ABSTRACT

The rates of elimination of N-nitrosodimethylamine (NDMA) and its fully deuterated analogue (N-nitrosodi[2H₆]methylamine, \([2H_6]NDMA\)) were studied in vivo to explore the origins of the difference in their carcinogenicity. Male Fischer 344 rats, 7.5 weeks of age, were given nitrosamine bolus doses of 1.35 \(\mu\)mol/kg by tail vein injection and 2.02 or 4.05 \(\mu\)mol/kg by p.o. gavage. Animals were sacrificed at various time points from 2.5 to 180 min after i.v. administration or 5 to 120 min after p.o. dosage, and their blood was analyzed for NDMA by gas chromatography-high resolution mass spectrometry. After i.v. injection, blood nitrosamine concentrations declined in an apparently biexponential manner with a terminal half-life of 10 min for NDMA and 12 min for \([2H_6]NDMA\). The apparent total systemic blood clearances for NDMA and \([2H_6]NDMA\) were 39 and 26 ml/min/kg, respectively. The apparent steady-state volumes of distribution were nearly identical (297 and 309 ml/kg, respectively). The areas under the curve after 2.02- and 4.05-\(\mu\)mol/kg p.o. doses were proportional to dose. The apparent bioavailability of NDMA was 8%, while that of \([2H_6]NDMA\) was 21%. Isotope effects calculated as the ratios of first-pass metabolism, total systemic clearances, bioavailabilities, and intrinsic hepatic clearances were 1.2, 1.5, 2.6, and 3.2, respectively. The isotope effect determined from blood concentrations measured after simultaneous administration of NDMA and \([2H_6]NDMA\) by steady-state infusion (each at 1.5 \(\mu\)mol/kg/h) was 2.6 ± 0.9 (SD). This study thus provides quantitative reference data on the time course of the disappearance of both N-nitrosodimethylamine and its deuterated analogue from blood (over 5 to 8 half-lives) after doses similar to those used to elicit liver tumors in chronic feeding studies, and permits estimation of their bioavailabilities from actual blood concentrations. The results suggest that elimination pathways not involving \(\alpha\)-hydroxylation are more important than is currently recognized.

INTRODUCTION

The metabolism of NDMA has been extensively studied since the initial discovery of its hepatotoxic and carcinogenic activities (1, 2). It is generally accepted that NDMA exerts its carcinogenic action after enzymatic carbon-hydrogen bond oxidation, which leads to a reactive methylating agent (2-4). The demonstration of a deuterium isotope effect on liver DNA methylation (5) and carcinogenicity (6) supports this concept. At doses similar to those used in chronic carcinogenicity studies, the in vivo metabolism of NDMA in rats has been studied only indirectly by examining byproducts of NDMA metabolism, such as carbon dioxide or DNA methylation products. These studies have shown that the hepatic DNA is virtually the exclusive site of DNA methylation after low p.o. doses of NDMA. However, after low i.v. doses or high p.o. doses, kidney DNA methylation is also observable, with the kidney/liver ratio of DNA methylation increasing with p.o. dose. After single, high p.o. doses, NDMA is primarily a kidney carcinogen, while with low chronic dosing, NDMA is primarily a liver carcinogen (1, 2, 7). The suggested origin of the influence of dose and route of administration on NDMA organotropism is extensive hepatic first-pass metabolism of NDMA (low bioavailability) after p.o. administration that is saturable at high doses (7). Thus, after low p.o. doses of NDMA, rapid metabolism of NDMA by the liver during transit of NDMA from the gut to the general circulation prevents systemic exposure to NDMA. The observation of increased kidney DNA methylation and decreased liver DNA methylation with fully deuterated NDMA suggests that deuterium substitution substantially decreases first-pass metabolism (5).

Despite the importance of these concepts, direct studies examining the pharmacokinetics of low-dose administration of NDMA and \(N\)-nitrosodi[2H₆]methylamine (\([2H_6]N\)DMDA) have not been performed. The purpose of this study was to examine the pharmacokinetics and deuterium isotope effect of NDMA at doses similar to those used to elicit liver tumors.

MATERIALS AND METHODS

Chemicals. \(N\)-Nitrosodimethylamine was purchased from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). \([2H_6]NDMA\) was prepared as previously described (6) and had an isotopic purity of 99.3 atom %. Solutions for animal treatments were prepared by dissolving weighed samples of the nitrosamines in various volumes of Dulbecco's phosphate buffered saline (Whittaker M.A. Bioproducts, Walkersville, MD). Concentrations were verified by UV spectrophotometry. Solutions were used within a few days of preparation; previous experiments showed that neither the isotopic purity of \([2H_6]NDMA\) (6) nor the absorbance of NDMA changes significantly with time under these conditions. For the infusion studies, a solution containing 10 units of heparin/ml as well as 30 \(\mu\)g each of NDMA and \([2H_6]NDMA\)/ml in normal saline was used.

Animals and Treatments. For the bolus administration studies, male Fischer (F344/NCr) rats were obtained from the Animal Production Area of the Frederick Cancer Research Facility and were used at 7 to 8 weeks of age. Their weights ranged from 129 to 221 g with a mean of 171 ± 19 g (SD). Rats were given free access to food and water throughout the experiment. Animals receiving i.v. doses were placed in restraining...
cages and were given injections of the 1.35 mm solution (1 ml/kg) via the tail vein without anesthetic. Typically, injections required no more than 30 s. The end of the injection period was taken as time zero. The p.o. doses were administered by gavage with each animal receiving 1.00 ml of a solution of 0.405 or 0.81 mm nitrosamine. Groups of 3 to 5 animals were killed by cervical dislocation at 2.5, 5, 10, 20, 40, 60, and 90 min after i.v. dosing, and 5, 10, 20, 30, 40, 50, 60, 90, and 120 min after p.o. dosing. Blood was collected from the aorta and inferior vena cava, and the time of collection was taken as the approximate midpoint of the roughly 30- to 100-s exsanguination procedure. Methoxyflurane anesthesia was used during blood collection from animals killed 90 min or longer after drug administration.

For the infusion studies, 5 male Fischer (CDF344/Cr/BR) rats were obtained from Charles River Breeding Laboratories and were housed as described (8). They were given free access to food (Certified Rodent Chow 5002, Ralston Purina Co., St. Louis, MO) and water at all times. Rats used in these experiments weighed 228 to 255 g. The jugular vein of each animal was cannulated under methoxyflurane anesthesia; 24 h later a continuous infusion of a solution containing 30 μg each of NDMA and [2H6]NDMA in 1 ml/kg/h of 0.9% saline was started. Isobutane pressure in the ion source was 20 pascals. A small volume of blood (50 μl) was drawn at 20 min after injection of the first dose and another aliquot of blood was drawn at 20, 5, 1.25, and 0.2 ng/ml, respectively.

Assay of NDMA in Blood. The concentration of NDMA in each blood sample was determined by gas chromatography-high resolution mass spectrometry using a previously described procedure (9) modified as follows. An aliquot of blood was mixed with 7.5 ng of [15N2]NDMA in 0.3 ml of water. The mixture was diluted with 1 ml of 1 M borate buffer (pH 10) and extracted with 7 ml of dichloromethane. The recovery of NDMA in the extraction step is 50-75%. The organic layer was separated and concentrated to 50 μl under a stream of argon further purified by passage through concentrated sulfuric acid. A 1-μl aliquot of the concentrated solution was injected onto a 50-m x 0.33-mm flexible quartz capillary column coated with 0.2 μm of Carbowax 20 M (Chrompack, Inc.). The column was mounted in a Carlo Erba gas chromatograph and operated with oven and injector temperatures of 100°C and 128°C, respectively. The retention time of NDMA at a hydrogen carrier gas pressure of 1 kg/cm2 was 180 s. The effluent from the gas chromatography column was diverted 60 s after injection directly into the chemical ionization ion source of a Kratos MS-50 high resolution mass spectrometer set to monitor, at a mass resolution of 10,000 (10% valley definition), the M+ ions of NDMA at 75.0558 (or [15N2]NDMA at 81.0935) and [2H6]NDMA at 77.0499 atomic mass units. The separator and ion source were at 150°C. Isobutane pressure in the ion source was 20 pascals. A small amount of isobutyl alcohol was added to the ion source for mass scale calibration.

The m/z 75 to m/z 77 ion ratio from the analysis of a blood sample was converted to a concentration of NDMA using the slope and intercept values from a weighted (1/y) linear least squares analysis of the ion ratio (ordinate) versus amount added (abscissa) data from the analysis in duplicate of 2-ml water samples fortified with 3.75 ng of [15N2]NDMA/ml and either 20, 5, 1.25, or 0.2 ng of NDMA/ml or of [2H6]NDMA. Various volumes of blood were analyzed in order to keep the ion ratios within the calibration range. The primary NDMA calibration solution was prepared from neat [2H6]NDMA. The primary [2H6]NDMA solution was prepared from neat [2H6]NDMA. Precision of the analyses from consideration of the calibration curve data is as follows: interassay (relative SD of the amount found), 3.8, 9.1, 10.9, and 15.3% at 20, 5, 1.25, and 0.2 ng/ml, respectively; intrassay (relative SD of the ratio of the duplicate analyses), 6.8, 12.7, 12.3, and 19.3% at 20, 5, 1.25, and 0.2 ng/ml, respectively.

In separate experiments, artifact formation could not be detected in any of 200 similarly prepared human plasma samples spiked with morpholine and stored at −20°C with sodium azide and morpholine for 18 months (detection limit, 2% of the theoretical yield).

Data Analysis. Each data point from the animals receiving i.v. doses was plotted and the results for each nitrosamine dose were fitted to a two-compartment open model with the aid of the PHARM pharmacokinetic parameter estimation program (10). Various weighting schemes were attempted with extended least squares nonlinear regression (11) yielding the best fit as judged by examination of the residuals. The reported pharmacokinetic parameters for the i.v. doses were calculated from the fitted data. Due to extremely high variability after p.o. doses, the observed blood concentrations were averaged for each time interval after the blood concentration was normalized to the 2.02- or 4.05-μmol/kg dose. The AUC was calculated with a linear trapezoidal rule using zero as a data point at time zero. The area after the last data point was obtained by dividing the concentration at that point by the rate constant for elimination determined from the corresponding i.v. data, then extrapolating to time infinity. The extrapolated area was less than 5% of the total AUC. The oral clearance and intrinsic clearance were calculated by dividing the dose by the AUC (12). The bioavailability was calculated from the dose-normalized AUC plots from the respective p.o. and i.v. administrations (12) using data from both the 2.02- and 4.05-μmol/kg p.o. doses.

Pharmacokinetic Considerations. Frequently the ability of hepatic enzymes to remove drug greatly exceeds the ability of the body to deliver drug in blood. In this case there is a blood flow limitation to drug removal, and the true expression of alterations in enzymatic activity (such as those associated with deuterium isotope effects) is masked because blood flow, not enzymatic activity, is the rate-limiting step in drug removal (12, 13). For a drug solely removed by the liver, this phenomenon can be expressed mathematically by the following equation (12-14):

\[
\frac{C_l}{\text{Dose}} = \frac{(Q_b)_o + (F_b)_o)(C_{Dose})}{(Q_b)_o + (F_b)_o + (C_{Dose})} = \frac{Dose}{AUC_o} \]

where \(C_l\) is the total systemic clearance, \(Q_b\) is the hepatic blood flow, \(F_b\) is the free fraction of drug in blood and \(C_{Dose}\) is the intrinsic clearance. The intrinsic clearance (see below) is a parameter which quantitates total, in this case, hepatic enzyme activity. When the intrinsic clearance is high, the total systemic clearance is a measure of organ blood flow rather than of enzyme activity. Similar to the masking of the intrinsic deuterium kinetic isotope effect by other rate-limiting steps in the enzymatic cycle (15-17), the true deuterium isotope effect on enzyme activity can be suppressed by blood flow limitations in vivo drug removal.

The suppression of the deuterium isotope effect at the enzyme level by organ blood flow limitations to metabolism can be eliminated by determining the drug AUC after a p.o. dose (12-14). In this case an increase in bioavailability directly compensates for blood flow limitations for drug removal and the full enzyme deuterium isotope effect (but not the intrinsic deuterium kinetic isotope effect) is expressed. For a drug that is completely absorbed, the total systemic clearance for a p.o. dose is

\[
C_{Dose} = \frac{Dose}{\text{AUC}_{p.o.}} = \frac{(C_{Dose})(F_b)}{\text{AUC}_{p.o.}}
\]

Since NDMA is completely unbound in plasma (18) (i.e., \(F_b = 1\) and completely absorbed (7, 19), the AUC after a p.o. dose is independent of hepatic blood flow and is inversely related to the intrinsic clearance. The intrinsic clearance is related to the enzyme kinetics for an unbound substrate by the following equation (12, 13, 20):

\[
C_{I} = \sum \frac{V_{max}}{K_m + S}
\]

When the substrate concentration, \(S\), is low compared to \(K_m\) (i.e., linear or first-order pharmacokinetics), the intrinsic clearance is simply the sum of the \(V_{max}/K_m\) values for the enzymes which remove NDMA. If \(K_m \gg S\), then the deuterium kinetic isotope effect on the AUC after p.o. NDMA administration is a direct measure of the \(V_{max}/K_m\) deuterium kinetic isotope effect at the enzyme level. The doses of NDMA used in this study were as low as possible to approach linear pharmacokinetics.
RESULTS

The individual NDMA and [2H6]NDMA blood concentration versus time curves obtained after i.v. injection of 1.35 μmol/kg are shown in Chart 1. Since an individual animal was used for each time point, the data points at equivalent time intervals were averaged (Chart 2) to accommodate statistical analysis. For the averaged data, the NDMA blood concentration was always lower than the corresponding [2H6]NDMA blood concentration. At five of the seven time points, the [2H6]NDMA blood concentrations were statistically different from the corresponding NDMA concentrations (two-tailed Student’s t test). Both NDMA and [2H6]-NDMA data sets were adequately described by a two-compartment open model with distribution phase half-lives of about 1 min and terminal half-lives of 10 min for NDMA and 12 min for [2H6]NDMA. Based on the fitted data, the early or distribution phase is pharmacokinetically important since approximately 50% of the NDMA and 34% of the [2H6]NDMA appeared to be irreversibly removed in this early phase. Unfortunately the first data point was taken 2.5 min after dosing and a large portion of the AUC had to be determined by extrapolation to time zero. Nevertheless the steady-state volume of distribution (Vss), determined from the fitted data, was 297 ml/kg for NDMA and 309 ml/kg for [2H6]NDMA. Since an isotope effect on drug distribution is unexpected, the similarity of the volume of distribution estimates indicates that the errors introduced by the extrapolation are minor. The total systemic clearances of NDMA and [2H6]-NDMA from blood were 39 and 26 ml/min/kg, respectively; these values approached hepatic blood flow in the rat.

The blood concentrations of NDMA and [2H6]NDMA after p.o. doses showed tremendous interanimal variation. The average coefficient of variation of the NDMA blood concentration was 25%. In contrast the average coefficient of variation of the blood concentration after i.v. administration was 10%. This variability greatly exceeded the variability in the analytical method. To suppress variability and test statistical significance, the concentrations at equivalent time intervals were averaged. As shown in Chart 3, the averaged blood concentrations of NDMA and [2H6]-NDMA still showed high variability. Nevertheless the AUC after the 2.02- and 4.05-μmol/kg p.o. doses (4270 nm·min and 8890 nm·min, respectively) were proportional to the dose. This indicates that NDMA metabolism in this dose range displays linear pharmacokinetics. The estimated bioavailability, or the fraction of drug which is absorbed, escapes first-pass metabolism, and reaches the general circulation, was calculated to be approximately 8% for NDMA and 21% for [2H6]NDMA. Assuming that absorption is complete (7, 19) and that nitrosamine is eliminated by hepatic metabolism (18), the ER was 92% for NDMA and 79% for [2H6]NDMA. Since NDMA is completely unbound in plasma (18), the intrinsic clearance can be calculated from the oral data. The intrinsic clearance is 467 ml/min/kg for NDMA and 147 ml/min/kg for [2H6]NDMA. As indicated in Chart 3, at seven of nine of the time points, the differences in the NDMA and [2H6]NDMA blood concentrations were statistically significant.

The deuterium kinetic isotope effect calculated from the ratio of the total systemic clearances from the i.v. bolus data (CIH/CID) is 1.5. The relative elimination rates of NDMA and [2H6]NDMA were also assessed by coadministration of these agents by steady-state i.v. infusion at low doses. The relative concentration
of [2H6]NDMA/NDMA at steady-state, corrected for small differences in the molar dose, was 2.6 ± 0.9 (mean ± SD, N = 5). The deuterium kinetic isotope effect estimated from the ratio of the hepatic extraction ratios (ERH/ERD) is 1.2. The isotope effects calculated as the ratios of bioavailabilities and of intrinsic clearances were 2.6 and 3.2, respectively.

DISCUSSION

Previous in vivo studies of NDMA metabolism in rats, in which the disappearance of NDMA from blood was measured, were conducted at relatively high doses (18, 21–25). The rate of metabolism of NDMA after administration of doses greater than 10 mg/kg is about 5 mg/kg/h and follows zero-order kinetics (22). We designed this study to examine the rates of metabolism of NDMA and [2H6]NDMA at doses relevant to the carcinogenicity studies and under first-order conditions, where the substrate concentration is small compared to the Michaelis-Menten constant for in vivo NDMA elimination. After a 1.35-μmol/kg (100 μg/kg) i.v. dose, the elimination of NDMA from blood was very rapid. The early phase (half-life, 1 min) of NDMA elimination accounted for approximately 50% of the metabolism of the administered dose. After drug distribution NDMA was eliminated with a half-life of 10 min. The metabolism of NDMA is so rapid that it was difficult to characterize the pharmacokinetics of the early phase. Future low-dose i.v. studies should use short-term i.v. infusions to help characterize the rapid phase of NDMA elimination. Nevertheless the apparent total systemic clearance of NDMA from blood, determined from the AUC of the fitted data, was 39 ml/min/kg. This clearance value approaches hepatic blood flow. Since the liver is the predominant organ responsible for NDMA elimination (18), this clearance value is consistent with a drug whose rate of in vivo metabolism is limited by delivery of drug to the eliminating organ (high hepatic extraction ratio) and not by hepatic enzymatic activity. Full appreciation of this observation is critical to proper interpretation of the influence of route of administration on the observed deuterium isotope effects.

The blood levels observed after p.o. administration of 2.02 and 4.05 μmol/kg of NDMA showed high variability. Nevertheless the AUCs of the averaged concentrations after these doses were proportional to dose. This indicates that, under the conditions used, the route constant of metabolism is constant and approaches first-order conditions (Km ≫ S). The blood concentration of NDMA after p.o. doses decreased more slowly than after i.v. doses. This indicates that the absorption of NDMA is slow relative to rates of elimination and suggests that the marked variability in the data after p.o. administration is due to erratic rates of absorption. A previous study by Pegg and Perry (7), using the extent of liver and kidney DNA methylation to examine the dose-dependent bioavailability of NDMA, indicated that the elimination of 2.02- and 4.05-μmol/kg doses (150 and 300 μg/kg) is characterized by nonlinear kinetics in fasted animals. In addition these investigators showed that in fasted animals the absorption of NDMA is very rapid from the intestine but not from the stomach. In our study the animals were not fasted and displayed a slower rate of absorption. Since the rate of absorption is expected to have a profound influence on the dosing of NDMA which saturates first-pass metabolism, it is likely that linear pharmacokinetics was observed in our study at 150- and 300-μg/kg doses because NDMA absorption was slow.

The bioavailability of NDMA estimated from the relative dose-normalized AUC in male Fischer 344 rats is approximately 8%. This result agrees well with previous indirect observations of NDMA bioavailability. Since the extent of 7-methylguanine formation is linearly related to increasing NDMA doses and is not subject to rapid repair, it is possible to estimate bioavailability from previous DNA alkylation data (7, 19, 26, 27). Based on the relative rates of 7-methylguanine formation in kidney after low i.v. and p.o. doses of NDMA at the lowest dose tested (approaching Km ≫ S), bioavailabilities of 6% (19) and 8% (7) can be estimated. Thus using direct studies of NDMA metabolism, our results confirm that low p.o. doses of NDMA are subject to extensive first-pass metabolism. These results also support the previous conclusion (2, 27) that the liver is the site of NDMA-induced lesions after low, chronic doses because it is virtually the exclusive site of metabolism after p.o. administration.

The terminal biological half-life of NDMA determined in this experiment agrees well with the value found in a previous study (11 min; Ref. 8) but it is substantially shorter than the half-lives of 45–60 min (28) used in a recent calculation (29) of the possible flux of NDMA formed in vivo in humans. The discussion of the latter paper (29) acknowledged that elimination half-life might be much more rapid than 45–60 min at low doses, however, and explicitly considered the influence of a higher elimination rate on the calculated endogenous exposure. The present work provides reference pharmacokinetic data that may prove useful in such risk assessment calculations.

The effect of substitution of deuterium for hydrogen in NDMA on its major disposition pathways in vivo was evaluated by comparing the NDMA and [2H6]NDMA blood concentrations after (a) independent bolus i.v. and (b) p.o. administration of equimolar doses of each agent and (c) by comparing the blood concentrations after concurrent steady-state i.v. infusion of each agent. At every time point the average [2H6]NDMA blood concentration after bolus administration was higher than the corresponding NDMA concentration, and in most cases the difference was statistically significant. That the magnitude of the observed deuterium isotope effect was dependent on the route of nitrosamine administration is readily explainable by fundamental pharmacokinetic principles.

The deuterium isotope effect estimated from the ratio of total systemic clearances after independent i.v. administration of NDMA and [2H6]NDMA is approximately 1.5. The magnitude of this observed deuterium isotope effect is not a measure of the true deuterium isotope effect at the enzyme level because of the blood flow limitation to enzymatic drug removal. This in vivo suppression of the isotope effect by blood flow limitations is directly analogous to the suppression of the enzymatic intrinsic deuterium isotope effect (kH/kD for the enzymatic bond-breaking step) by other rate-determining steps in the enzymatic cycle (15–17). The observation of a deuterium isotope effect after i.v. nitrosamine administration in the presence of blood flow limitations to metabolism (nonenzymatic rate-determining step) indicates that the true enzymatic isotope effect is much larger than 1.5 (see below).

The suppression of the enzymatic deuterium isotope effect by blood flow limitations to metabolism can be partially eliminated by concurrent i.v. administration of NDMA and [2H6]NDMA. In this experimental design the protium and deuterium variants...
directly compete for enzymatically active sites, and the competitive nature of the experiment provides an opportunity for the further expression of the enzymatic isotope effect (5, 15–17, 30). As expected, the deuterium isotope effect estimated from the relative concentrations of NDMA and \(^{[\text{H}_6]}\)NDMA after concurrent steady-state infusions is larger than that observed on total systemic clearance after independent administration of the nitrosamines (2.6 versus 1.5). However, the full inherent deuterium isotope effect at the enzyme level is still partially suppressed by blood flow limitations of \textit{in vivo} drug elimination.

For a drug which is completely absorbed after a p.o. dose and is eliminated predominantly by the liver, the AUC after a p.o. dose is a direct measure of the efficiency of hepatic enzymes that irreversibly remove drug. Thus the AUC under these conditions is independent of hepatic blood flow and can be used to estimate the \(V_{\text{max}}/K_m\) deuterium kinetic isotope effect. Our oral experiment was designed to estimate this isotope effect. The p.o. doses used were extremely low for this type of experiment and the AUCs after 2.02- and 4.05-\(\mu\)mol/kg doses were proportional to dose. The observation of dose proportionality indicates that the substrate concentration is low relative to the \(K_m\) for metabolism and that this experiment approached \(K_m \gg S\) conditions. Since NDMA is completely absorbed (7, 19), metabolized predominantly in the liver (18), and displays linear pharmacokinetics at the doses used, an estimate of the enzymatic \(V_{\text{max}}/K_m\) deuterium isotope effect can be made from the ratio of the unbound AUCs (ratio of intrinsic clearances). The ratio of the unbound AUCs after p.o. doses of \(^{[\text{H}_6]}\)NDMA and NDMA is 3.2. This value is the best measure of the deuterium kinetic isotope effect (but not the intrinsic enzyme isotope effect) at the enzyme level \textit{in vivo} since it is not subject to suppression by blood flow limitation of metabolism. Thus as predicted for a drug with a high hepatic extraction ratio, the magnitude of the observed deuterium isotope effect increases when the experiment is designed to factor out blood flow limitations to metabolism. If \(K_m \gg S\) conditions were not truly achieved in this experiment, the \(V_{\text{max}}/K_m\) isotope effect calculated in this experiment would be an underestimate of the true \(V_{\text{max}}/K_m\) effect.

In a previous study (5) of the deuterium isotope effect on \textit{in vivo} NDMA metabolism, which used large doses (40 mg/kg) and measured the rate of carbon dioxide formation rather than NDMA disappearance, only a small isotope effect (1.2) was observed on the maximal velocity of NDMA metabolism. However, when large doses \((V_{\text{max}}/S\) conditions, no blood flow limitation) of NDMA and \(^{[\text{H}_6]}\)NDMA were concurrently administered, the isotope effect on the initial rate of nitrosamine metabolism was 3.9 (5). Competitive experiments do not permit determination of an isotope effect on maximal velocity since neither substrate can truly saturate the enzyme in the presence of the other. Consequently this competitive experiment measured \(V_{\text{max}}/K_m\) isotope effects and is in quantitative agreement with the \(V_{\text{max}}/K_m\) isotope effect measured at low p.o. doses in the present experiment. The present study thus confirms the previous finding with direct measurements of NDMA disappearance after independent, low-dose nitrosamine administration.

The estimated bioavailability of \(^{[\text{H}_6]}\)NDMA is 21%, compared to 8% for NDMA. The deuterium isotope effect on the bioavailability of NDMA is 2.6. Thus 2.6 times more \(^{[\text{H}_6]}\)NDMA than NDMA reaches the general circulation after equimolar p.o. doses. This result is in good agreement with the observation that alkylation of kidney DNA is 3 times greater with \(^{[\text{H}_6]}\)NDMA than with NDMA after low p.o. doses (5). The isotope effect on the percentage of p.o. administered NDMA that is metabolized before it reaches the general circulation is much lower. Approximately 92% of p.o. administered NDMA is metabolized during its transit from the gastrointestinal tract to the general circulation at these doses. With deuterium substitution this percentage is reduced to 79%. Thus the ratio of the hepatic extraction, or the isotope effect on the percentage of p.o. administered nitrosamine that is metabolized on the first pass through the liver, is only 1.2. Even then the relatively small percentage of nitrosamine that escapes first-pass metabolism is expected to be predominantly metabolized in the liver. Thus the percentage of a p.o. dose that is ultimately metabolized in the liver should be essentially the same with and without deuterium substitution, and the deuterium isotope effect on the extent of NDMA metabolism by the liver should be essentially 1.0. However, previous studies have shown that deuterium substituted NDMA gives rise to only two-thirds of the extent of liver DNA alkylation as NDMA (5), yielding an isotope effect on alkylation of 1.5. The origin of this decreased extent of liver DNA alkylation with \(^{[\text{H}_6]}\)NDMA is unclear. One possibility is that the rate, rather than the extent, of formation of methylating species influences the extent of liver DNA alkylation. For example a threshold concentration of methylating species may be needed before DNA alkylation processes can occur. This is unlikely since it is known that the alkylation of liver DNA is proportional to dose over a wide range of doses studied (7, 19, 26).

A second possibility is that there is a deuterium isotope effect on reactions occurring after \(\alpha\)-hydroxylation of NDMA, which decreases the efficiency of DNA alkylation. For example further oxidation of the carbinolamine may be an important step in the biological action of NDMA, and deuterium substitution may slow such further oxidation steps. There is currently no evidence for such a mechanism for NDMA, however. A third explanation is that there is an alternative pathway of NDMA metabolism (such as nitrogen oxidation) that becomes kinetically important when the rate of the \(\alpha\)-hydroxylation pathway is decreased 3-fold by deuterium substitution. Some support for this last hypothesis comes from studies of the stoichiometry of carbon dioxide and nitrogen gas formation from NDMA (31–34), as well as findings that nitrosamines can be metabolically denitrosated (35–37). These studies indicate that \(\alpha\)-hydroxylation to produce an alkylating intermediate is not the only pathway of NDMA metabolism. Thus it appears that the origin of the deuterium isotope effect on liver DNA alkylation, and perhaps on liver carcinogenicity, is not simply due to a decreased rate of \(\alpha\)-hydroxylation, but arises instead from metabolic switching to pathways of metabolism that do not lead to DNA methylation.

The present study provides quantitative reference data on the time course of the disappearance of low-dose NDMA and \(^{[\text{H}_6]}\)NDMA from blood over 5 to 8 half-lives, confirms the first-pass effect on their metabolism with direct blood measurements, and demonstrates that NDMA systemic clearance is blood flow limited. A \(V_{\text{max}}/K_m\) deuterium kinetic isotope effect on low dose NDMA metabolism \textit{in vivo} was documented. The magnitude of the isotope effect is dependent on the route of administration and increases when the experimental design reduces the blood flow limitation of rates of enzymatic drug removal.

The present results are in good agreement with previous studies of low dose NDMA and \(^{[\text{H}_6]}\)NDMA metabolism based...
on DNA alkylation measurements. However, the present results indicate that a reduced rate of α-hydroxylation does not explain the suppression of liver DNA alkylation and liver carcinogenicity observed with deuterium substitution. Indeed, the present data strongly indicate that metabolic pathways not involving α-hydroxylation may be more important than are currently recognized.

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Low-Dose in Vivo Pharmacokinetic and Deuterium Isotope Effect Studies of N-Nitrosodimethylamine in Rats

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