Articles

Microsome-Mediated Oxidation of *N*-Nitrosodiethanolamine (NDELA), a Bident Carcinogen

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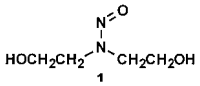
Received December 29, 2000

N-Nitrosodiethanolamine (NDELA), an environmentally prevalent, potent carcinogen, undergoes competitive rat liver microsome-mediated oxidation at both the α (adjacent to N)- and *β*-positions of the 2-hydroxyethyl chains. The former process, α-hydroxylation, is detected by the formation of glycolaldehyde (determined as its 2,4-dinitrophenylhydrazone DNP) that is assumed to arise from the decomposition of the corresponding α-hydroxynitrosamine, which is also the progenitor of the 2-hydroxyethyldiazonium ion. This finding refutes prior published work that states that the α-hydroxylation of NDELA does not occur. Competitive microsomal oxidation at the *β*-position gives the hemiacetal *N*-nitroso-2-hydroxymorpholine (NHMOR) at a rate 1.5 times α-hydroxylation. Glycolaldehyde is oxidized in this system to glyoxal at a rate 39 times the conversion of NDELA to glycolaldehyde. The α-hydroxylation of NHMOR at either C-3 or C-5 to give glyoxal or glycolaldehyde, respectively, occurs at respective rates 3−6 times that of the α-hydroxylation of NDELA. Ethylene glycol, a hydrolysis product of the 2-hydroxyethyldiazonium ion is shown to undergo microsome mediate oxidation to glyoxal. Ethyl-2-hydroxyethylnitrosamine (NEELA) undergoes a similar set of microsome-mediated oxidations at α-position of the ethyl (fastest) and 2-hydroxyethyl groups, as well as *β*-oxidation of the 2-hydroxyethyl group, a process which is slightly more rapid than α-hydroxylation of the same chain. Comparisons of oxidations rates of these substrates, as manipulated by preinducers, isoniazid, streptozocin, and phenobarbital, and enzyme inhibitors diethyldithiocarbamate and 4-methylpyrazole, with that of dimethylnitrosamine, a substrate for cytochrome P450 2E1, strongly suggest that this isozyme is also responsible for the oxidations reported here. α-Deuteration of NDELA practically eliminates its α-hydroxylation by microsomes from isoniazid induced rats, but doubles *β*-oxidation, while *β*-deuteration of this substrate significantly reduces *β*-oxidation and enhances α-hydroxylation. Since both glyoxal-guanine and 2-hydroxyethyl-DNA base adducts are known to arise from the in vivo administration of NDELA and because this work demonstrates that these two fragments can come from the microsomal oxidation of a single nitrosamine molecule containing the 2-hydroxyethyl group, NDELA and related nitrosamines are bident (two-toothed) carcinogens, a process which is likely to enhance their carcinogenic potency.

Introduction

In developed countries, *N*-nitrosodiethanolamine (NDELA),[1] **1**, a potent animal carcinogen (*1*−*5*), is a ubiquitous



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trace contaminant in many commercial products. It arises from di- and triethanolamine, and their various derivatives by inadvertent nitrosation processes that occur during product formulation. It has been found in cosmetics, shampoos, and personal care items (*6*−*12*), but most notably in metalworking fluids (*13*−*18*) where concentrations as high as 2−3% have been detected. To better estimate the human health hazards of this carcinogen, an understanding of its mechanism of activation is necessary. In recent publications we have documented some of the difficulties that have plagued searches for the NDELA mechanism(s) (*19*, *20*). While most nitrosamines are activated by initial P450 mediated α-hydroxylation, this process is unknown for NDELA (*20*−*22*). Nevertheless, recent studies in our laboratory utilizing

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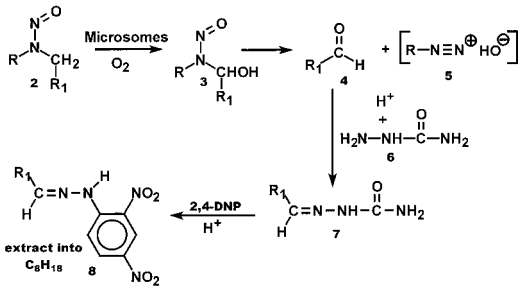
[1] Abbreviations: ADH, alcohol dehydrogenase; DCC, diethyldithiocarbamate; DMN, dimethylnitrosamine; DNP, 2,4-dinitrophenyhydrazone; DNPH, 2,4-dinitrophenylhydrazine; MEPY, 1-methylpyrazole; NDELA, *N*-nitrosodiethanolamine; NEEALA, *N*-nitrosoethylethanalamine; NEELA, ethyl-2-hydroxyethylnitrosamine; NHMOR, *N*-nitroso-2-hydroxymorpholine; SSB, single strand breaks.

10.1021/tx000267b CCC: $22.00
Published on Web 03/20/2002

specifically α- and β-deuterated isotopomers of NDELA have produced evidence for in vivo DNA damage produced by the scission of the α-CH bond (breakage of the CH bond adjacent to the N) (*20*). Because these data strongly suggest the operation of an α-hydroxylation pathway for NDELA, we have reinvestigated the research which claims that this process does not occur (*21*, *22*).

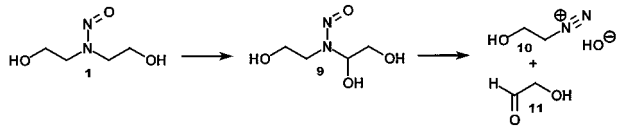
Numerous in vitro investigations of nitrosamine metabolism by either S-9000 cell fractions or microsomes have relied on the detection of aldehydes which are produced by the decomposition of an unstable α-hydroxynitrosamine intermediate **3**, as is shown in Scheme 1.

Scheme 1



The aldehyde **4** is commonly trapped as a semicarbazone **7** and then converted into a 2,4-dinitrophenylhydrazone (DNP) **8**, which is extracted with isooctane and detected by HPLC−UV−vis in comparison with known standards (*23*). In the case of NDELA, α-hydroxylation would result in the generation of glycolaldehyde **11** (Scheme 2).

Scheme 2



Farrelly et al. attempted to detect the α-hydroxylation of NDELA by both rat liver microsomes and rat hepatocytes using the analytical method above and failed to observe formation of the DNP of glycolaldehyde or other aldehydes (*21*, *22*). Because our deuteration experiments (*20*) with NDELA in vivo pointed toward an α-hydroxylation pathway for this compound, we reconsidered the applicability of the method shown in Scheme 1 to the detection of glycolaldehyde, a very polar aldehyde, which is also susceptible to osazone reactions with hydrazines to give hydrazones of glyoxal. Isooctane is a very nonpolar organic solvent, and the possibility that it would not extract either the DNP of glycolaldehyde or glyoxal was considered prior to the initiation of the work presented here. We report that through a modification of the analytical method shown in Scheme 1 we have been able to observe the α-hydroxylation of NDELA, as well as other significant biotransformations.

Materials and Methods

General. Microsomes were purchased from Xenotech, Kansas City, KS. Male Sprague−Dawley rats (8 weeks old) were induced with isoniazid (200 mg/kg every day for 4 days), phenobarbital (80 mg/kg every day for 4 days), and streptozocin (single injection 100 mg/kg). Rat liver microsomes were prepared on day 5 by differential centrifugation in a suspension of 250 mM sucrose with 10 mg of protein/mL. NADP, NAD, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 2,4-dinitrophenylhydrazine (DNPH), glycolaldehyde (dimer) and glyoxal (40% solution) were purchased from Sigma Chemical (St. Louis, MO). Ethylene glycol was obtained from Fisher (St. Louis, MO). The inhibitors, 4-methylpyrazole and diethyldithiocarbamate were obtained from Aldrich Chemical (Milwaukee, WI). Nitrosamines (dimethylnitrosamine, ethylethanolnitrosamine, *N*-nitrosodiethanolamine, ethylethanalnitrosamine, *N*-nitroso-2-hydroxymorpholine) are all known compounds prepared in our laboratory according to literature procedures. The isotopic purity of the deuterated NDELA was 98% (*24*).

Incubations. Different substrate concentrations were incubated with microsomes (1 mg protein/mL), 10 mM $MgCl_2$, 7.5 mM G-6-P (glucose6-phosphate), 0.75 mM NADP, and 5 units/mL glucose 6-phosphate dehydrogenase in a 100 mM phosphate buffer, pH 7.4. Samples (volume 500 μL) were incubated in a shaking thermomixer for 20 min to 1 h at 37 °C. The reaction was terminated by the addition of 100 μL trichloroacetic acid (10%), and then 500 μL of 2,4-dinitrophenyl hydrazine (2 mM in 18% phosphoric acid) was added. After 5 min reaction time the hydrazones and the remaining hydrazine were extracted into 500 μL of methylene chloride. The organic phase (200 μL) was transferred into a new vial, evaporated with N_2, and redissolved in 200 μL of acetonitrile. To ensure an excess of hydrazine, only an aliquot of each of the incubation mixtures containing glycolaldehyde and NHMOR was reacted with DNPH. Inhibitors were added at concentrations of 1 mM before the addition of the microsomes.

A typical run involved the addition of the following substances to give the final concentration noted: 20 μL of NDELA (0.5 M) = 20 mM; 50 μL of microsomes (10 mg/mL) = 1 mg/mL; 50 μL of cofactor (20 mg of $MgCl_2$ = 100 mM, 20 mg of glucose 6 phosphate = 75 mM, 6 mg of NADP = 7.5 mM in 1 mL of water); 5 μL of glucose-6-phosphate dehydrogenase (500 units/mL) = 5 units/mL; 375 μL of phosphate buffer, pH 7.4, 0.1 M.

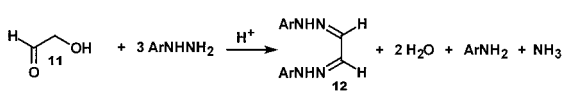
HPLC. The samples were separated on a Zorbax C18 column (4.6 mm × 25 cm), flow 1 mL/min, with a gradient of acetonitrile (B) in water (A): 0 min 90% A/10% B, 34 min 60/40, 50 min 20/80, 65 min 20/80, 75 min 90/10. Detection: UV−vis at 390 nm.

DNPs. Hydrazone standards were prepared by refluxing the respective aldehydes (1 mmol) with 2,4-dinitrophenylhydrazine (1 mmol) in ethanol, containing 10% phosphoric acid. The precipitated hydrazones were filtered, washed and dried, and dissolved in acetonitrile. To quantify the recovery, of the respective hydrazone, a known amount of aldehyde was added to a sample of heat deactivated microsomes, derivatized with DNPH, and immediately extracted according to the procedure described above. Microsomal and glycolaldehyde blanks were performed in each case. Recoveries were as follows: glyoxal, 100%; glycolaldehyde, 32%, acetaldehyde and formaldehyde, 45%; NHMOR, 15%; and *N*-nitrosoethylethanalamine (NEEALA), 45%. Low recoveries in some cases resulted from incomplete derivatization and/or extraction, but were very reproducible.

Results

Modification of the α-Hydroxylation Assay. Investigation of the assay used by Farrelly et al. (*23*) and Yoo et al. (*25*) to detect the α-hydroxylation of NDELA showed that it could not detect glycolaldehyde or glyoxal. As a result, several steps in these similar procedures were changed. Difficulties with the assay arise because the DNP of glycolaldehyde can be oxidized by DNPH under acidic conditions (the osazone reaction, Scheme 3)

Scheme 3



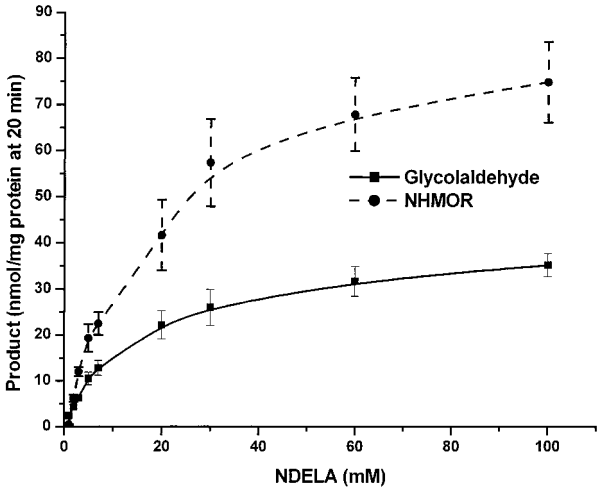


Figure 1. Products, glycoladehyde and NHMOR, from the microsomal oxidation of NDELA are shown to increase as the substrate concentration is increased. All incubations were stopped at 20 min.

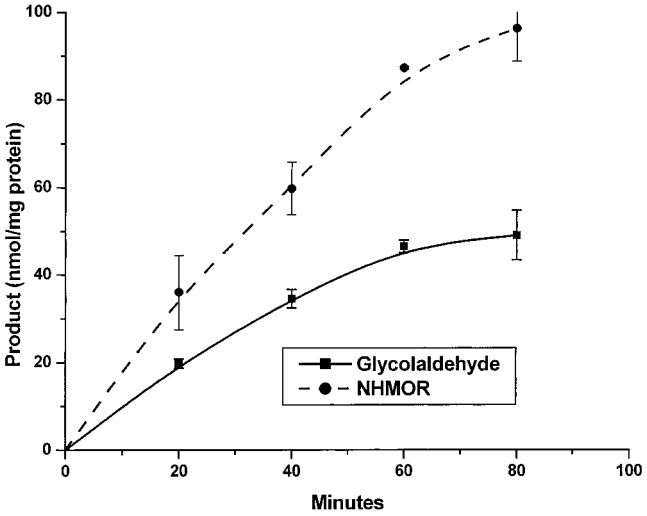


Figure 2. Time course of product formation from the incubation of NDELA (20 mM) with rat liver microsomes (isoniazid induction).

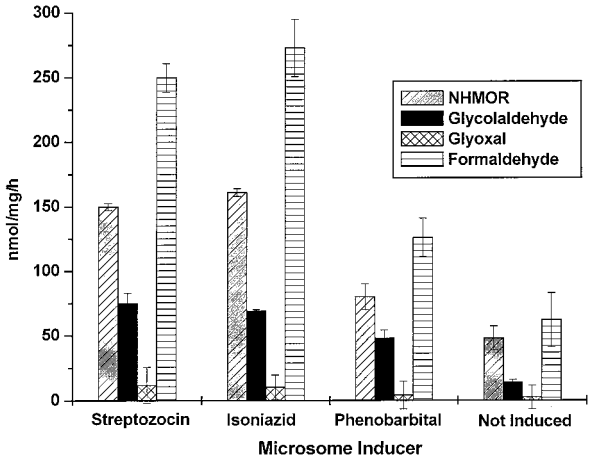


Figure 3. A comparison of the production of NHMOR, glycolaldehyde, and glyoxal from the incubation of NDELA with the same microsomes that were used to separately produce formaldehyde from the incubation with dimethylnitrosamine (DMN).For each set of microsomes, each induced by a different agent, the quantity of NDELA metabolites per milligram of protein is represented by the three left bars and the right bar shows the quantity of formaldehyde produced from DMN under identical conditions.

to the di-DNP of glyoxal. Since glyoxal itself is a possible product of the NDELA metabolism, selective determination of the two aldehydes is important. Therefore, the addition of semicarbazide to trap the aldehydes immediately in the incubation mixture was omitted because this step significantly increased osazone formation. The application of milder conditions (higher pH, and lower temperatures) with the semicarbazide trapping did not help, and resulted in a mixture because of incomplete conversion of the semicarbazone to the DNP. Direct addition of DNPH and a short reaction time of 5 min at room-temperature minimized the oxidative conversion of glycolaldehyde to glyoxal via the osazone reaction to about 1%. Isooctane was found to be an ineffective solvent for the extraction of the polar DNPs of glycolaldehyde and glyoxal. It was replaced by the more polar methylene chloride. With this solvent, the unreacted DNPH is extracted too, but it can be separated from the products by HPLC on RP-18 column using a gradient of acetonitrile and water. With these modifications, the recovery for glycolaldehyde was about 30%.

Metabolism of NDELA by Rat Liver Microsomes. Incubation of NDELA with isoniazid-induced rat liver microsomes and a NADPH generating system resulted in the formation of glycolaldehyde, NHMOR, and glyoxal. Concentrations ranging from 0.5 to 80 mM NDELA produced increasing amounts of aldehyde finally reaching saturation at 120 mM (Figure 1). Product formation was nearly linear with time up to 30 min before leveling off at about 60 min (Figure 2). At 20 mM NDELA, the rate of NHMOR formation (1.8−2.0 nmol/mg/min) exceeded glycolaldehyde formation (0.9−1.0 nmol/mg/min) by a factor of 2 while glyoxal was only a minor product with 0.1 nmol/mg min. While we cannot be absolutely certain at this stage, the reaction appears to be catalyzed by a cytochrome P450 enzyme since controls without glucose-6-phosphate dehydrogenase, as well as controls without microsomes, showed no aldehyde formation. There was no evidence for an ADH (alcohol dehydrogenase)-mediated oxidation of NDELA (*20*, *26*−*30*) under these conditions as replacement of the cofactor NADPH by NAD did not result in any NHMOR formation. In a control reaction, we observed that microsomes from isoniazid-induced rats, with the NADPH generating system, produced formaldehyde and acetaldehyde without the addition of any specific substrate. Therefore, the possible formation of small amounts of these compounds from NDELA could not be detected. The same system also showed formation of glyoxal, but the amounts were very small and did not prevent the determination of glyoxal in the sample.

To further investigate the possible involvement of cytochrome P450, microsomes from animals that had been induced by different drugs were compared. Isoniazid and streptozocin, which are known to be inducers of 2E1 (*25*, *31*−*34*), and phenobarbital an inducer of both 2E1 and 2B1 (*32*), were selected and the activity of the respective microsomes was measured by determining the demethylation of dimethylnitrosamine DMN (Figure 3). The incubation time was extended to 1 h to increase product formation. Normal microsomes formed formaldehyde at a rate of 62 μM/h/mg protein which is in the

Table 1. Michaelis−Menten Parameters for the Microsomal[a]-Catalyzed Oxidation of NDELA and Its Products

substrate	product	V_{max} (nmol/min/ mg protein)	K_m (mM)	V_{max}/K_m	rel. rate
NDELA	glycolaldehyde	3.84 ± 0.07	15 ± 0.01	0.256 ± 0.005	1
NDELA	NHMOR	10.4 ± 0.6	27 ± 2 0.8	0.38 ± 0.03	1.5
NHMOR	glycolaldehyde	7.4 ± 0.6	5.2 ± 0.5	1.4 ± 0.3	5.6
NHMOR	glyoxal	1.5 ± 0.4	1.8 ± 0.5	0.8 ± 0.4	3.3
glycolaldehyde	glyoxal	3.2 ± 0.5	0.32 ± 0.05	10 ± 3	39.1

[a] Isoniazid induced rat liver microsomes.

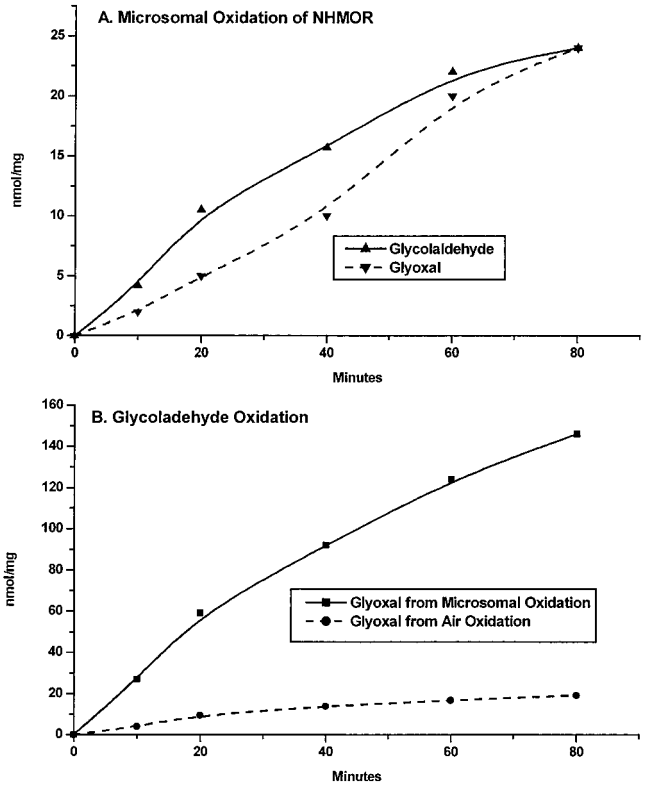


Figure 4. Yield of oxidation products from the microsomal oxidation of NDELA is shown as a function of time. Panel A shows that both glycoladehyde and glyoxal are formed from the isoniazid induced microsomal oxidation of NHMOR at a substrate concentration of 0.4 mM. Panel B shows that glyoxal is formed from both the microsomal and nonenzymatic oxidation of glycolaldehyde (0.4 mM).

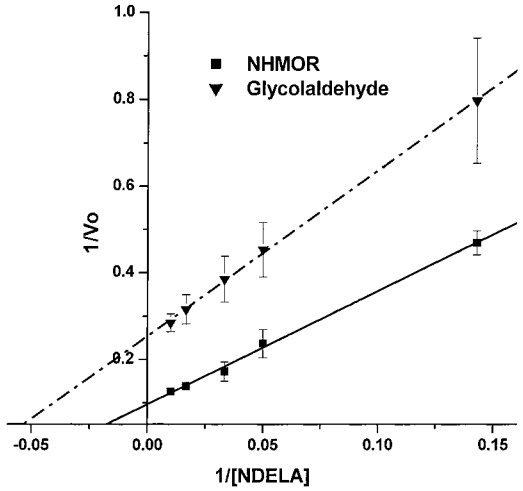


Figure 5. Michelis−Menten plots of the formation of NHMOR and glycolaldehyde from NDELA where catalysis is provided by isoniazid induced microsomes. Each line represents the average of three determinations.

expected range (*31*, *32*, *35*). Phenobarbital induction increased the formaldehyde production by a factor of 2 while isoniazid and streptozocin inductions resulted in an increase of about 4 times. With NDELA, the various microsomes showed an increase in aldehyde formation pattern similar to the demethylation of DMN. Microsomes from isoniazid and streptozocin-induced rats produced the highest yields of both glycolaldehyde and NHMOR, while phenobarbital-induced rat microsomes only showed an increase of a factor of 2 over those from noninduced rats. Comparing the ratio of glycolaldehyde to NHMOR, induction has a slightly stronger effect on glycolaldehyde formation than on NHMOR.

Oxidation of Metabolites from NDELA by Rat Liver Microsomes. The metabolites NHMOR and glycolaldehyde themselves can be further oxidized by rat liver microsomes. NHMOR is metabolized to glycolaldehyde and glyoxal (Figure 4A). At a concentration of 0.4 mM NHMOR, microsomes from isoniazid induced rats formed glycolaldehyde at a rate of 0.58 nmol/mg/min while its conversion rate to glyoxal was 0.26 nmol/min/mg. Without the addition of microsomes concentrations up to 2 mM NHMOR showed neither formation of glycolaldehyde nor glyoxal within 60 min. Figure 4B shows that glyoxal can also be generated by the oxidation of glycolaldehyde under these conditions. Incubation of 0.4 mM glycolaldehyde with microsomes from isoniazid-induced rats gave glyoxal at a rate of 1.36 nmol/mg/min. This value was corrected for the nonenzymatic autoxidation of glycolaldehyde (0.44 nmol/min). Enzymatic oxidation of glycolaldehyde to glyoxal is about 10 times faster than glyoxal formation from NHMOR (Table 1). With microsomes from noninduced rats the reaction is much slower for both NHMOR and glycolaldehyde. After incubating 0.4 mM NHMOR for 1 h with these microsomes, 3 nmol/mg glyoxal and 3.3 nmol/mg glycolaldehyde form, compared to 22 nmol of glyoxal and 24 nmol of glycolaldehyde, when microsomes from induced rats are used. A similar result was obtained for glycolaldehyde where microsomes from noninduced animals produced only 15% of the glyoxal generated by the induced rat microsomes.

Another product of the microsome-mediated oxidation of glycolaldehyde is formaldehyde, which was formed at a rate of 0.42 nmol/mg/min. Because formaldehyde is also formed in the microsomal blank, we have estimated that only about 50% of the total formaldehyde arises from the microsomal oxidation of glycolaldehyde. Formaldehyde was not found in the glycolaldehyde substrate blank, nor was it present in the incubation of glyoxal with microsomes, showing that it is a result of enzymatic

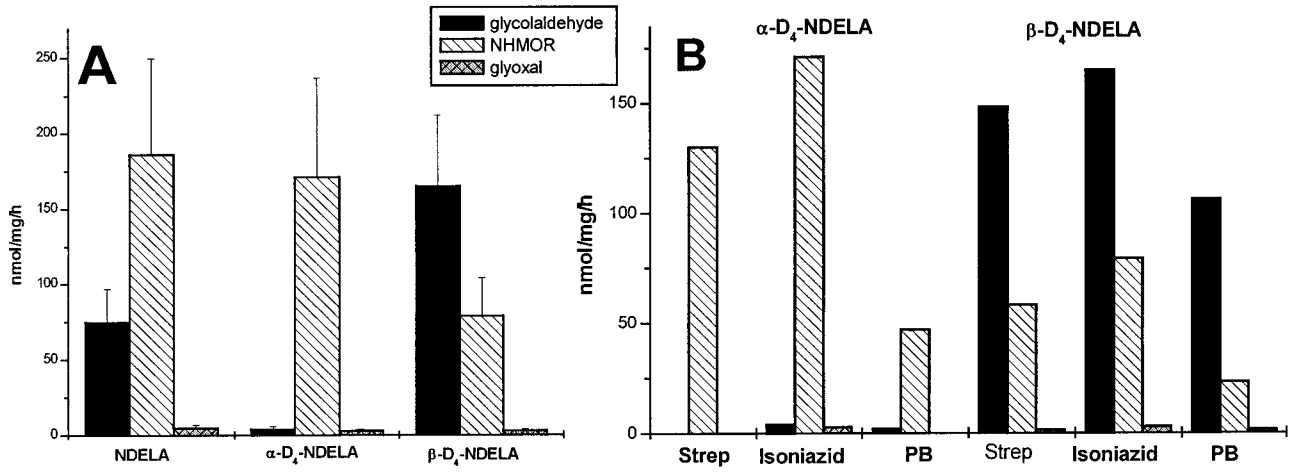


Figure 6. Deuterium isotope effects on the microsomal oxidation of isotopomers of NDELA. In panel A the affect of specific deuterium substitution on the rates of glycolaldehyde, NHMOR, and glyoxal formation from the isoniazid induced microsome mediated oxidation are shown. In panel B rates are given as sets for each isotopomer and all of the induced rat liver microsomes used in this work. (Inducers: Strep = streptozocin, PB = phenobarbital).

oxidation of glycolaldehyde. After 1 h incubation time, 45% of the glycolaldehyde was recovered unchanged, 28% was converted to glyoxal and 10% to formaldehyde.

From other experiments, not reported here, we know that ethylene glycol is a product of the decomposition of the α-hydroxynitrosamine **9** through the hydrolysis of the 2-hydroxyethyldiazonium ion **10** (Scheme 2). Accordingly, we have investigated the microsomal oxidation of ethylene glycol and shown that it produces glycolaldehyde. Rates are less than that observed for the comparable glycolaldehyde oxidation. Incubation of 2 mM ethylene glycol with microsomes from isoniazid-induced rats results in the formation of glycolaldehyde at a rate of 120 nmol/mg/h. The NADPH-generating system was necessary to catalyze this reaction, suggesting an involvement of a cytochrome P 450.

The Michaelis−Menten parameters (V_{max} and K_m) for the isoniazid-induced rat liver microsomal oxidation of NDELA and its oxidation products are given in Table 1. In all cases, data comparable to that shown for the oxidation of NDELA in Figure 5 were obtained. Initial rates were determined from data obtained in the first 20 min of reaction. Because of the different reaction rates, variable concentrations were used and ranged from 1 to 120 mM for NDELA, 0.2−10 mM for NHMOR and 0.04−2 mM for glycolaldehyde, respectively.

Oxidation of Specifically Deuterated NDELA Substrates. As mentioned in the Introduction, the impetus for this work was the finding that deuterium substitution at the α-position of NDELA inhibited DNA single strand breaks in vivo (*20*). It was therefore important to determine whether the same phenomena could be observed in the microsome-mediated oxidation of NDELA. In general, rate reductions can be expected upon deuterium substitution of hydrogen when the C−H bond is broken in a rate-determining step. The rates of production of the spectrum of metabolites arising from NDELA were determined as a function of deuterium substitution in the substrate and the results are shown in Figure 6. With microsomes from isoniazid-induced rats, all three compounds formed the same three metabolites yet the ratio of metabolites depended on the deuteration pattern. Deuteration at the C-2 reduced NHMOR formation by a factor of 2 and increased the rate of production of glycolaldehyde by more than a factor of 2 over the control. On the other hand, the C-1 deuteration did not change the rate of NHMOR formation significantly, but nearly eliminated the α-hydroxylation product glycolaldehyde. Glyoxal formation was decreased for both deuterated compounds because it requires oxidation at both carbons. In Figure 6B, we compare the rates of metabolite formation as a function of the inducer. Although there are some differences in relative amounts, the same general trends are observed.

Comparison of NDELA with NEELA. To study the influence of the hydroxyl group in the β-position (to the N), we compared the metabolism of ethyl-2-hydroxyethylnitrosamine (NEELA **26**) and NDELA. With microsomes from isoniazid-induced rats, NEELA formed both glycolaldehyde **11** and acetaldehyde. Oxidation also occurs at the β-position resulting in the formation of ethylethanalnitrosamine (NEEALA) **29**. The rate of acetaldehyde formation (361 nmol/h/mg) was greater than that of glycolaldehyde formation (139 nmol/h/mg) demonstrating a preference for oxidation of the ethyl group over the hydroxyethyl moiety. As was the case with NDELA, the rate oxidation of the 2-hydroxyethyl group is slightly more rapid (153 nmol/h/mg) at the β-position to produce **29** than at the α-position which gives **11**. β-Oxidation of the ethyl chain would produce NDELA, but this process, which is known for other nitrosamines, cannot be detected by our assay because the product is not an aldehyde.

As is shown in Figure 7, those inducers that are most effective in increasing the rate of DMN oxidation are also the ones that are most effective in catalyzing the α-hydroxylation of NEELA suggesting that the same isoenzyme is involved. Oxidation at the β-position of the 2-hydroxyethyl chain shows the same pattern.

Effect of Enzyme Inhibitors. Diethyldithiocarbamate (DDC) and methylpyrazole (MEPY) are relatively selective inhibitors for P450 2E1 in rat liver microsomes (*31*, *36*). Methylpyrazole is also an inhibitor for alcohol dehydrogenase. With our isoniazid-induced microsomes both inhibitors showed a strong inhibition of DMN demethylation as described by Yamazaki et al. (*31*). The data for the effect of these inhibitors on the generation of aldehydes from NDELA and NEELA are shown in

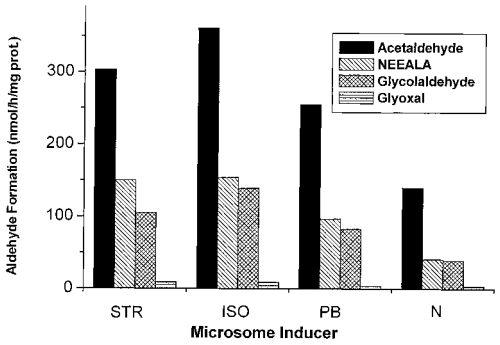


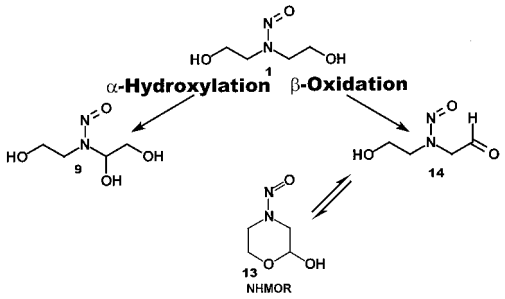
Figure 7. Rates of production of the aldehyde metabolites from the rat liver microsome meditated oxidation of NEELA are shown as a function of the inducing agent (STR = streptozocin, ISO = isoniazid, PB = phenobarbital, and N = no induction (control).

Figure 8. In incubations with NDELA, DDC and MEPY inhibit the formation of glycolaldehyde, glyoxal, and NHMOR, exhibiting a slightly greater effect on glycolaldehyde and glyoxal production. Similar results were obtained with NEELA, where DDC and MEPY also produced a greater inhibition of glycolaldehyde and acetaldehyde formation than that observed for the β-oxidation as detected by production of **29**. As can be seen from inspection of panels A and B of Figure 8, the two inhibitors produced similar effects with each of the substrates. We also found both inhibitors to decrease the rate of NDELA metabolism by microsmes from phenobarbital-induced animals (data not shown), but MEPY, in particular was a significantly less effective inhibitor with these microsomes.

Discussion

The mechanism of bioactivation of NDELA **1** has been in doubt for many years (*19*−*22*, *26*−*30*, *37*−*51*). The work presented here reveals two major findings that significantly clarify this problem and lead to a sound hypothesis for the initiation of carcinogenesis by NDELA. First, NDELA undergoes microsome-mediated α-hydroxylation to generate an unstable α-hydroxynitrosamine **9**, as shown in Scheme 4. Evidence for the existence of the pathway is provided by (1) the detection of the α-hydroxynitrosamine decomposition product, glycolaldehyde; (2) the demonstration that α-deuteration of NDELA practically eliminates the formation of glycolaldehyde; (3) model chemistry experiments which show that the hydrolysis of α-acetoxy NDELA, a progenitor of α-hydroxy NDELA, generates glycoladehyde in addition to other products (*52*); and (4) O^6-hydroxyethylguanine adducts, produced from the reaction of the diazonium ion derived from α-hydroxy NDELA which were found in rat liver DNA following the administration of NDELA (*30*, *53*). Second, microsomes mediate the oxidation of NDELA at the β-position to give NHMOR, and the rate of this process is greater than that of the α-hydroxylation. Both glycolaldehyde and NHMOR are substrates for further microsomal oxidation yielding glyoxal and other electrophiles that bind DNA. *Thus, both α- and β-oxidation are important in the carcinogenic activation of NDELA.*

Scheme 4



α-Hydroxylation of NDELA. The thinking that α-hydroxylation was not involved in the bioactivation of NDELA arose from three types of experimental data. Numerous mutagen assay experiments involving S-9000 fractions as an activating media failed to show mutagenicity for NDELA (*19*, *20*). Neither glycolaldehyde nor glyoxal, the expected products from the α-hydroxylation of NDELA or its metabolite NHMOR, respectively, could be detected from the incubation of either rat liver microsomes or hepatocytes with NDELA or NHMOR (*22*). Using high specific activity [^{14}C]NDELA, Lijinsky and Farrelly found only low levels of radio-carbon incorporation into rat liver DNA and this observation is contrary to that observed for numerous other nitrosamines for which evidence of bioactivation by α-hydroxylation exists (*21*). On the other hand, *N*-2-hydroxyethyl-*N*-nitrosoglycine is a urinary metabolite of NDELA and NHMOR, its precursor, has been detected in the rat liver S-9000 mediated metabolism of NDELA (*45*, *46*). Both of these

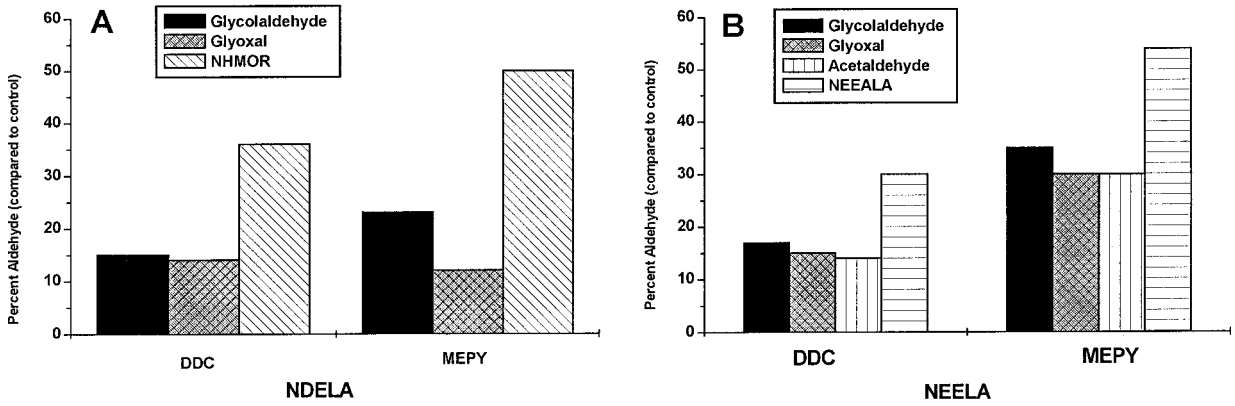


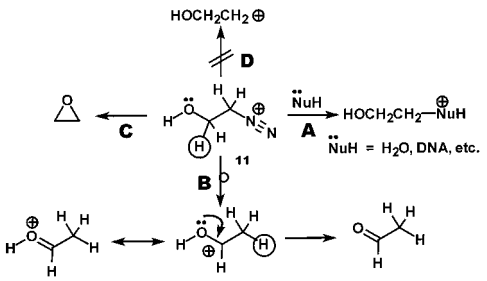
Figure 8. Effect of enzyme inhibitors, DCC and MEPY, on the percentage of each aldehyde, based on controls, formed during the isoniazid induced rat liver microsome incubation with (A) NDELA and (B) NEELA, respectively, is displayed in the two panels.

substances are β-oxidation products. NHMOR is a modestly reactive compound that reacts with DNA in vitro (*54*).

The most important evidence for the α-hydroxylation of NDELA is the observation of the production of glycolaldehyde and that this transformation is significantly inhibited by the α-deuteration of NDELA. As discussed earlier in this paper, previous assays for the DNP of this compound relied on the extraction of this very polar compound into nonpolar isooctane in which it has very low solubility and did not take into account the osazone reaction of the hydrazine and the α-hydroxyaldehyde which result in the even less soluble glyoxal di-DNP (an osazone) (*22, 23*). Numerous investigations of the microsome-mediated metabolism of nitrosamines have employed the isooctane extraction of the poorly soluble DNPs produced from the aldehydes resulting from the decomposition of the α-hydroxynitrosamines. Unless, careful recovery and control experiments are or were performed, the extent of metabolic activation by this pathway could be significantly underestimated. Here we extracted the DNPs with CH_2Cl_2 in which they are much more soluble. Our improvements in the assay procedure are undoubtedly responsible for our ability to detect the α-hydroxylation of NDELA and metabolites derived from it.

If NDELA is activated by α-hydroxylation to generate a reactive α-hydroxynitrosamine that then decomposes to the 2-hydroxyethy-diazonium ion (see Scheme 2), a putative potent electrophile, then why was no mutagenicity observed for NDELA in numerous Ames assays and modifications thereof. In fairness, several groups report low levels of mutagenicity for NDELA in S-9000 mediated Ames assays (*51*), but the vast majority of reports show negative mutagenicity for this compound (*55*). There are probably several reasons for the failure to observe S-9000-mediated mutagenicity for NDELA (*56*). As we have shown (Table 1), NDELA is not a particularly good substrate even with the microsomes we used. In an S-9000 preparation, the microsome quantity, and thereby the concentration of the active enzyme(s), is diluted compared to the preparations used by us. Moreover, anecdotal reports of failures to observe mutagenicity for various known mutagenic nitrosamines are common. Much more is known now about the P450 enzymes in microsomal preparations and how to keep them active than was the case when many of the mutagenicity experiments were performed several decades ago. Moreover, the compounds generated by the α-hydroxylation of nitrosamines are often much less stable than the electrophiles produced from other carcinogens. α-Hydroxynitrosamines have short half-lives and the diazonium ions and carbocations derived from them are even more reactive. Primary diazonium ions, such as the methyl- or ethyldiazonium ion, mainly react by very low activation energy Sn-2 nucleophilic displacement reactions, rather than by carbocation generation (*57*). In the case of the 2-hydroxyethyldiazonium ion, other pathways also play an important role in the chemistry of this species. These are depicted in Scheme 5. The two predominant reaction pathways for this diazonium ion are **A** and **B**. Nucleophilic displacement of N_2 is principally governed by the relative concentration of nucleophile, which in most cases is H_2O, a transformation which generates ethylene glycol (**15** NuH = OH) here, and, from the perspective of carcinogenesis, mostly results in detoxification. Another detoxification pathway is represented by path **B**, which involves an intramolecular rearrangement via a 1,2-hydride shift (sigmatropic transformation) to **17** and generates acetaldehyde **18**. Independent estimates show that this pathway accounts for at least 35% of the chemistry of the 2-hydroxyethyldiazonium ion, based on product studies (*52*). Pathway C is relatively minor (8−10%) and generates the relatively unreactive ethylene oxide **19**. It is unlikely that the free carbocation **20** forms. Thus, the intrinsic chemistry of **10** severely limits its reaction with DNA, because highly reactive electrophiles must be produced nearby for reaction and the mutagenesis that results from that process to occur. In summary, then, failure to observe mutagenicity for NDELA in numerous experiments during 1970s was likely due to the relatively inactive S-9000 toward nitrosamines, the poor substrate quality of NDELA, and the "self-detoxifying chemistry" of a significant fraction of the 2-hydroxyethyldiazonium ions produced.

Scheme 5



As mentioned in the Introduction, the level of DNA single strand breaks in vivo is significantly reduced by the α-deuteration of NDELA. The data of Figure 6 demonstrate that only very small amounts of glycolaldehyde are produced from the microsomal oxidation of α-D_4NDELA compared to controls. Thus, these two sets of observations are complementary and support the hypothesis that α-oxidation is involved in the carcinogenic bioactivation of NDELA. The preinduction history of the microsomes was relatively unimportant in this context. α-Deuteration of NDELA reduced glycolaldehyde production with all microsomes, although the levels of metabolic transformation were a function of the inducing drug. The rate of NHMOR formation (Figure 6A) is not increased above the control when the α-position is deuterated, as might be expected if oxidation at the two sites were competitive in roughly the same time realm. The data basically represents "one-point kinetic experiments." Evidence for mechanistic switching by α-deuteration, as evidenced by enhanced NHMOR production, must await more detailed kinetic experiments utilizing reconstituted enzyme preparations. On the other hand, evidence for mechanistic switching as a result of β-deuteration is clearly evident. β-Deuteration of NDELA enhances the α-oxidation of NDELA with microsomes from isoniazid-induced animals by a factor of 2. While quantitation is more problematic in the DNA SSB (single strand break) work, we did observe a modest enhancement in DNA SSB by β-D_4NDELA (*20*) that is also indicative of mechanistic switching in vivo as a result of D for H substitution in NDELA.

A possible indicator of α-hydroxylation would be the observation of the production of DNA hydroxyethyl

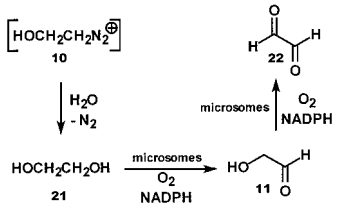
adducts in vivo. There are two relevant literature reports. Farrely et al. reported evidence for the formation O^6- and N^7-2-hydroxyethylguanine in DNA and RNA obtained from the livers of rats administered high specific activity [^{14}C]NDELA. For DNA, the counts eluting at the same place as the standards were so low as to raise questions about the validity of the observation (*21*). RNA, however, yielded higher levels of coeluting adducts upon acidic hydrolysis. Through the use of an imuno-slot blot analysis, Scherer et al. showed that O^6-2-hydroxyethylguanine formed in a dose responsive manner upon administration of NDELA (*30*). It was also demonstrated that this same adduct was formed in rat liver DNA following the administration of methyl- or ethylethanolnitrosamine. In both cases, however, the authors attributed these observations to a metabolic activation path other than the α-hydroxylation of NDELA.

***β*-Oxidation.** Others and we have shown that NDELA undergoes *β*-oxidation, but the focus of this transformation has been on alcohol dehydrogenase (ADH) mediated processes (*20*, *26*−*30*, *58*). The data presented here clearly show that microsomes are also capable of mediating the *β*-oxidation of NDELA to NHMOR (see Scheme 4). Evidence that ADH is not responsible for this phenomenon rests on the requirement for NADPH and not NAD, the required cofactor of ADH enzymes. Moreover ADHs are found in the cytosol and should not be in the microsomal preparations used here. As is evident from Table 1, and Figures 1, 2, and 6, the rate of microsomal *β*-oxidation of NDELA to NHMOR is 1.5 times more rapid than the α-hydroxylation of NDELA. While we have not made k_H/k_D measurements for the microsomal oxidation for either the *β*- or α-oxidation of NDELA, the data of Figure 6 clearly show that NHMOR production is reduced by about 50% by *β*-deuteration of NDELA. This effect is not as great as that observed for α-deuteration, however. We did not observe a significant isotope effect on the DNA SSB produced by *β*-D_4NDELA, but there are several possible explanations for these observations. NDELA is not a particularly good substrate for horse liver ADH, but a comparison of *β*-oxidation rates for NDELA by ADH and the P450 enzymes, likely responsible for the microsomal oxidation reported here must await further work. Ethanol is oxidized to both acetaldehyde and acetic acid by P450 2E1 (*59*, *60*), so it is not unusual that the alcohol functional group of NDELA should be oxidized by microsomes. As is discussed below, this *β*-oxidation process leads to further DNA damaging species.

Nature of the Enzyme. Dimethylnitrosamine is known to be α-hydroxylated by cytochrome P450 2E1 (*25*, *35*, *61*, *62*). This enzyme can be induced in rat liver by the preadministration of isoniazid or streptozocin (*31*). Using our assay we compared the activation of DMN with that of NDELA and other nitrosamines by different microsome preparations (no induction, isoniazid, streptozocin, or phenobarbital-induced). We have taken the level of product formation (aldehyde) to be indicative of microsome/enzyme(s) capacity to metabolize the substrate. Comparisons of formaldehyde production from DMN with aldehyde production from NDELA are shown in Figure 3. Some experimentation supports the idea that there are two DMN metabolizing enzymes present in microsomes, a low K_m enzyme presumed to be 2E1 and a higher K_m enzyme. While the concentrations of DMN used in our experiments are significantly lower than the concentration of NDELA, the DMN concentrations are still higher than those used by others in some of the 2E1 work. Nevertheless, we believe the good correlations of activity observed here, are meaningful. In all cases the levels of glycolaldehyde and NHMOR formation correlated with the amount of formaldehyde formation from DMN. With respect to the preinduction protocol, the conversion level was in the order isoniazid ≈> streptozocin > phenobarbital > no preinduction. This order and correlation with DMN metabolism strongly suggests that cytochrome P450 2E1 is the microsomal enzyme responsible for the metabolism of NDELA at both the α- and *β*-positions. Further support for this conclusion is obtained from our work on the effect of inhibitors. DCC and MEPY are both effective inhibitors of cytochrome P450 2E1. Both of these inhibitors were demonstrated to effectively inhibit the metabolism of DMN in our microsomes and they also inhibited the metabolism of NDELA. Because the *β*-oxidation of NDELA was not inhibited as much as was the α-hydroxylation, it is possible that other enzymes or P450s may be involved in this process as well.

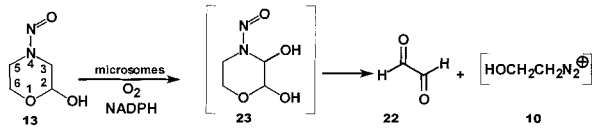
Oxidation of Related Substrates. The data presented in Figure 3 and Table 1 shows that glyoxal is produced in small amounts from the microsomal oxidation of NDELA. Incubation of guanine nucleosides, nucleotides, or DNA in vitro with NHMOR produces glyoxal-guanine (gG) adducts by means of an unknown mechanism which does not involve any other activation process (*29*, *53*, *58*, *63*, *64*). Moreover, as is demonstrated in the following paper in this issue (*64*), gG adducts are formed in rat liver DNA upon administration of either NDELA or NHMOR, as well as structurally related nitrosamines (*53*). Glyoxal has been known to be an effective mutagen for many years, but it is not a carcinogen, probably because its oral administration results in its metabolic detoxication. But, because it is a significant DNA adduct from the carcinogenic NDELA and because it is found in small amounts in the microsome-mediated transformations of NDELA, we have investigated its formation from other substrates. The formation of glyoxal from the microsomal metabolism of *N*-nitrosomorpholine, which also gives NHMOR has been reported (*65*, *66*), but it is also known and we show here that the air oxidation of glycolaldehyde produces glyoxal. A comparison of the rate of this process, however, with the microsomal oxidation of the same substrate (see Figure 4B and Scheme 6)

Scheme 6



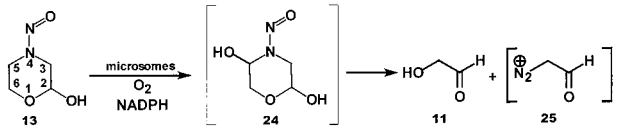
shows that it is slow. The microsomal oxidation of NHMOR also results in the generation of glycoladehyde and glyoxal (Figure 4A). As shown in Scheme 7, α-hy-

Scheme 7



droxylation of NHMOR at C-3 leads to the direct formation of glyoxal **22** through the decomposition of the α-hydroxynitrosamine **23**. This process also results in the formation of the potent alkylating agent **10**. On the other hand, α-hydroxylation at C-5 produces the unstable α-hydroxynitrosamine **24**, the decomposition of which results in the formation of glycolaldehyde **11** and the diazonium ion **25** (Scheme 8). The rate of glycolaldehyde

Scheme 8

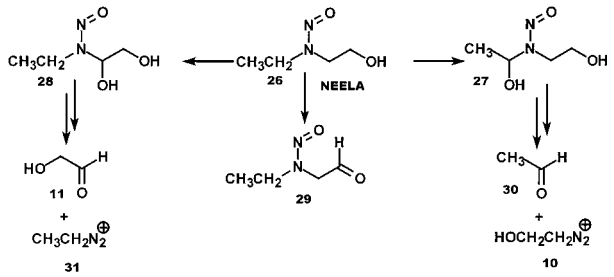


formation from NHMOR is greater than the rate of formation of glyoxal, but these data can only be considered to be "apparent rates". The reason for this is also revealed in the data of Table 1, where we see that the rate of microsomal oxidation of glycoladehyde to glyoxal is more than 10 times faster than the conversion of NHMOR to this aldehyde. In short, glycoladehyde produced from either NDELA or NHMOR can be further oxidized to glyoxal. Not only that, the hydrolysis of the diazonium ions **10** or **25** resulting from the α-hydroxynitrosamines **9**, **23**, or **24** produced from the microsomal oxidation of NDELA or NHMOR can lead to glyoxal by oxidation of glycoladehyde. This is even true for ethylene glycol **21** (Scheme 6), the hydrolysis product of the diazonium ion **10**. We demonstrated that **21** is oxidized to glycoladehyde and that this process requires NADPH not NAD. In fact, based on our work with inhibitors and preinducers all of the oxidations discussed in this paragraph appear to be mediated by a P450 enzyme, probably 2E1 (Figure 8). The properties of the diazonium ion **25** have not been explored in our laboratory yet, but its hydrolysis is expected to give glycolaldehyde, and thence, by further oxidation, glyoxal.

A reading of the literature on the mechanism of ethylene glycol toxicity leads one to believe that the primary bio-oxidative path for glycolaldehyde, presumed to arise principally from ADH mediated metabolism, is its oxidation to glycolic acid through aldehyde dehydrogenase catalysis (*67*). The possible role of glyoxal in ethylene glycol toxicity has been ignored. But, the enzymatic oxidation of ethylene glycol through glycolaldehyde to glyoxal has been reported recently and is the basis of an industrial process (*68*, *69*). We have been unable to find any previous reports of the microsomal oxidation of glycolaldehyde to glyoxal. Our work demonstrates that microsomal oxidation of ethylene glycol, and glycolaldehyde to glyoxal may be important in the toxic action of these compounds.

Microsomal Oxidation of Ethylethanolnitrosamine. Ethylethanolnitrosamine NEELA **26**, a compound that differs from NDELA only in the absence of a terminal hydroxyl group, is a potent rat renal carcinogen, yet, despite numerous carcinogenesis experiments, relatively little is known about its mode of bioactivation. In this study, it has served as a convenient comparison to NDELA and provides us with valuable knowledge about the activation processes of nitrosamines carrying the 2-hydroxyethyl group. The microsomal oxidation of NEELA produces the aldehydes shown in Scheme 9, as has been discussed above. Like NDELA, NEELA is oxidized at both the α- and β-positions of the 2-hydroxyethyl chain. The former process gives the α-hydroxynitrosamine **28** while the latter gives the nitrosamino aldehyde **29**. It is also α-hydroxylated on the ethyl chain to give the α-hydroxynitrosamine **27** that decomposes to acetaldehyde **30** and the diazonium ion **10**. Compared to NDELA,

Scheme 9



NEELA is a better substrate for α-hydroxylation (acetaldehyde and glycolaldehyde) by a factor of 5 as measured by product yields under comparable conditions. As is evident from Figure 7, α-hydroxylation on the ethyl chain predominates. Although glycolaldehyde, produced by α-hydroxylation on the other chain, is formed only from one chain, rates are still nearly twice the glycolaldehyde formation from NDELA. This demonstrates the fact that this more lipophilic nitrosamine is a much better substrate for P450.

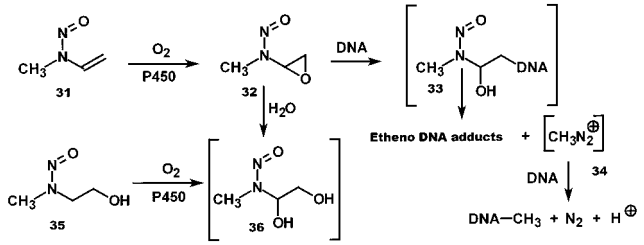
The data of Figures 7 and 8 provide good evidence that P450s responsible for the metabolism of NEELA and NDELA are likely the same since the effect of inducers and inhibitors are similar and once again implicate cytochrome P450 2E1 as the major enzyme responsible for the oxidative metabolism. The processes depicted in Scheme 9 provide evidence for pathways leading to DNA damage produced upon the in vivo administration of **26** to rats. It has been shown to give both gG and O^6-2-hydroxyethylguanine adducts.

Simultaneous Production of Two DNA-Binding Fragments from the Same Molecule (A Bident Carcinogen). A simple nitrosamine such as dimethylnitrosamine or ethylmethylnitrosamine, can undergo α-hydroxylation at either carbon next to the nitrogen, but decomposition of these α-hydroxynitrosamines will give nonbindings aldehydes (formaldehyde or acetaldehyde) and a single diazonium ion which leads to DNA adducts. The data described and discussed above clearly show that all four of the carbon atoms from a single molecule of NDELA can become bound to DNA as two adducts. One two-carbon fragment binds as glyoxal to form gG adducts. The other two-carbon fragment, arises from the 2-hydroxyethyldiazonium ion, which alkylates various sites in DNA, giving, for example, O^6-2-hydroxyethylguanine adducts. We define a bident (two-toothed) carcinogenic compound as one which simulataneaously gives two DNA-binding fragments from the same molecule upon metabolic activation. In the case of NDELA, and related β-oxidized nitrosamines, the process requires both α- and β-oxidation of one of the two carbon fragments. This can

occur by the processes depicted in Schemes 6, 7, and 8 or combinations thereof. The route to the "alkylating" fragments differs depending upon whether NDELA is α-hydroxylated or *β*-oxidized to give the aldehyde (NHMOR) first. α-Hydroxylation gives **10** which decomposes to the 2-hydroxyethyldiazonium ion and glycolaldehyde, which we have shown to be more rapidly oxidized to glyoxal by microsomes than the parent nitrosamine is α-hydroxylated. Hydrolytic decomposition of the 2-hydroxyethyldiazonium gives ethylene glycol, which we have shown can be oxidized to glyoxal by microsomal preparations. Microsomal or ADH-mediated oxidation of NDELA at the primary alcohol produces NHMOR. This compound can be α-hydroxylated at either of two positions. α-Hydroxylation at C-3 (Scheme 7) leads ultimately to the 2-hydroxyethyldiazonium ion and glyoxal after decomposition of the α-hydroxynitrosamine. If oxidation occurs at C-5 (Scheme 8), then the α-hydroxynitrosamine will give glycolaldehyde and the 2-oxoethyldiazonium ion **25**. We have not yet determined whether this latter pathway is operative, but the hydrolysis of **25** gives glycolaldehyde that can be further oxidized to glyoxal. Thus, there are a number of biochemical routes leading to the formation of glyoxal from NDELA.

While bident DNA-binding capacity has been designed into several well-known anti-cancer drugs such as bischloroethylnitrosourea, certain nitrogen mustards, and other compounds, the "double alkylation", bident carcinogenic, capacity of NDELA, and related *β*-oxidized nitrosamines, has been observed for one other carcinogen that we know of, the highly potent carcinogen methylvinylnitrosamine **31** (Scheme 10). Good evidence has been

Scheme 10



presented for its P450-mediated oxidation to the epoxide **32** (*70*). Attack of a nucleophilic nitrogen atom in a DNA base will result in the formation of an unstable DNA bound α-hydroxynitrosamine **33**. Decomposition will lead to etheno adducts and the methyldiazonium ion **34** which will readily alkylate other nucleophilic sites in DNA. This chemistry has been demonstrated in vitro by Okazaki et al. (*70*). Even if the epoxide is hydrolyzed (**32** → **36**) prior to reaching DNA it still has the capacity of being a bident carcinogen because the α-hydroxynitrosamine **36** can ultimately give rise to both glyoxal and the methyldiazonium ion. The metabolite **36** is produced by the α-hydroxylation of *N*-nitrosomethylethanolamine **35**, as well. The bident carcinogenic nature of methylvinylnitrosamine may be responsible for its extreme carcinogenicity.

An interesting feature of the carcinogenicity of NDELA compared to other nitrosamines is that, even at the lowest dose studied, a large percentage of the administered dose is excreted unchanged in the urine (*47*, *49*, *71*−*74*). Yet several dose response studies have shown NDELA to be a very potent carcinogen (*1*, *3*, *4*). The "double alkylating" capacity of NDELA could be responsible for its "bident carcinogenic" character and its high effective potency. The two teeth (bident) in this case are the two DNA adducts arising from the metabolic conversion of each two-carbon fragment into a DNA binding moiety. Our work with NEELA **26**, shows that this bident carcinogenic capacity is not limited to NDELA or NHMOR. Reference to Scheme 9 shows that the pathways going from **26** → **28** → **11** + **31**, and from **26** → **29** (followed by α-hydroxylation on the 2-oxoethyl group) can be bident, but the more prevalent process which involves the α-hydroxylation of the ethyl chain (**26** → **29**) cannot. In principle, any nitrosamine carrying a 2-hydroxyethyl chain (an ethanol nitrosamine) bound to N can be a bident carcinogen. This is also true of *N*-nitrosomorpholine, because it is known to be metabolized in part to NHMOR (*65*, *66*, *75*).

The emphasis of this discussion has been on the microsomal oxidation pathways that lead to the DNA binding of carbon fragments from NDELA, NHMOR, and NEELA. But, we have demonstrated through in vitro experiments that NHMOR and other α-nitrosaminoaldehydes, e.g., **25** can deaminate the amino groups of guanine, adenine, and cytosine bases in DNA (*54*, *76*) by a process which is believed to involve NO transfer (*37*, *58*). This process does not lead to adducts, per se, but modified bases which will mispair. At present, we have no method available to assess the importance of this pathway in vivo compared to the metabolic transformations that we have delineated here.

Of course, further oxidative or reductive metabolism of the molecules generated from the microsomal oxidation of NDELA can enter detoxification pathways. *N*-Nitroso-2-hydroxyethylglycine, the oxidation product of the masked aldehyde group of NHMOR, does not appear to be genotoxic and is excreted in the urine of animals fed NDELA. NHMOR is also known to undergo ADH mediated reduction to NDELA. We can reasonably expect that both glycolaldehyde and glyoxal, once they diffuse or are transported to the cytosol, will be substrates for aldehyde dehydrogenase enzymes which will convert them into their more easily excreted carboxylic acids. Additionally further microsomal oxidation of these compounds to nontoxic products is highly likely. P450 2E1 has been shown to catalyze the oxidation of ethanol to acetaldehyde and further to acetic acid without loss of acetaldehyde from the enzyme pocket (*59*, *60*). A similar process could be occurring with NHMOR arising from NDELA, but competitive oxidation at carbons 3 or 5 is likely to yield DNA binding fragments as shown here. The latter paths are expected to lead to carcinogenesis. Such a phenomenon, that is, further oxidation of NHMOR at carbons 3 or 5 before it escapes from the P450 active site, could explain why Hecht et al. failed to observe carcinogenic activity for orally administered NHMOR (*77*). It is possible that most of this material is detoxified by cytosolic enzymes before it reaches the endoplasmic reticulum where high concentrations of P450 are located. Although the carcinogenesis experiments can be criticized because of the relatively low doses used, competitive detoxifying oxidation of NHMOR and its excretion as *N*-nitroso-2-hydroxyethylglycine could prevent its reach-

ing the same cell sites which permit its microsomal oxidation to produce reactive electrophiles.

Conclusion

Contrary to all previous reports, we have unequivocally demonstrated that NDELA undergoes microsome-mediated α-hydroxylation producing glycolaldehyde and presumably the short-lived 2-hydroxyethyldiazonium ion. This process competes slightly less favorably with the β-oxidation of the alcohol group to give the masked aldehyde NHMOR, a hemiacetal. In this system, glycolaldehyde is further oxidized to glyoxal, a known DNA-binding molecule. Since NDELA is known to produce both 2-hydroxyethyl- and gG DNA adducts in vivo, we propose that it is a bident carcinogen where all four carbons of the molecule can become bound to DNA as two separate adducts and this process may account for the potency of this carcinogen. NHMOR has also been shown to undergo microsome-mediated oxidation to give glyoxal and glycolaldehyde in addition to diazonium ions. Thus, this compound can also act as a bident carcinogen, as can any alkyl-2-hydroxyethylnitrosamine, as we have shown here with NEELA.

Acknowledgment. We gratefully acknowledge the support of this research by a grant, RO1 ES03953, from the National Institute of Environmental Health Sciences (NIH, PHS).

References

(1) Preussmann, R., Habs, M., Habs, H., and Schmaehl, D. (1982) Carcinogenicity of *N*-nitrosodiethanolamine in rats at five different dose levels. *Cancer Res.* **42**, 5167−5171.

(2) Lijinsky, W., Reuber, M. D., and Manning, W. B. (1980) Potent carcinogenicity of nitrosodiethanolamine in rats. *Nature (London)* **288**, 589−590.

(3) Lijinsky, W., and Reuber, M. D. (1984) Dose−response study with *N*-nitrosodiethanolamine in F344 rats. *Food Chem. Toxicol.* **22**, 23−26.

(4) Hoffmann, D., Rivenson, A., Adams, J. D., Juchatz, A., Vinchkoski, N., and Hecht, S. S. (1983) Effects of route of administration and dose on the carcinogenicity of *N*-nitrosodiethanolamine in the Syrian golden hamster. *Cancer Res.* **43**, 2521−2524.

(5) Hilfrich, J., Schmeltz, I., and Hoffmann, D. (1978) Effects of *N*-nitrosodiethanolamine and 1,1-diethanolhydrazine in Syrian golden hamsters. *Cancer Lett.* **4**, 55−60.

(6) Fan, T. Y., Goff, U., Song, L., Fine, D. H., Arsenault, G. P., and Biemann, K. (1977) *N*-Nitrosodiethanolamine in cosmetics, lotions and shampoos. *Food Cosmet. Toxicol.* **15**, 423−430.

(7) Havery, D., C, and Chou, H. J. (1994) Nitrosamines in sunscreens and cosmetic products. In *Nitrosamines and related N-nitroso compounds: Chemistry and biochemistry* (Loeppky, R. N., and Michejda, C. J., Eds.) pp 20−33, American Chemical Soc., Washington, DC.

(8) Gettings, S. D., Dressler, W. E., Franz, T. J., Kelling, C. K., Howes, D., and Walters, K. A. (1995) Assessing risk from *N*-nitrosamines in cosmetic products. *Cosmet. Toiletries* **110**, 72−80.

(9) Fine, D. H. (1983) HPLC-TEA determination of *N*-nitrosodiethanolamine (NDELA) and similar compounds in cosmetics. *International Agency for Research in Cancer Scientific Publication*, p 45.

(10) Rollmann, B., Lombart, P., Rondelet, J., and Mercier, M. (1981) Determination of *N*-nitrosodiethanolamine in cosmetics by gas chromatography with electron capture detection. *J. Chromatogr.* **206**, 158−163.

(11) Sommer, H., Blankart, M., and Eisenbrand, G. (1989) Determination of *N*-nitrosodiethanolamine in cosmetics and in alkanolamines. Results of collaborative studies. *Z. Lebensm. Unters. Forsch.* **189**, 144−146.

(12) Westin, J. B., Spiegelhalder, B., Preussmann, R., and Shani, J. (1990) Assay of suntan lotions for the carcinogenic, non-volatile *N*-nitrosamine *N*-nitrosodiethanolamine. *Cancer Lett.* **50**, 157−160.

(13) Rappe, C., and Zingmark, P. A. (1978) Formation of *N*-nitrosamines in cutting fluids. *Int. Agency Res. Cancer Sci. Publ.* **19**, 213-217.

(14) Fan, T. Y., Morrison, J., Rounbehler, D. P., Ross, R., Fine, D. H., Miles, W., and Sen, N. P. (1977) *N*-Nitrosodiethanolamine in synthetic cutting fluids: a part-per-hundred impurity. *Science* **196**, 70−71.

(15) Monarca, S., Scassellati Sforzolini, G., Spiegelhalder, B., Pasquini, R., and Fatigoni, C. (1993) Monitoring nitrite, *N*-nitrosodiethanolamine, and mutagenicity in cutting fluids used in the metal industry. *Environ. Health Perspect.* **101**, 126−128.

(16) Keefer, L. K., Goff, U., Stevens, J., and Bennett, E. O. (1990) Persistence of *N*-nitrosodiethanolamine contamination in American metal-working lubricants. *Food Chem. Toxicol.* **28**, 531−534.

(17) Fadlallah, S., Cooper, S. F., Fournier, M., Drolet, D., and Perrault, G. (1990) Determination of *N*-nitroso compounds in metalworking fluids. *J. Chromatogr. Sci.* **28**, 517−523.

(18) Fuchs, J., Burg, J., Hengstler, J. G., Bolm-Audorff, U., and Oesch, F. (1995) DNA damage in mononuclear blood cells of metal workers exposed to *N*-nitrosodiethanolamine in synthetic cutting fluids. *Mutat. Res.* **342**, 95−102.

(19) Loeppky, R. N. (1999) The mechanism of bioactivation of *N*-nitrosodiethanolamine. *Drug Metab. Rev.* **31**, 175−193.

(20) Loeppky, R. N., Fuchs, A., Janzowski, C., Humberd, C., Goelzer, P., Schneider, H., and Eisenbrand, G. (1998) Probing the mechanism of the carcinogenic activation of *N*-nitrosodiethanolamine with deuterium isotope effects: In vivo induction of DNA single strand breaks and related in vitro assays. *Chem. Res. Toxicol.* **11**, 1556−1566.

(21) Farrelly, J. G., Thomas, B. J., and Lijinsky, W. (1987) Metabolism and cellular interactions of *N*-nitrosodiethanolamine. *IARC Scientific Pub.* **84**, 87−90.

(22) Farrelly, J. G., Stewart, M. L., and Lijinsky, W. (1984) The metabolism of nitrosodi-*N*-propylamine, nitrosodiallylamine and nitrosodiethanolamine. *Carcinogenesis (London)* **5**, 1015−1019.

(23) Farrelly, J. G. (1980) A new assay for the microsomal metabolism of nitrosamines. *Cancer Res.* **40**, 3241−3244.

(24) Loeppky, R. N., and Xiong, H. (1994) The synthesis of deuterium-labeled *N*-nitrosodiethanolamine and *N*-nitroso-2-hydroxymorpholine. *J. Labelled Compd. Radiopharm.* **24**, 1099−1110.

(25) Yoo, J. S. H., Ishizaki, H., and Yang, C. S. (1990) Roles of cytochrome P450IIE1 in the dealkylation and denitrosation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in rat liver microsomes-9. *Carcinogenesis (London)* **11**, 2239−2243.

(26) Eisenbrand, G., Denkel, E., and Pool, B. (1984) Alcohol dehydrogenase as an activating enzyme for *N*-nitrosodiethanolamine (NDELA): in vitro activation of NDELA to a potent mutagen in Salmonella typhimurium. *J. Cancer Res. Clin. Oncol.* **108**, 76−80.

(27) Denkel, E., Pool, B. L., Schlehofer, J. R., and Eisenbrand, G. (1986) Biological activity of *N*-nitrosodiethanolamine and of potential metabolites which may arise after activation by alcohol dehydrogenase in Salmonella typhimurium, in mammalian cells, and in vivo. *J. Cancer Res. Clin. Oncol.* **111**, 149−153.

(28) Denkel, E., Sterzel, G., and Eisenbrand, G. (1987) In-vivo activation of *N*-nitrosodiethanolamine and other *N*-nitroso-2-hydroxyalkylamines by alcohol dehydrogenase and sulfotransferase. *IARC Scientific Pub. No.* **84**, 83−86.

(29) Loeppky, R. N., Tomasik, W., Denkel, E., and Eisenbrand, G. (1987) α-Nitrosaminoaldehydes: Highly reactive metabolites. *IARC Sci. Publ.* **84**, 94−99.

(30) Scherer, G., Ludeke, B., Kleihues, P., Loeppky, R. N., and Eisenbrand, G. (1991) Mutagenicity, DNA damage and DNA adduct formation by *N*-nitroso-2-hydroxyalkylamine and corresponding aldehydes. *IARC Sci. Publ.* **105**, 339−342.

(31) Yamazaki, H., Oda, Y., Funae, Y., Imaoka, S., Inui, Y., Guengerich, F. P., and Shimada, T. (1992) Participation of rat liver cytochrome P 450 2E1 in the activation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine to products genotoxic in an acetyltransferase-overexpressing Salmonella typhimurium strain (NM2009). *Carcinogenesis* **13**, 979−983.

(32) Shu, L., and Hollenberg, P. F. (1997) Alkylation of cellular macromolecules and target specificity of carcinogenic nitrosodialkylamines: metabolic activation by cytochromes P450 2B1 and 2E1. *Carcinogenesis* **18**, 801−810.

(33) Chen, T. L., Chen, S. H., Tai, T. Y., Chao, C. C., Park, S. S., Guengerich, F. P., and Ueng, T. H. (1996) Induction and suppression of renal and hepatic cytochrome P450-dependent monooxygenases by acute and chronic streptozotocin diabetes in hamsters. *Arch. Toxicol.* **70**, 202−208.

(34) Sohn, O. S., Ishizaki, H., Yang, C. S., and Fiala, E. S. (1991) Metabolism of azoxymethane, methylazoxymethanol and *N*-nitrosodimethylamine by cytochrome P450IIE1. *Carcinogenesis* **12**, 127−131.

(35) Yang, C. S., Yoo, J. S. H., Ishizaki, H., and Hong, J. (1990) Cytochrome P450IIE1: roles in nitrosamine metabolism and mechanisms of regulation. *Drug Metab. Rev.* **22**, 147−159.

(36) Lauriault, V. V., Khan, S., and O'Brien, P. J. (1992) Hepatocyte cytotoxicity induced by various hepatotoxins mediated by cytochrome P-450IIE1: protection with diethyldithiocarbamate administration. *Chem.-Biol. Interact.* **81**, 271−289.

(37) Loeppky, R. N., Tomasik, W., Kovacs, D. A., Outram, J. R., and Byington, K. H. (1984) Alternative bioactivation routes for β-hydroxynitrosamines. Biochemical and chemical model studies. In *N-Nitroso Compd: Occurrence, Biol. Eff. Relevance Hum. Cancer* (Bartsch, H., O'Neill, I., and VanBorstal, Eds.) pp 429−436, IARC, Lyon.

(38) Loeppky, R. N., Srinivasan, A., and Erb, E. (1992) Putative proximate carcinogens derived from ethanolnitrosamines: Chemical properties. In *Nitroso Compounds: Biological Mechanisms, Exposures and Cancer Etiology* (Bartsch, H., and O'Neill, I., Eds.) pp 42a−42b, IARC, Lyon.

(39) Eisenbrand, G., and Janzowski, C. (1994) Potential mechanism of action of nitrosamines with hydroxy, oxo, or carboxy groups. In *Nitrosamines and Related N-nitroso Compounds: Chemistry and Biochemistry* (Loeppky, R. N., and Michejda, C., J, Eds.) pp 179−194, American Chemical Society, Washington, DC.

(40) Sterzel, W., and Eisenbrand, G. (1986) *N*-Nitrosodiethanolamine is activated in the rat to an ultimate genotoxic metabolite by sulfotransferase. *J. Cancer Res. Clin. Oncol.* **111**, 20−24.

(41) Michejda, C. J., Koepke, S. B., Kroeger, M. B., and Hernandez, L. (1994) Activation of β-hydroxyalkylnitrosamines. Evidence for involvement of a sulfotransferase. In *Nitrosamines and related N-nitroso compounds: Chemistry and Biochemistry* (Loeppky, R. N., and Michejda, C. J., Eds.) pp 195−210, Am. Chem. Soc., Washington, DC.

(42) Koepke, S. T. R., Kroeger-Koepke, M. B., Born, W., Thomas, B. J., Alvord, W. G., and Michejda, C. J. (1988) Alkylation of DNA in rats by *N*-nitrosomethyl-(2-hydroxyethyl)amine: Dose respose and persistence of the alkylated lesions in vivo. *Cancer Res.* **48**, 1537−1542.

(43) Kroeger-Koepke, M. B., Koepke, S. R., Hernandez, L., and Michejda, C. J. (1992) Activation of a β-hydroxyalkylnitrosamine to alkylating agents: Evidence for the involvement of a sulfotransferase. *Cancer Res.* **52**, 3300−3305.

(44) Koepke, S. R., Cresia, D. R., Knutsen, G. L., and Michejda, C. J. (1988) Carcinogenicity of hydroxyalkylnitrosamines in F344 rats: Contrasting behavior of β- and γ-hydroxyalkyl nitrosamines. *Cancer Res.* **48**, 1533−1536.

(45) Airoldi, L., Bonfanti, M., Benfenati, E., Tavecchia, P., and Fanelli, R. (1983) Identification of an acidic metabolite of *N*-nitrosodiethanolamine isolated from rat urine. *Biomed. Mass Spectrom.* **10**, 334−337.

(46) Airoldi, L., Bonfanti, M., Fanelli, R., Bove, B., Benfenati, E., and Gariboldi, P. (1984) Identification of a nitrosamino aldehyde and a nitrosamino acid resulting from β-oxidation of *N*-nitrosodiethanolamine. *Chem.-Biol. Interact.* **51**, 103−113.

(47) Airoldi, L., Macri, A., Bonfanti, M., Bonati, M., and Fanelli, R. (1984) Kinetics and bioavailability of *N*-nitrosodiethanolamine after intravenous and cutaneous administration to rats. *Food Chem. Toxicol.* **22**, 133−138.

(48) Bonfanti, M., Castelli, M. G., Fanelli, R., and Airoldi, L. (1985) Enterohepatic recycling of *N*-nitrosodiethanolamine in rats. *Food Chem. Toxicol.* **23**, 1011−1013.

(49) Bonfanti, M., Magagnotti, C., Fanelli, R., and Airoldi, L. (1986) Comparative in vitro and in vivo β-oxidation of *N*-nitrosodiethanolamine in different animal species. *Chem.-Biol. Interact.* **59**, 203−210.

(50) Suzuki, E., Iiyoshi, M., and Okada, M. (1981) Metabolic fate of *N*-alkyl-*N*-(3-hydroxypropyl and 2- hydroxyethyl)nitrosamines in the rat in relation to the induction of bladder cancer by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine and its homologs. *Gann* **72**, 254−258.

(51) Mori, Y., Yamazaki, H., and Konishi, Y. (1987) Mutagenicity of *N*-nitrosodiethanolamine in the Salmonella/microsome test. *Mutat. Res.* **192**, 91−94.

(52) Park, M., Gu, F., and Loeppky, R. N. (1998) The synthesis of highly reactive multifunctional α,β-epoxy- and α-acetoxy-nitrosamines. *Tetrahedron Lett.* **39**, 1287−1290.

(53) Loeppky, R. N., Cui, W., Goelzer, P., Park, M., and Ye, Q. (1999) Glyoxal-guanine DNA adducts: Detection, stability and formation from nitrosamines. In *Cyclic DNA Adducts II* (Singer, B., and Bartsch, H., Eds.) pp 155−168, IARC, Lyon.

(54) Park, M., and Loeppky, R. N. (2000) In Vitro DNA Deamination by α-Nitrosaminoaldehydes Determined by GC/MS−SIM Quantitation. *Chem. Res. Toxicol.* **13**, 72−81.

(55) Rao, T. K., Young, J. A., Lijinsky, W., and Epler, J. L. (1979) Mutagenicity of aliphatic nitrosamines in Salmonella typhimurium. *Mutat. Res.* **66**, 1−7.

(56) Lijinsky, W., and Andrews, A. W. (1983) The superiority of hamster liver microsomal fraction for activating nitrosamines to mutagens in Salmonella typhimurium. *Mutat. Res.* **111**, 135−144.

(57) McGarrity, J. F., and Smyth, T. (1980) Hydrolysis of diazomethane−Kinetics and mechanism. *J. Am. Chem. Soc.* **102**, 7303−7308.

(58) Loeppky, R. N., Tomasik, W., and Kerrick, B., E. (1987) Nitroso transfer from α-nitrosamino aldehydes: Implications for carcinogenesis. *Carcinogenesis (London)* **8**, 941−946.

(59) Bell-Parikh, L. C., and Guengerich, F. P. (1999) Kinetics of cytochrome P450 2E1-catalyzed oxidation of ethanol to acetic acid via acetaldehyde. *J. Biol. Chem.* **274**, 23833−23840.

(60) Bell, L. C., and Guengerich, F. P. (1997) Oxidation kinetics of ethanol by human cytochrome P450 2E1. Rate-limiting product release accounts for effects of isotopic hydrogen substitution and cytochrome b5 on steady-state kinetics. *J. Biol. Chem.* **272**, 29643−29651.

(61) Tu, Y. Y., Peng, R., Chang, Z. F., and Yang, C. S. (1983) Induction of a high affinity nitrosamine demethylase in rat liver microsomes by acetone and isopropanol. *Chem.-Biol. Interact.* **44**, 247−260.

(62) Tu, Y. Y., and Yang, C. S. (1983) High-affinity nitrosamine dealkylase system in rat liver microsomes and its induction by fasting. *Cancer Res.* **43**, 623−629.

(63) Chung, F. L., and Hecht, S. S. (1985) Formation of the cyclic 1,-N2-glyoxal-deoxyguanosine adduct upon reaction of *N*-nitroso-2-hydroxymorpholine with deoxyguanosine. *Carcinogenesis (London)* **6**, 1671−1673.

(64) Loeppky, R. N., Ye, Q., Goelzer, P., Chen, Y., and Li, E. (2002) Detection of glyoxal-guanine DNA adducts produced in vivo from β-oxidized nitrosamines by ^{32}P-postlabeling. *Chem. Res. Toxicol.* **15**, 470−482.

(65) Manson, D., Cox, P. J., and Jarman, M. (1978) Metabolism of *N*-nitrosomorpholine by the rat in vivo and by rat liver microsomes and its oxidation by the Fenton system. *Chem.-Biol. Interact.* **20**, 341−354.

(66) Jarman, M., and Manson, D. (1986) The metabolism of *N*-nitrosomorpholine by rat liver micrsomes and ists oxidation by the Fenton system. *Carcinogenesis* **7**, 559−565.

(67) Wiener, H. L., and Richardson, K. E. (1988) The metabolism and toxicity of ethylene glycol. *Res. Commun. Subst. Abuse* **9**, 77−87.

(68) Isobe, K. (1995) Oxidation of ethylene glycol and glycolic acid by glycerol oxidase. *Biosci., Biotechnol., Biochem.* **59**, 576−581.

(69) Isobe, K., and Nishise, H. (1995) A new enzymic method for glycolaldehyde production from ethylene glycol. *J. Mol. Catal. B: Enzymol.* **1**, 37−43.

(70) Okazaki, O., Persmark, M., and Guengerich, F. P. (1993) N-Nitroso-N-methylvinylamine: Reaction of the epoxide with guanyl and adenyl moieties to yield adducts derived from both parts of the molecule. *Chem. Res. Toxicol.* **6**, 168−173.

(71) Lethco, E. J., Wallace, W. C., and Brouwer, E. (1982) The fate of *N*-nitrosodiethanolamine after oral and topical administration to rats. *Food Chem. Toxicol.* **20**, 401−406.

(72) Preussmann, R., Wuertele, G., Eisenbrand, G., and Spiegelhalder, B. (1978) Urinary excretion of *N*-nitrosodiethanolamine administered orally to rats. *Cancer Lett.* **4**, 207−209.

(73) Preussmann, R., Spiegelhalder, B., Eisenbrand, G., Wuertele, G., and Hofmann, I. (1981) Urinary excretion of *N*-nitrosodiethanolamine in rats following its epicutaneous and intratracheal administration and its formation in vivo following skin application of diethanolamine. *Cancer Lett.* **13**, 227−231.

(74) Spiegelhalder, B., Eisenbrand, G., and Preussmann, R. (1982) Urinary excretion of *N*-nitrosamines in rats and humans. *IARC Sci. Publ.* **41**, 443−449.

(75) Hecht, S. S. (1984) *N*-Nitroso-2-hydroxymorpholine, a mutagenic metabolite of *N*-nitrosodiethanolamine. *Carcinogenesis (London)* **5**, 1745−1747.
(76) Loeppky, R. N., Lee, M. P., and Mueller, S. (1993) DNA modification by α-nitrosamino aldehydes in *The Chemistry of DNA Adducts* (Hemminki, K., and Bartsch, H., Eds.) pp 429−432, International Agency for Research on Cancer.
(77) Hecht, S. S., Lijinsky, W., Kovatch, R. M., Chung, F. L., and Saavedra, J. E. (1989) Comparative tumorigenicity of *N*-nitroso-2-hydroxymorpholine, *N*-nitrosodiethanolamine, and *N*-nitrosomorpholine in A/J mice and F344 rats. *Carcinogenesis (London)* **10**, 1475−1477.

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