% A; from 2.0 to 3.5 min, linear gradient to 100% B; from 3.5 to 4.5 min, held at 100% B; from 4.5 to 5.0 minm linear gradient to 100% A; from 5.0 to 7.0 min, held at 100% A. The temperature of the column was maintained at 40 °C. Samples (15 μL) were infused with an autosampler. MS was conducted in positive APCI mode, and the products (d_0 and d_4) were detected using SIM mode. The mass spectrometer was tuned using an authentic β-hydroxy DEN standard.[15]

Time Course Assays. For DMN oxidation time course assays, 0.5 nmol of P450 2E1, 1.0 nmol of NADPH-P450 reductase, 0.5 nmol of cytochrome b_5, 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and [^{14}C]DMN (1 mM, 0.1 mCi/mmol) were incubated in 100 μL of potassium phosphate buffer (50 mM, pH 7.4). A 60 μL aliquot of an NADPH-generating system was used to initiate the reactions (final concentrations of 10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 1 IU/mL yeast glucose-6-phosphate dehydrogenase).[28] Incubations were generally conducted for 0−10 min in a shaking water bath at 37 °C and terminated by the addition of 40 μL of a 17% $ZnSO_4{\cdot}7H_2O$ solution (w/v). To measure formation of formaldehyde, 100 μL of potassium phthalinate buffer (250 mM, pH 4.0) was added and samples were centrifuged ($2 \times 10^3 \times g$) for 10 min. The supernatants (200 μL) were transferred to new vials; 50 μL of freshly prepared aqueous *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (15 mg/mL) was added, and the reaction mixtures were incubated at 37 °C for 45 min. Derivatized products were analyzed by HPLC and online scintillation counting using a Zorbax (Santa Clara, CA) cyano (CN) column (150 mm × 4.6 mm, 5 μm). HPLC conditions were as follows. Buffer A was H_2O, and buffer B was CH_3CN. The following gradient program was used, with a flow rate of 1.0 mL/min: from 0 to 6.0 min, linear gradient from 95% A (v/v) to 100% B; from 6.0 to 10 min, held at 100% B; from 10 to 11 min, linear gradient to 95% A (v/v); from 11 to 15 min, held at 95% A (v/v). Quantitation of the product was performed using a standard curve generated with [^{14}C]HCHO (0−20 nmol).

To measure the formation of formic acid, reactions were terminated by the addition of 40 μL of a 17% (w/v) $ZnSO_4{\cdot}7H_2O$ solution and centrifuged ($2 \times 10^3 \times g$) for 10 min. The supernatants were loaded onto Bakerbond quaternary amine SPE columns that had been prewashed with 6 mL of CH_3OH and then equilibrated with 10 mL of H_2O.[21] After being loaded, the columns were washed with 10 mL of H_2O and 10 mL of 20% CH_3OH (v/v) to remove residual aldehyde or nitrosamine substrate. The bound [^{14}C]HCO_2H was eluted with 1.0 mL of 1 M HCl, and radioactivity was measured by liquid scintillation spectrometry in Fisher ScintiVerse III cocktail (Fisher, Pittsburgh, PA).

For DEN, time course assays were performed essentially as reported previously,[15] with the following exceptions. d_3d_3-DEN [labeled at the methyl groups, i.e., $CD_3CH_2N(N{=}O)$-CH_2CD_3] was used as the substrate, and the internal standards used for the aldehyde and acid assays were propionaldehyde and propionic acid, respectively.

Pulse−Chase Experiments. Experiments were conducted with [^{14}C]DMN (1 mM, 0.2 mCi/mmol) in the case of DMN and with d_3d_3-DEN (0.5 mM) in the case of DEN. In both cases, the reaction (37 °C) was initiated with 2.5 μM P450 2E1, 5 μM NADPH-P450 reductase, 2.5 μM cytochrome b_5, 30 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and an NADPH-generating system (vide supra). After 1 or 2 min, either (unlabeled) HCHO (1.3 mM, in the case of DMN) or CH_3CHO (0.12 mM, in the case of the substrate DEN) was added and the reaction was allowed to proceed for a total of 20 min (at 37 °C) (the concentrations used were calculated to give a 20- or 10-fold excess, respectively, compared to the amount of labeled aldehyde known to be produced at that time point). In the case of DMN, the product [^{14}C]HCO_2H was measured as described for the [^{14}C]HCO_2H time course assays (vide supra). A similar experiment was conducted with [^{14}C]DMN and rat P450 2B1. In the case of DEN, the product (CD_3CO_2H/CH_3CO_2H) was derivatized with 4-nitrophenacyl bromide and analyzed by LC−MS, using the procedure described above. Assays were run in triplicate, and the mean results were compared, with the extent of decrease due to the presence of the added aldehyde being indicative of the fraction of the unlabeled aldehyde (or its equivalent) that exchanged.

RESULTS

Steady-State Catalytic Activities of Human P450 2E1.

Human P450 2E1 was approximately twice as efficient in catalytically oxidizing DEN to an aldehyde compared with DMN (Table 1). With MEN, P450 can catalyze hydroxylation of an ethyl group or a methyl group; the former process was

Table 1. Steady-State Kinetic Parameters for Human P450 2E1 Oxidations[a]

substrate	product	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
DMN	HCHO	0.23 ± 0.01	0.92 ± 0.09	250 ± 30
DEN	CH_3CHO	0.27 ± 0.02	0.49 ± 0.07	506 ± 90
MEN	HCHO	0.023 ± 0.007	0.50 ± 0.06	46 ± 5
MEN	CH_3CHO	0.077 ± 0.003	0.76 ± 0.07	100 ± 20
HCHO	HCO_2H	0.012	1.3	9.2
CH_3CHO	CH_3CO_2H	0.077	0.12	640

[a]All assays involved HPLC−UV analysis of 2,4-dinitrophenyl hydrazones except the oxidations of the aldehydes, which were conducted using ^{14}C-labeled substrates and ion-exchange chromatography of the carboxylic acid products. Values are from hyperbolic plots (±SE) made using GraphPad Prism, with further analysis of quotients.

twice as efficient as the latter, consistent with the catalytic efficiencies measured for DMN and DEN (Table 1). However, the overall rate (sum of formation of HCHO and CH_3CHO) was $<^1/_3$ of that for DEN conversion to CH_3CHO (Table 1).

In the case of DEN, there is the possibility of β-oxidation (formation of β-hydroxy DEN). We searched for this using a synthetic standard[15] and LC−MS, but no product was detected [from d_0- or d_4-DEN, i.e., limit of 0.0013 nmol formed min^{-1} (nmol P450 2E1)$^{-1}$].

HCHO and CH_3CHO were both oxidized to carboxylic acids (Table 1). The ratio of catalytic efficiencies for CH_3CHO to HCHO oxidation was ~70 (compared to 19 for P450 2A6[15]).

Kinetic Deuterium Isotope Effects for DMN Oxidation in Rat Liver Microsomes. Wade et al.[20] used liver microsomes prepared from rats treated with acetone to induce P450 2E1 and reported that a kinetic deuterium isotope effect was expressed in K_m (value of 5)[41] but not V_{max}, using a noncompetitive intermolecular experiment (d_0- vs d_6-DMN) and a Nash assay[35] to measure formaldehyde colorimetrically. We repeated this work with liver microsomes prepared from rats treated with ethanol, which also induces P450 2E1.[42] We utilized two assay procedures to measure V_{max} and K_m for the cleavage of the C−H and C−D bonds, because of the problematic issue of HCHO contamination in reagents. [^{13}C]DMN was utilized to measure the rate of oxidation of d_0-DMN (ignoring the small ^{13}C isotope effect), using derivatization of the [^{13}C]formaldehyde with dansyl hydrazine and LC−MS analysis. (As pointed out in Experimental Procedures, [^{14}C]DMN was no longer commercially available at this point in the work.) The ^{13}C-labeled material was used to minimize issues with exogenous HCHO. The rate of cleavage of the C−D bond (of d_6-DMN) was analyzed by LC−MS of the released DCDO, following derivatization with dansyl hydrazine.

Measurement of noncompetitive intermolecular kinetic deuterium isotope effects provides insight into the extent to which C−H bond breaking is rate-limiting in the overall steady-state reaction.[43] The noncompetitive intermolecular kinetic deuterium isotope effects measured in rat liver microsomes were as follows: ^DV = 12.5 ± 1.2, and $^D(V/K)$ = 10.9 ± 6.0 (Figure 1). A separate experiment involved d_4-DMN [CD_2H-N(NO)-CD_2H], in which HCDO and DCDO were detected by LC−MS following derivatization with dansyl hydrazine.[15] This estimated intrinsic kinetic deuterium isotope effect (^Dk) was 8.1 ± 0.1 (n = 3), without correction for any secondary isotope effect.[44] The values measured in the noncompetitive intermolecular experiment (i.e., 12.5 and 10.9) are as high (within experimental error) as the estimated intrinsic isotope effect (vide supra), supporting the conclusion that C−H bond breaking is largely rate-limiting. These results contrast with those in a previous report,[20] in that the deuterium isotope effect was expressed in V_{max} but not in K_m in our work.

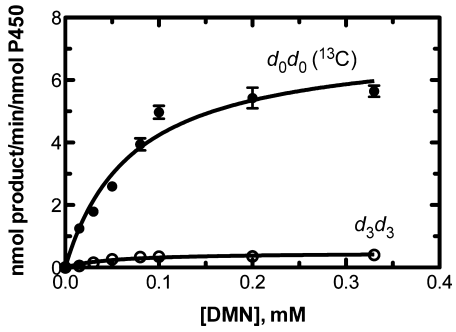


Figure 1. Noncompetitive intermolecular kinetic deuterium isotope effects for oxidation of DMN to formaldehyde by rat liver microsomes containing P450 2E1 (d_6 vs d_0). The assays were conducted with d_0-[^{13}C]DMN (●), and the d_6 assays were conducted with $CD_3N(NO)$-CD_3 (○). In these experiments, the dansyl hydrazone samples were analyzed by LC−MS.

Noncompetitive Intramolecular and Competitive Kinetic Deuterium Isotope Effects with Purified Recombinant Human P450 2E1. The noncompetitive kinetic deuterium isotope effects were measured (MS) by competition at -CHD- sites (Table 2): 8.4−8.8 for DMN and 6.3−7.0 for DEN. Both sets of values are subject to possible contributions of secondary isotope effects (possibly as high as 1.4^2 ~ 2 for DMN[44]) but are still relatively high. With DEN, there is a possible stereoselective contribution.[45] The intramolecular competitive kinetic isotope effect for DEN was 5.6, and that for DMN was 12 (Table 2). The intermolecular competitive kinetic isotope effect for DMN was 12 (Table 2).

Table 2. Intramolecular and Competitive Intermolecular Kinetic Deuterium Isotope Effects for Human P450 2E1-Catalyzed N-Dealkylation of DMN and DEN[a]

substrate	product	[substrate][b]	$^D(V/K)$
	DMN		
CHD_2-N(N=O)-CHD_2	formaldehyde	0.5 × K_m	8.4 ± 0.3
	formaldehyde	1.0 × K_m	8.8 ± 0.1
	formaldehyde	1.5 × K_m	8.7 ± 0.1
CH_3-N(N=O)-CD_3	formaldehyde	1.0 × K_m	12 ± 1
CH_3-N(N=O)-CH_3/CD_3-N(N=O)-CD_3	formaldehyde	1.0 × K_m	12 ± 4
	DEN		
CH_3-CHD-N(N=O)-CHD-CH_3	acetaldehyde	0.5 × K_m	6.3 ± 0.6
	acetaldehyde	1.0 × K_m	7.0 ± 0.2
	acetaldehyde	1.5 × K_m	6.7 ± 0.5
CH_3CH_2-N(N=O)-CD_2CH_3	acetaldehyde	1.0 × K_m	5.6 ± 0.1

[a]All measurements were taken with 2,4-dinitrophenyl hydrazones (MH^+) separated by HPLC online. n = 3; values are means ± the standard deviation. [b]See Table 1 and Figure 2 regarding K_m values.

A series of different deuterated MEN derivatives all yielded relatively high kinetic deuterium isotope effects (Table 3). Most were >4, with the only one lower being 2.9 for a competitive experiment with CD_3-N(NO)-CD_2CH_3.

Noncompetitive Intermolecular Kinetic Deuterium Isotope Effects with Recombinant Human P450 2E1. The noncompetitive intermolecular kinetic deuterium isotope effect was considerably higher for the oxidation of DMN than DEN (Figure 2). Further analysis of the DMN results showed components of the kinetic deuterium isotope effect mainly in the k_{cat} component (Table 4), as in the case of rat liver microsomes containing P450 2E1 (Figure 1).

The differences in the rates of formation of formaldehyde and acetaldehyde from d_0-MEN and $CH_3CD_2N(NO)CD_3$ were significant but ≤2-fold (Table 4 and Figure 3). However, closer analysis (Table 4) of the results in Figure 4 revealed more striking differences when MEN molecules with individually deuterated sites were compared. Deuterium substitution of the methylene group of MEN (d_2) increased the isotope effect (^DV) for demethylation 4.3-fold, in considering d_0d_2- and d_3d_2-MEN. The ^DV was ~5 in comparing d_3d_0- and d_0d_0-MEN

Table 3. Kinetic Isotope Effects for Human P450 2E1-Catalyzed N-Dealkylation of MEN[a]

substrate	product	$^{D}(V/K)$
CD_2H-N(N=O)-CH_2CH_3	formaldehyde	7.6 ± 0.2
CH_3-N(N=O)-CH_2CH_3/CD_3-N(N=O)-CD_2CH_3	formaldehyde	4.1 ± 0.1
CH_3-N(N=O)-CH_2CH_3/CD_3-N(N=O)-CH_2CH_3	formaldehyde	7.6 ± 0.5
CH_3-N(N=O)-$CHDCH_3$	acetaldehyde	6.0 ± 0.2
CH_3-N(N=O)-CH_2CH_3/CH_3-N(N=O)CD_2CH_3	acetaldehyde	4.2 ± 0.1
CH_3-N(N=O)-CH_2CH_3/CD_3-N(N=O)-CD_2CH_3	acetaldehyde	2.9 ± 0.1

[a]All measurements were taken with 2,4-dinitrophenyl hydrazones (MH^+) separated by HPLC online. Substrate concentrations were 1 mM. n = 3; values are means ± the standard deviation.

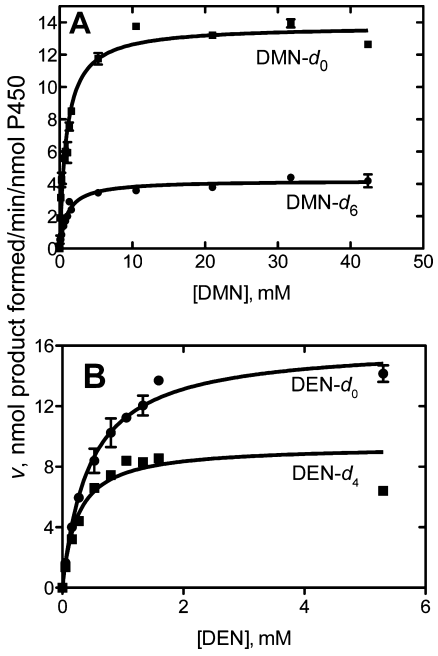


Figure 2. Noncompetitive intermolecular kinetic deuterium isotope effects for oxidation of DMN and DEN by purified human P450 2E1. (A) DMN: d_6 (●) vs d_0 (■). (B) DEN: d_4 (■) vs d_0 (●), i.e., CH_3CD_2N(N-O)CD_2CH_3. See Table 4. 2,4-Dinitrophenyl hydrazones were analyzed by an HPLC−UV method.

(Figure 3A). Similarly, an increase (~2-fold) in the level of MEN deethylation was seen upon deuteration of the methyl group (Figure 3B). The value of ^{D}V was 2.4 in comparing d_0d_2- and d_0d_0-MEN, considerably more than for comparing d_0d_0-

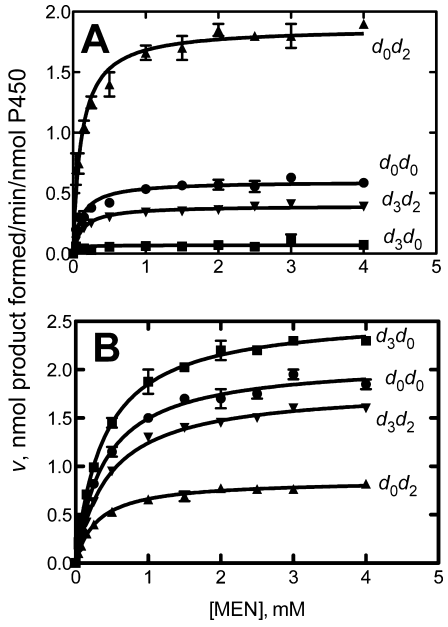


Figure 3. Noncompetitive intermolecular kinetic deuterium isotope effects for oxidation of MEN by P450 2E1: (A) N-demethylation and (B) N-deethylation. d_3 indicates CD_3-N(N=O)-, and d_2 indicates -N(N=O)CD_2CH_3. 2,4-Dinitrophenyl hydrazones were analyzed by an HPLC−UV method.

and d_2d_3-MEN (Figure 3B). These results can be attributed to "metabolic switching"; i.e., blocking one part of a molecule causes the enzyme to oxidize another site.[46] Although increases in some reactions were observed by blocking other oxidations (Figure 3), it should be noted that the total amount of dealkylation did not increase because of deuteration. The metabolic switching observed here (Figure 3) is entirely consistent with the results listed of Tables 2 and 3, in which competitive experiments did not show an attenuation of the kinetic isotope effect because of any restriction of tumbling or exchange of the substrate with the medium.

Oxidations of Nitrosamines to Carboxylic Acids by P450 2E1. P450 2E1 oxidized HCHO and CH_3CHO to carboxylic acids, as reported previously,[47] with the conversion of CH_3CHO to CH_3CO_2H being much more efficient (Table 1). The nitrosamines (DMN and DEN) were also shown to form the carboxylic acids (Figure 4). Careful analysis of the products showed the lack of a lag phase for the oxidation of nitrosamine to carboxylic acids, which would have been expected to occur if the aldehydes freely dissociated and bound again to P450 2E1 for further oxidation.

Table 4. Noncompetitive Intermolecular Isotope Effects for Human P450 2E1-Catalyzed Oxidations of Nitrosamines to Aldehydes[a]

substrate	isotope location	product	^{D}V	$^{D}(V/K)$
DMN	CD_3N(N=O)CD_3 (d_3d_3 vs d_0d_0)	formaldehyde	3.3 ± 0.1	3.7 ± 1.4[b]
DEN	CH_3CD_2N(N=O)CD_2CH_3 (d_2d_2 vs d_0d_0)	acetaldehyde	1.4 ± 0.1	1.2 ± 0.1
MEN	CH_3CD_2N(N=O)CD_3 (d_3d_2 vs d_0d_0)	formaldehyde	2.0 ± 0.1	1.5 ± 0.4
MEN	CH_3CD_2N(N=O)CD_3 (d_3d_2 vs d_2d_0)	formaldehyde	4.7 ± 0.1	5.3 ± 0.9
MEN	CH_3CD_2N(N=O)CD_3 (d_3d_2 vs d_3d_0)	acetaldehyde	0.48 ± 0.03	0.77 ± 0.07
MEN	CH_3CD_2N(N=O)CD_3 (d_3d_2 vs d_0d_0)	acetaldehyde	1.3 ± 0.1	1.8 ± 0.5

[a]From Figures 2 and 3. Values are from hyperbolic plots (±SE) made using GraphPad Prism, with further analysis of quotients. [b]The large SE is a reflection of the uncertainty in the estimation of the K_m for the d_6 substrate [CD_3N(N=O)CD_3] (SE is 38% of K_m).

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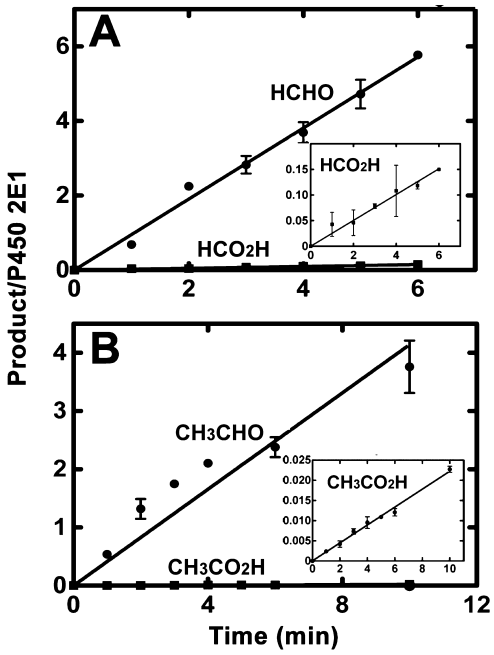

Figure 4. Kinetics of conversion of *N*,*N*-dialkyl nitrosamines to aldehydes (●) and carboxylic acids (■) by P450 2E1: (A) DMN and (B) DEN. The procedures are described in Time Course Assays. For each experimental time point, the mean (■) and range (n = 2) are shown. Fits were made using linear regression. The insets are expansions of the results for formation of the carboxylic acids, which lack a lag phase in both cases.

A model constructed using a simplified kinetic scheme for the sequential oxidations (Figures S3 and S4 of the Supporting Information) showed (Figure 5) (i) an expected lag in the

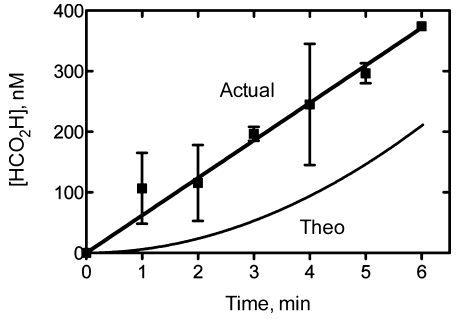

Figure 5. Comparison of the human P450 2E1 experimental ("Actual") time course of product formation (Figure 4) with prediction ["Theo" (theoretical)] based on k_{cat} and K_m values for individual reactions (Table 1). For each experimental time point, the mean (■) and range (n = 2) are shown. The experimental fit used linear regression analysis. See Figure S4 of the Supporting Information for the DynaFit script and differential equations used in the modeling.

production of the second oxidation product (HCO_2H) and inconsistency with the experimentally observed rapid, immediate formation of HCO_2H (Figure 4) and (ii) the fact that the level of HCO_2H product formation is considerably greater than expected on the basis of the measured k_{cat} and K_m values for oxidations of DMN and HCHO. However, because of the much higher efficiency of P450 2E1 conversion of acetaldehyde to acetic acid (vs that of formaldehyde to formic acid) (Table 1), this latter relationship (Figure 5) did not apply with DEN (results not presented).

Pulse−Chase Experiments. Pulse−chase experiments were conducted with P450 2E1. Reactions were initiated with labeled DMN (^{14}C) or DEN [d_6, i.e., $CD_3CH_2N(N{=}O)CH_2CD_3$], and after 1 or 2 min, excess unlabeled aldehyde was added and the reactions were continued for a total of 20 min (Figure 6). If the aldehyde had completely exchanged with

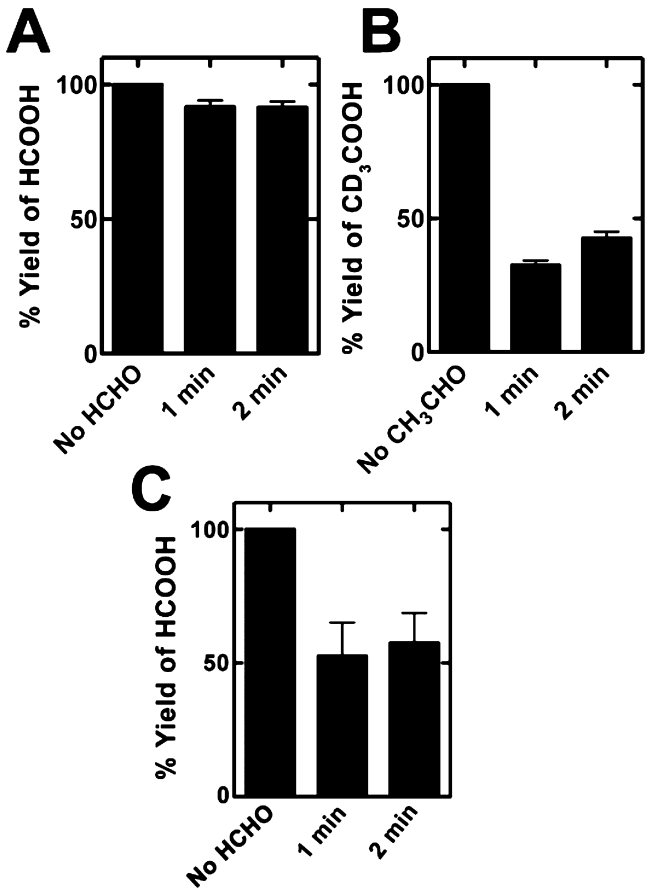

Figure 6. Pulse−chase experiments. (A) P450 2E1 and DMN oxidation to formic acid. (B) P450 2E1 and DEN oxidation to acetic acid. (C) P450 2B1 and DMN oxidation to formic acid. Results are shown as means (n = 3) ± the standard deviation. In the case of complete equilibration of the intermediate aldehyde with the medium during the 20 min incubation, the expected yield of labeled carboxylic acid would be ~5% for a 1 min preincubation and ~10% for a 2 min initial incubation with labeled substrate, based on the expected production of the product calculated for other assays. The procedures are described in Pulse−Chase Experiments.

the intermediate (aldehyde) bound to the P450 2E1, the yield of (labeled) carboxylic acid would have been reduced to 5−10%. Contrary to the expected 5−10% yield reduction if complete equilibration were to occur, the carboxylic acid yields with P450 2E1 remained as high as 30−40% for DEN and 90% with DMN and, with P450 2B1, ~50% for DMN (Figure 6). Thus, limited equilibration occurred with the added aldehydes in the medium.

Denitrosation of DMN and DEN by P450 2E1. The denitrosation of DMN has been demonstrated in rat liver microsomes and in vivo[20,39,48,49] and proposed to follow a sequence similar to that of N-demethylation (Scheme 2).[20] The released nitric oxide is oxidized (nonenzymatically) to nitrite, and another product of the reaction is the aldehyde (HCHO from DMN and CH_3CHO from DEN) (Scheme 2).

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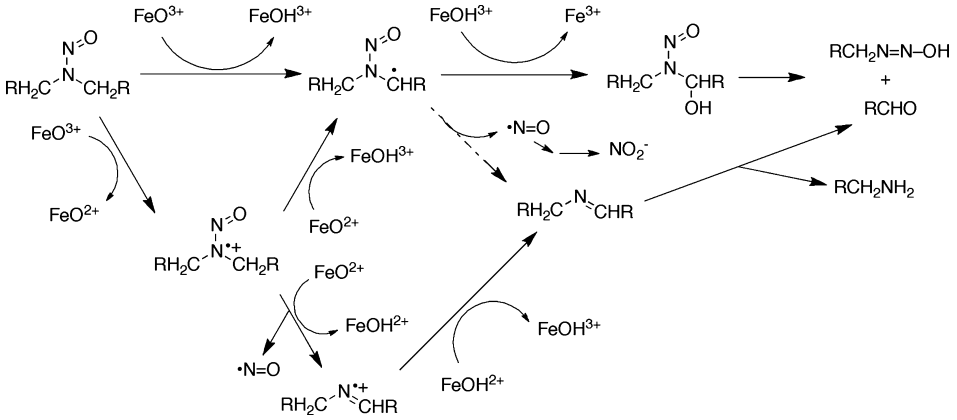
Scheme 2. Possible Mechanisms for N-Dealkylation and Denitrosation of Dialkyl Nitrosamines[20]



We analyzed the products with recombinant human P450 2E1 (Figure S5 of the Supporting Information). Nitrite was measured colorimetrically,[39] and the k_{cat} values with DMN and DEN were 1.0 and 1.1 min^{-1}, respectively (0.017 and 0.018 s^{-1}, respectively). CH_3NH_2 and $CH_3CH_2NH_2$ were assayed (as dansyl derivatives) by LC–MS and were found to be generated (from DMN and DEN, respectively) with k_{cat} values of 2.8 and 3.6 min^{-1}, respectively (0.047 and 0.06 s^{-1}, respectively). In line with the microsomal results,[20,39,48] this pathway accounts for ≤15% of the formation of aldehydes from d_0-DMN and -DEN (Figure 2). Our measurements showed approximately half as much NO_2^- formation as alkylamine from DMN and DEN, possibly because of the complexity of NO• oxidation to NO_2^- (Scheme 2).[20] The complexity of the denitrosation pathways may also be responsible for the variation in the K_m values, compared with the N-dealkylation reactions (Table 1 and Figure 2).

The kinetic deuterium isotope effects on the denitrosation reaction were also measured (Figure S5 of the Supporting Information). The formation of nitrite from DMN showed a ^{D}V of 0.69 ± 0.10 and a $^{D}(V/K$ of 8.8 ± 3.3. With DEN, nitrite production yielded a ^{D}V of 0.77 ± 0.09 and a $^{D}(V/K)$ of 2.0 ± 0.6. The analysis of the alkylamines (LC–MS) yielded a ^{D}V of 0.76 ± 0.24 and a $^{D}(V/K)$ of 4.2 ± 3.4 for DMN and a ^{D}V of 1.6 ± 0.3 and a $^{D}(V/K)$ of 1.3 ± 0.5 for DEN. Although the errors for $^{D}(V/K)$ were large, inspection of the plots (Figure S5 of the Supporting Information) clearly shows the kinetic isotope effects on the initial slopes in the DMN series (and the small isotope effects with DEN).

Although NO• is apparently produced in the denitrosation of dialkyl nitrosamines and can complex P450 heme, the concentration was apparently not great enough to be significant in that the reactions (dealkylation) were linear for ≥10 min (Figure 4). Further, ferric P450–NO• complexes have strong 430 nm bands,[50,51] and we were unable to detect such a complex when steady-state spectra were recorded [350–600 nm (data not presented)].

Oxidation of DMN by Rat P450 2B1. Although P450 2E1 and 2A6 appear to be the enzymes most involved in the oxidations of small alkyl nitrosamines,[11–14,16,17] other P450s can oxidize nitrosamines to aldehydes, including DMN.[25,26,52,53] In many earlier studies, relatively high concentrations of DMN were used, but P450 enzymes in what is now known as subfamily 2B were active.[25,26] We selected (rat) P450 2B1, which had been studied before (then termed "P450 PB-B").[25,26]

P450 2B1 oxidized DMN to HCHO with a k_{cat} of 0.11 ± 0.01 s^{-1} and a K_m of 2.5 ± 0.5 mM (Figure S6 of the Supporting Information), a catalytic efficiency 6-fold lower than that of P450 2E1. The intrinsic kinetic deuterium isotope effect (^{D}k) was estimated to be 2.0 ± 0.1, using LC–MS analysis of DCDO and HCDO formed from d_4-DMN (DMN with -CD_2H groups).[a] The enzyme formed HCO_2H, as well as HCHO, with the former not showing a lag in formation (Figure 7).

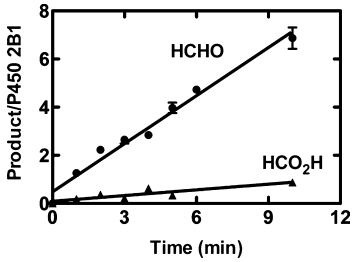
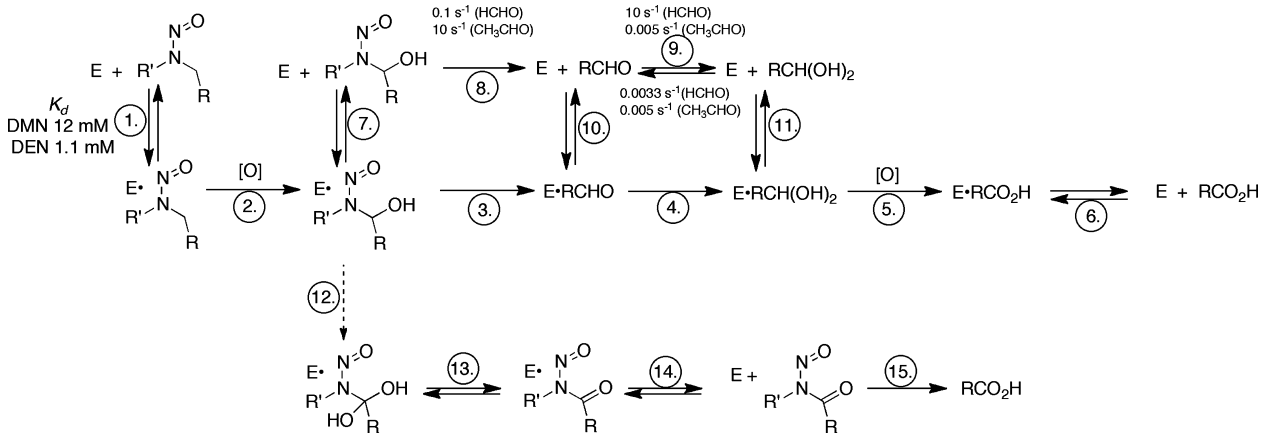


Figure 7. Kinetics of conversion of [^{14}C]DMN to formaldehyde and formic acid by P450 2B1. Results are means of duplicate experiments, with the means (● for HCHO and ▲ for HCO_2H) and ranges shown. Fits were made by linear regression analysis. Note the lack of a lag phase for carboxylic acid formation.

Attempts To Implicate *N*-Nitrosamides as Secondary Oxidation Products. One potential explanation for the processivity of P450 2E1 reactions is that the initial α-hydroxylation product is further oxidized to an *N*-nitrosamide (Scheme 3, step 12), circumventing (at least in part) decomposition to an aldehyde. The nitrosamide derivatives of DMN and DEN are previously synthesized compounds[34] and are known to decompose to HCO_2H and CH_3CO_2H, respectively. To address the possibility that these compounds are formed, we synthesized and characterized both *N*-nitrosamides (Figure S7 of the Supporting Information). The methyl and ethyl nitrosamides had respective $t_{1/2}$ values of 56 s (determined by scanning UV spectroscopy, at 23 °C) and 114 min (determined by HPLC) [in 0.10 M potassium phosphate buffer (pH 7.4) at 37 °C].

Both *N*-nitrosamides had poor MS ionization characteristics, and accordingly, searches for the production of these compounds as oxidation products were conducted with a UPLC–UV method (diode array detection), using the wavelength maxima at 240 nm (for both *N*-nitroso-*N*-methylformamide and *N*-nitroso-*N*-ethylacetamide). Incubations were conducted with DMN and DEN under the usual

Scheme 3. Oxidations of *N*,*N*-Dialkyl Nitrosamines to Aldehydes and Acids, Including the Possibility of *N*-Nitrosamides[a]



[a]Reported rates of nonenzymatic reactions in steps 8 and 9 are from the indicated references.[55,61,63,64]

reaction conditions (1 mM substrate and 37 °C), with UPLC−UV analysis from the steady state (reaction for 5 min). The retention times for *N*-nitroso-*N*-methylformamide and *N*-nitroso-*N*-ethylacetamide were 5.9 and 4.6 min, respectively, under these HPLC conditions, and we estimate that the limit of detection was ∼10 pmol of product. No *N*-nitrosamide products could be detected from either DMN or DEN in P450 2E1 reactions under these conditions, and we conclude that any production of these *N*-nitrosamides, if formed, is below or near the detection limit of our UPLC−UV detection method.

In other efforts related to this hypothesis, we added 1 mM α-acetoxy DMN (gift of L. A. Peterson, University of Minnesota, Minneapolis, MN)[54] and an excess of hog liver esterase (to generate α-hydroxy DMN, which has a $t_{1/2}$ of 7 s)[55] to P450 2E1, NADPH-P450 reductase, cytochrome b_5, and NADPH. No evidence of the production of *N*-nitroso-*N*-methylformamide was obtained in these experiments either.

DISCUSSION

The oxidation of the *N*-alkyl nitrosamines DMN, DEN, and MEN was examined with rat and human P450 2E1, the major enzyme involved in DMN oxidation. Kinetic deuterium isotope effects were measured, and the C−H bond breaking step was found to be at least partially rate-limiting in DMN oxidation. As in the case of the metabolism of DMN and DEN by P450 2A6,[15] P450 2E1 formed not only the aldehyde products but also the corresponding carboxylic acids. The lack of a lag phase in the production of the acids (Figures 4 and 7) and the results of the pulse−chase experiments (Figure 6) indicate a largely processive nature of the conversion of DMN and DEN to the respective carboxylic acids, which was observed previously in the human P450 2E1 oxidation of CH_3CH_2OH to CH_3CO_2H.[47]

P450 2E1 showed a preference for the oxidation of the ethyl moiety relative to methyl, as judged by the catalytic efficiencies (k_{cat}/K_m), which showed an ∼2-fold difference (Table 1). The same difference was also seen in comparing the two alkyl substituents of MEN (Table 1). However, the ethyl selectivity (i.e., DEN > DMN) was not as great as with P450 2A6 (∼40-fold selectivity).[15]

An intramolecular noncompetitive deuterium isotope effect (Table 2) is an estimate of the intrinsic isotope effect, with the caveat that secondary kinetic isotope effects might be included.[44] The estimated isotope effects were 8.4−8.8 for DMN and 6.3−7.0 for DEN for human P450 2E1 (Table 2), values that are consistent with many other P450 reactions.[21,56−58] In experiments with intermolecular competition (requiring substrate exchange, i.e., dissociation and reassociation) or competition between the two alkyl groups on a single substrate (requiring substrate flipping), little attenuation was observed (Table 2). Thus, we conclude that substrate exchanges freely in the active site of P450 2E1. In a series of kinetic isotope effect experiments with MEN, similar conclusions could be reached (although the value of 2.9 seen for the competition between d_0- and d_5-MEN was weaker) (Table 3).

Comparisons between the kinetic isotope effects measured in noncompetitive intermolecular experiments (Table 4 and Figures 1 and 2) with the higher values in Table 2 are useful. In the simplest case, the values [$^D(V/K)$ = 3.7 and 1.2 for DMN and DEN, respectively (Table 4 and Figure 2)] may be compared with values of 8.4−8.8 and 6.3−7.0, respectively, in Table 2. These values indicate that (i) C−H bond breaking is partially rate-limiting in the oxidation of DMN and (ii) C−H bond breaking is not rate-limiting in DEN oxidation.

The results with MEN in the remainder of Table 4 and Figure 3 are more complex. These are indicative of metabolic switching, i.e., a shift in oxidation from one alkyl group (ethyl or methyl) to the other caused by deuteration.[46] The overall yields of product formation (acetaldehyde and formaldehyde) are approximately constant when one of the alkyl chains is deuterated (but less when both are). A simplified overall conclusion is that the overall rate of oxidation of MEN is not changed by deuteration at a particular carbon, but the intramolecular site of oxidation is. These results are consistent with the lack of attenuation of the (apparent) intrinsic kinetic deuterium isotope effect. Thus, for MEN and DEN (Table 2), a step(s) other than C−H bond breaking is largely rate-limiting and deuteration at one part of the molecule will block oxidation, but tumbling or exchange occurs to change the site of oxidation but not the overall rate. However, the results with DMN showed a lack of a switch from N-dealkylation to denitrosation caused by deuteration, consistent with the proposal (Scheme 2)[20] that both pathways involve initial hydrogen abstraction. The possibility of an initial one-electron abstraction from the nitrogen atom is considered unlikely with *N*-nitrosamines in light of the limited electron density of the

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nitrogen atom (as shown by ^{15}N NMR)[59] and the strong kinetic isotope effects (Tables 2−4). With DEN, there is a lack of a switch to β-hydroxylation, based on the lack of detection of that product with or without deuteration of the methylenes of DEN (vide supra).

The reason for the greater attenuation of the kinetic isotope effect in DEN compared to DMN is not clear. The same pattern was seen with P450 2A6.[15] The C−H bond in a methylene group should be ∼6 kcal/mol weaker than in a methyl[60,61] (without consideration of the influence of the nitrosamine), because of an inductive effect. Oxidation is more efficient at the methylene carbon [i.e., DEN > DMN (Table 1)], but the difference is only ∼2-fold (cf. 40-fold for P450 2A6).[15] Which P450 step(s) in catalysis (with DEN) is rate-limiting is not clear. We previously reported that P450 2E1 reduction (of the ferric iron) is rapid even in the absence of a substrate.[62] A step following product formation is limiting in the oxidation of ethanol, as judged by the burst kinetics.[21]

One of the reasons for initiating this study, as well as those with P450 2E1 and ethanol[21] and P450 2A6, DMN, and DEN,[15] was the initial observation by Keefer et al.[19] of the protective effect of DMN deuteration against liver cancer in rats. Later work with rat liver microsomes showed a kinetic deuterium isotope effect (for DMN N-demethylation) only on K_m but not V_{max}.[20] Such a pattern was observed in the oxidation of ethanol, in our own work, and rationalized in the context of burst kinetics and a rate-limiting step following product formation.[21] We did not find such a burst for aldehyde formation with DMN or DEN (Figure 4). P450 2E1 is now recognized to be the major enzyme involved in DMN oxidation in rat[16,17] and human[11] liver. However, the kinetic deuterium isotope effects we measured for N-dealkylation were for k_{cat} and not K_m (Figures 1 and 2 and Table 4).

Our N-dealkylation results (Figure 1) clearly differ from those of Wade et al.[20] We propose that the use of the Nash colorimetric assay[35] is not sensitive enough to accurately measure low rates of formaldehyde formation, because of the issues with background formaldehyde, which have complicated our own studies. We found that we could only reliably assay formaldehyde with HPLC-based methods (with derivatives), preferably using isotopically labeled DMN (^{14}C, ^{13}C, or ^{2}H). Even (LC−MS) assays generating H^{13}CHO were problematic under sensitive conditions and required numerous controls.

Processivity was observed in the conversion of DMN and DEN to the respective carboxylic acids (HCO_2H and CH_3CO_2H, respectively), i.e., the intermediate did not readily exchange with added aldehydes. Evidence of processivity is based on the lack of any lag in the formation of acids from nitrosamines (Figure 4), which would have been expected with free dissociation of aldehyde (Figure 5). Further, pulse−chase experiments did not show complete equilibration of the aldehydes with the P450-bound intermediate (Figure 6).

Several possibilities can be considered regarding the observed processivity. One is that the intermediate aldehydes are inherently tightly bound to P450 2E1. However, this was not found to be the case previously.[47] Another possibility we considered was that the α-hydroxynitrosamines might be directly oxidized, in part, to yield nitrosamides (Scheme 3, step 12). This explanation is attractive, in that the relevant nitrosamides are known to be mutagens[34] and also to break down to carboxylic acids (Scheme 3, step 15). However, we could not detect either *N*-nitroso-*N*-methylformamide or *N*-nitroso-*N*-ethylacetamide using UV or MS detection. Another possibility is that some aspect of the aldehyde hydration−dehydration process is missing in the kinetic analysis; e.g., several of the rate constants for hydration and rehydration of aldehydes are either slow [in solution, i.e., <0.1 s^{-1} (Scheme 3)[63,64]] or unknown in the presence of the enzyme. At least three mechanisms are possible for P450 oxidation of an aldehyde,[65] and depending on which is used, the aldehyde might require a certain pathway (Scheme 3).

Another possibility for the processivity has been proposed, with kinetic modeling, for the same phenomenon observed in P450 2A6.[15] The gist of the mechanism is that the P450 undergoes an initial conformation change (for which there is general evidence based upon substrate binding[66]), α-oxidation of the nitrosamine occurs, and then the second oxidation occurs while the enzyme is still in the "reactive" conformation (before it relaxes). We did not do the kinetic modeling with P450 2E1, although the P450 2A6 model can be readily adapted with some numerical changes. The modeling admittedly has arbitrary rate constants and does not in itself prove the mechanism. Nevertheless, a model similar to that proposed for P450 2A6[15] is certainly viable and can explain the phenomenon.

In light of some of the unresolved issues regarding the P450 oxidation of nitrosamines, we also considered the oxidation of DMN by rat P450 2B1. The structures of P450 2A6[22] and 2E1[23] indicate small active sites, with some substrates (including an *N*-nitrosamine) fitting rather tightly.[24] The features of oxidation of small alkyl nitrosamines considered here (kinetic deuterium isotope effects and processivity) are of interest in the sense that they may be related to the size of the active site. Earlier studies indicated that other P450s can oxidize DMN to HCHO, including rat P450 2B1.[25,26] Crystal structures of the closely related rabbit P450 2B4[27] and human P450 2B6[67] enzymes have been determined and indicate much larger binding sites, which probably apply to rat P450 2B1 as well in light of similar catalytic selectivities. We did some investigations with P450 2B1 and found, as expected,[17,26] a higher K_m (2.5 mM). Nevertheless, several of the phenomena observed with P450 2A6 and 2E1 were also present with P450 2B1, including the oxidation of DMN to both HCHO and HCO_2H without a lag (Figure 7) and the lack of equilibration of added HCHO with the enzyme in the pulse−chase experiments (Figure 6). We suggest that (i) the small size of the active sites of P450 2A6 and 2E1 is not the major factor in at least some of the observed processive phenomena and (ii) these phenomena may be more widespread among the P450s that can conduct these reactions.

In summary, we have examined several aspects of the oxidation of DMN and related alkyl *N*-nitrosamines by P450 2E1, the major enzyme involved in the activation of this substrate. A large intrinsic kinetic deuterium isotope effect was observed for DMN N-demethylation, which was only partially attenuated in noncompetitive intermolecular studies with rat and human P450 2E1 and indicative of a partial rate-limiting contribution of C−H bond cleavage. The results can explain the attenuated hepatocarcinogenicity of deuterated DMN in rats.[19] As in the case of P450 2A6,[15] oxidation of DMN and DEN occurs to give aldehydes and carboxylic acids in a rather processive manner. Several explanations can be considered, but the most likely involve conformational changes or possibly slow carbonyl hydration−dehydration steps.[65] We were unable to demonstrate postulated oxidation to *N*-nitrosamides, although

the possibility should not be dismissed in light of the technical limitations of our work.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of deuterated MEN substrates and ^{1}H NMR spectra, LC−MS of 2,4-dinitrophenyl hydrazone of HCHO formed from DMN, binding spectra of P450 2E1 with DMN and DEN, Dynafit modeling of theoretical time courses of formation of HCHO and HCO_2H, steady-state kinetics of denitrosation products, steady-state kinetics of N-demethylation of d_4-DMN by rat P450 2B1, and HPLC−UV data for *N*-nitroso-*N*-methylformamide. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

DEN, *N*,*N*-diethylnitrosamine (*N*-nitrosodiethylamine); DMN, *N*,*N*-dimethylnitrosamine (*N*-nitrosodimethylamine); HPLC, high-performance liquid chromatography; HPLC−MS, high-performance liquid chromatography with MS detection; HPLC−UV, HPLC with UV detection; HRMS, high-resolution mass spectrometry; LC, liquid chromatography; MEN, *N*-methyl-*N*-ethylnitrosamine (*N*-nitroso-*N*-methylethylamine); MS, mass spectrometry; P450, cytochrome P450; SE, standard error; UPLC, ultraperformance liquid chromatography; UPLC−MS, ultraperformance liquid chromatography with MS detection; UPLC−UV, ultraperformance liquid chromatography with UV detection; ^DV and $^D(V/K)$, kinetic deuterium isotope effects on V_{max} (or k_{cat}) and V_{max}/K_m (or k_{cat}/K_m), respectively, using the nomenclature of Northrop.[41]

■ ADDITIONAL NOTE

[a]These assays were conducted with d_4-DMN [HD_2C-N(N=O)CD_2H] to reduce the contribution of background HCHO in the LC−MS assays and because [^{14}C]DMN was no longer available at the time that these studies were conducted. The intrinsic kinetic deuterium isotope effect was low for P450 2B1 (2.0, vide supra). We corrected the apparent k_{cat} [0.053 s^{-1} (Figure S6 of the Supporting Information)], measured for the production of DCHDO, by multiplying it by 2 because the statistical chance of breaking a C−D bond (in -CD_2H) is twice that for breaking a C−H bond, but the apparent intrinsic kinetic isotope effect of 2.0 makes this half as rapid, thus suggesting a yield of HCDO equal to that of DCDO.

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