^{1}H NMR Spectroscopic Studies on the Reactions of Haloalkylamines with Bicarbonate Ions: Formation of *N*-Carbamates and 2-Oxazolidones in Cell Culture Media and Blood Plasma

Maria L. Anthony,*,† Elaine Holmes,† Peter C. R. McDowell,† Tim J. B. Gray,‡ Melanie Blackmore,‡ and Jeremy K. Nicholson†

Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H 0PP, U.K., and Department of Toxicology, Sanofi Research Division, Willowburn Avenue, Alnwick, Northumberland NE66 2JH, U.K.

Received March 20, 1995⊗

^{1}H NMR spectroscopic methods have been applied to compare the in vitro reactivity of the renal papillary nephrotoxin 2-bromoethanamine (BEA) with those of selected halide-substituted nephrotoxic analogues, 2-chloroethanamine (CEA), 2-fluoroethanamine (FEA), and 1-phenyl-2-iodoethanamine (PIEA). The primary ^{1}H NMR-detectable transformation during a 24 h incubation of confluent Madin Darby canine kidney (MDCK) cells with BEA, CEA, and FEA (at concentrations up to the IC_{50} determined by neutral red uptake) was the appearance in cell culture media of 2-oxazolidone (OX). Additional novel signals assigned as FEA carbamate (*N*-carbamoyl-2-fluoroethanamine) were observed in media collected following incubation of cells with FEA. We propose that *N*-carbamate intermediates are formed from the spontaneous reaction of these haloalkylamines with HCO_3^--buffered growth media and that OX is formed from the carbamate via elimination of the hydrogen halide. Further ^{1}H NMR experiments, conducted for up to 8 h at 25 °C on 5 mM solutions of BEA, CEA, and FEA in ^2H_2O containing a 20-fold excess of HCO_3^- at pH 7.6, demonstrated a time-dependent decrease in the concentration of the free haloalkylamines accompanied by the production of *N*-carbamate intermediates and OX. Under these pseudo-first-order reaction conditions, the formation of OX from BEA was complete within approximately 6 h. In similar reaction conditions OX formation from CEA (24 h after initiation) had reached 54% of its final equilibrium concentration. Equivalent experiments demonstrated that PIEA was almost completely converted to 4-phenyl-2-oxazolidinone (PHOX) within 2 h. These observations reveal the strong disposition of this series of haloalkylamines toward reaction with HCO_3^- and indicate that the compounds in this family may exist only transiently as free amines in vivo, where there will virtually always be excess HCO_3^-. The physiological relevance of the in vitro findings is further indicated by the NMR-detectable conversion of BEA to OX and also an alkylating aziridine (AZ) moiety in rat plasma containing BEA. The ability to form carbamoylated species and OX (or PHOX) may mediate the toxicity of this series of haloalkylamines and hence is potentially of considerable significance.

Introduction

Administration of 2-bromoethanamine hydrochloride (BEA[1]) induces renal papillary necrosis (RPN) in experimental animals (*1*, *2*). For this reason, BEA has been used as a model compound to mimic the RPN characteristic of long-term human analgesic abuse (*3*, *4*). Functional changes reported following treatment with BEA include diuresis, a decrease in urinary osmolality (*1*, *5*), and enzymuria (*N*-acetyl-β-D-glucosaminidase) (*6*). However, comparatively little is known about the metabolic transformations of BEA itself or other related haloalkylamines. This is in part due to the fact that BEA is highly reactive, eliminating HBr and cyclizing readily in aqueous solutions to form the stable three-membered cyclic imine, aziridine (AZ; see Scheme 1 where X = Br), which is itself a direct-acting alkylating agent (*7*, *8*). The effect of the structure variations on the nephrotoxic potential of haloalkylamines has been studied previously, however, in an attempt to relate the mechanism of RPN to direct alkylating ability (*9*, *10*). Powell *et al.* (*9*, *10*) examined nine structural analogues of BEA that differed by halide substitution, alkyl chain elongation, or amine substitution. These workers reported a correlation between the RPN produced by BEA and three RPN-inducing analogues (2-chloroethanamine (CEA), 3-bromopropanamine, and 2-chloro-*N*,*N*-dimethylethanamine) and biological alkylating potential evaluated by the Ames plate incorporation assay (*11*). In contrast, poor mutagenic capacity was displayed by six analogues of BEA that either directed their toxicity to the proximal tubule

* To whom correspondence should be addressed, at the Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H OPP, U.K. Phone: (0171) 380-7519; Fax: (0171) 380-7464; E-mail: maria@chem.bbk.ac.uk.

† Department of Chemistry, Birkbeck College, University of London.

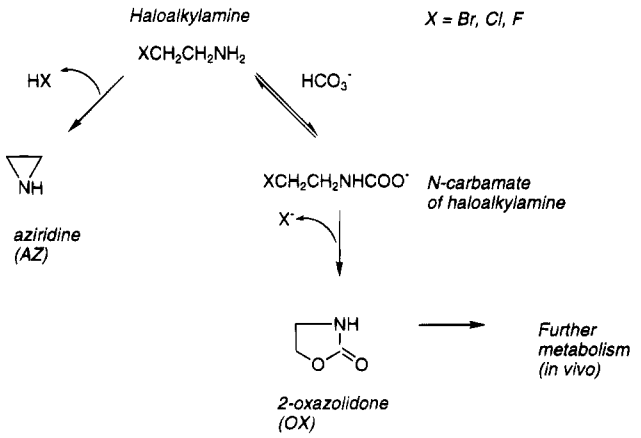
‡ Department of Toxicology, Sanofi Research Division.

⊗ Abstract published in *Advance ACS Abstracts*, August 15, 1995.

[1] Abbreviations: AZ, aziridine; BEA, 2-bromoethanamine hydrobromide; CEA, 2-chloroethanamine monohydrochloride; FEA, 2-fluoroethanamine hydrochloride; FID, free induction decay; F344, Fischer 344; GTA, glutaric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOX, 5-hydroxy-2-oxazolidone; MDCK, Madin Darby canine kidney; OX, 2-oxazolidone; PHOX, 4-phenyl-2-oxazolidinone; PIEA, 1-phenyl-2-iodoethanamine hydrochloride; RPN, renal papillary necrosis; TSP, 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]-1-propionic acid.

0893-228x/95/2708-1046$09.00/0

Scheme 1



(2-fluoroethanamine (FEA) 1-phenyl-2-iodoethanamine (PIEA) 2-hydroxy-3-bromopropanamine, and 1-amino-3-bromopropan-2-one) or did not produce renal abnormalities (2-bromoethyl-*O*-hydroxylamine and 3-chloro-*N*,*N*-dimethylpropanamine). These observations suggested that the in vivo formation of reactive alkylating species and their accumulation in papillary tissue may be a prerequisite for haloalkylamine-induced RPN (*11*).

High resolution ^{1}H NMR spectroscopic methods provide an alternative way of exploring both the metabolism and toxicological effects of xenobiotics (*12*) and have also been applied to the investigation of BEA-induced RPN in the Fischer 344 (F344) rat (*13*–*15*). Previous ^{1}H NMR urinalysis studies demonstrated that the acute in vivo effects of BEA administration included early (within 8 h of dosing) elevations in dimethylamine and trimethylamine *N*-oxide excretion, followed by a decrease in trimethylamine *N*-oxide excretion and rises in acetate and succinate levels up to 48 h post-dosing (*13*). Additional multiplets observed (at δ 1.82 and 2.23) in ^{1}H NMR spectra of urine for up to 24 h following BEA treatment were originally speculated to be from novel BEA metabolites (*13*, *15*) and were more recently identified as being from glutaric acid (GTA), the excretion of which is greatly elevated following BEA treatment (*16*). ^{2}H NMR spectroscopic analysis of urine from F344 rats exposed to ^{2}H-labeled (methylene groups) BEA showed that the GTA was of endogenous origin, probably due to BEA-induced inhibition of fatty acid metabolism caused by the selective suppression of mitochondrial fatty acyl CoA dehydrogenase activity (*16*). In addition, the BEA dissociation product AZ and novel deuterated BEA-related species that were tentatively identified as 2-oxazolidone (OX; at δ ^{2}H 3.68 and δ ^{2}H 4.52) and 5-hydroxy-2-oxazolidone (HOX; at δ ^{2}H 4.27, δ ^{2}H 4.61, and δ ^{2}H 5.52) were detected (*16*). The formation of OX was suggested to arise from the spontaneous reaction of BEA with endogenous bicarbonate (to form an *N*-carbamate intermediate) followed by a rapid cyclization reaction eliminating HBr (Scheme 1; X = Br).

In the current ^{1}H NMR studies we have investigated the reactivity of BEA in several in vitro systems, including renal cell cultures, blood plasma, and model solutions containing known HCO_3^- concentrations, and show that OX is produced from the *N*-carbamate of BEA (*N*-carbamoyl-2-bromoethanamine). Additional studies have been conducted on selected halide-substituted analogues of BEA in which the Br atom attached to the α-carbon was replaced either with more strongly electronegative Cl or F atoms (CEA and FEA, respectively) or with the less electronegative I atom in the form of a more stable phenyl-substituted iodo derivative (PIEA). By investigating the propensity of these haloalkylamines to react with HCO_3^-, we have examined whether the production of *N*-carbamates and OX from haloalkylamines is a general phenomenon that could be an important determinant of in vivo haloalkylamine toxicity.

Materials and Methods

Chemicals. Caution: *The haloalkylamines (and related species) used in these studies should be considered to be both potentially mutagenic and nephrotoxic.* BEA, CEA, FEA, OX, and PHOX were purchased from the Aldrich Chemical Co. (Gillingham, U.K.) and were used as supplied. PIEA, synthesized from iodocyanate and phenylethene (*10*), was kindly provided by Dr. C. J. Powell, Department of Health Toxicology Unit, St. Bartholomew's Hospital Medical College, London, EC1 7ED, U.K.

Renal Cell Line Derivation, Culture Conditions, and Haloalkylamine Transformation Studies. The Madin Darby canine kidney (MDCK) cell line (Flow Laboratories, Ricksmanworth, U.K.) was used between passage numbers 69 and 71. For all experiments cells were maintained in HEPES-free Eagle's minimal essential medium (Gibco BRL, Paisley, U.K.) supplemented with 10% calf serum, 1% L-glutamine, and 1% nonessential amino acids (L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glycine, L-proline, L-serine). Routinely, cells were grown in 175 cm^2 flasks, incubated at 37 °C in a humidified atmosphere containing 5% CO_2/95% air, and subcultured by conventional methods using trypsin–EDTA. For haloalkylamine transformation studies, confluent MDCK cells were exposed over a 24 h period to toxin concentrations of up to the IC_{50} determined by neutral red (*17*) staining assays. More specifically, cells (5 flasks per treatment group) were incubated with BEA, CEA, FEA, or PIEA in growth media corresponding to concentrations of 1, 5, 20, and 1.25 mM, respectively. Control cells were incubated with growth medium alone. In all cases, dosing solutions were administered within 30 min of formulation.

Sample Preparation and ^{1}H NMR Analysis of Cell Culture Media. Aliquots of cell culture medium (3 mL) were removed from flasks after incubation times of 1, 3, 6, 9, and 24 h, and protein was extracted by the addition of an equal volume of acetone. Precipitated cellular debris were removed by centrifugation at 3000 rpm for 10 min at 4 °C. Media supernatants were frozen immediately after separation and maintained at −20 °C. ^{1}H NMR measurements were made on a JEOL GSX500 spectrometer operating at 500.14 MHz ^{1}H resonance frequency at ambient probe temperature (ca. 298 K). Media supernatants were analyzed by NMR after lyophilization and reconstitution in ^2H_2O (750 μL). Sixty-four free induction decays (FIDs) were collected into 32K computer points using a 45° pulse width, a spectral width of 6000 Hz, an acquisition time of 2.73 s, and an additional T_1 relaxation delay of 2.27 s. Water suppression was achieved by gated secondary irradiation at the water resonance frequency (off during acquisition). Exponential weighting functions, corresponding to a line broadening of 0.37 Hz, were applied to the FIDs prior to FT. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]-1-propionate (TSP; δ 0; 1 mM), and resonance assignments were confirmed by a consideration of chemical shift, spin–spin couplings, literature values (*16*), and where appropriate, the addition of authentic compounds.

^{1}H NMR Studies on the Reactions of Haloalkylamines with Bicarbonate Ions in Aqueous Solution. The reactivity of BEA, CEA, FEA, and PIEA was investigated further by preparing model solutions (750 μL) containing 5 mM of each haloalkylamine and 100 mM sodium bicarbonate in ^2H_2O. The pH of each solution was recorded. ^{1}H NMR spectra (64 FIDs) were measured immediately after haloalkylamine addition, and further spectra were acquired at frequent intervals (in "blocks" of 64 FIDs) over the next 2–8 h, or until no additional spectral

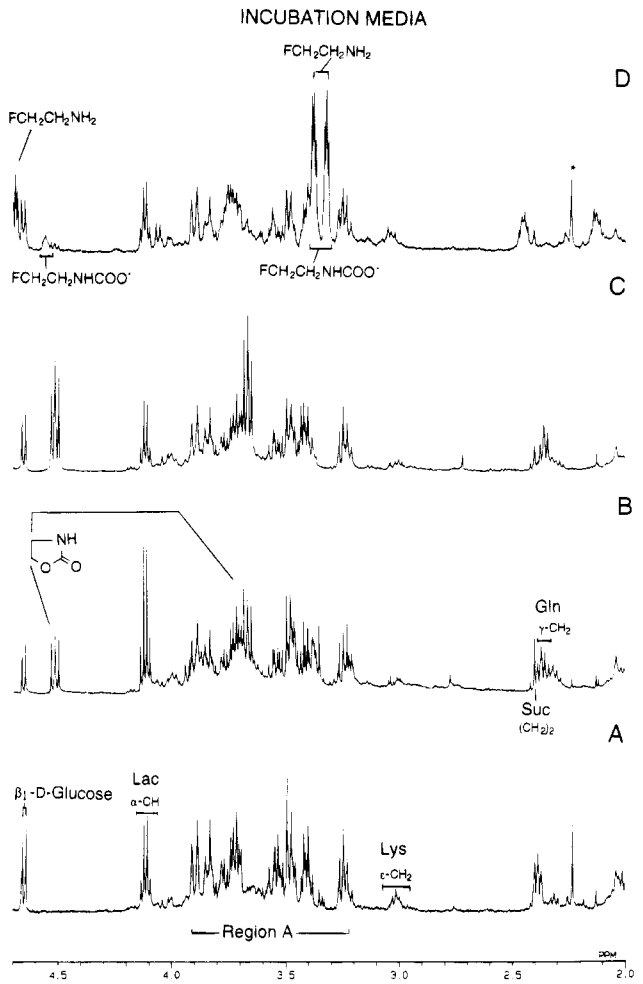


Figure 1. Partial 500 ^{1}H NMR MHz spectra (δ 2.0−4.7) of Eagle's minimal essential growth medium collected from control MDCK cells (A) and media obtained 9 h following incubation of MDCK cells with 1 mM BEA (B), 5 mM CEA (C), or 20 mM FEA (D). Abbreviations: Region A contains overlapped resonances from α- and β-D-glucose and the α hydrogens of amino acids; Lac, lactate; Suc, succinate; *, acetone impurity from sample preparation procedure. Experimental procedures are described in Materials and Methods.

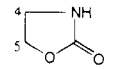
changes were observed. Solutions were then stored, sealed in NMR tubes, for several days to allow full equilibration, prior to measurement of a final NMR spectrum. As HCO_3^- was present in a 20-fold excess over haloalkylamine concentration, the data were analyzed in terms of pseudo-first-order kinetics. The data were plotted as log $(A_\infty - A_t)$ versus time, where A_∞ and A_t were the concentrations of haloalkylamine, OX, or *N*-carbamate present at equilibrium and at time t, respectively. Concentration data were determined from a consideration of the NMR peak height intensities of the haloalkylamines, *N*-carbamates, and OX that were expressed relative to the corresponding peak intensity of TSP (taking account of the known TSP concentration and the number of protons contributing to each resonance).

^{1}H NMR Studies on the Reactions of BEA in Whole Rat Blood Plasma. Plasma samples (500 μL) each obtained from two control F344 rats were incubated at room temperature, in the presence and absence of 3 mg of BEA, for 16 h. After this time, samples were diluted with 250 μL of ^2H_2O (to provide a field-frequency lock), and ^{1}H NMR spectra (128 FIDs) were recorded as described above.

Results

^{1}H NMR Detection of OX Formation in Renal Cell Culture Media. Partial 500 MHz ^{1}H NMR spectra of growth media (δ 2.0−4.7) obtained 9 h following incubation of MDCK cells at 37 °C with BEA, CEA, or FEA are illustrated in Figure 1. Of prime interest in this study was the evidence supporting the HCO_3^--mediated transformation of BEA and its halide-substituted analogues. ^{1}H NMR spectra of growth media obtained following incubation of MDCK cells with BEA revealed a pair of multiplets centred at δ 3.70 and 4.50 (Figure 1B) that were assigned to OX (Table 1). Strong signals for OX were also seen in media collected following incubation of cells with CEA (Figure 1C). However, signals from the parent haloalkylamines were not detected. In contrast, spectra of media obtained at all time points following incubation of cells with FEA were dominated by resonances from the parent haloalkylamine (at δ 3.35 and 4.63), and OX was present in minor amounts relative to that produced following incubation of cells with BEA or CEA, as illustrated for the 9 h time point (Figure 1D; Table 1). Additional novel resonances, not observed in control media or media obtained following incubation of cells with BEA or CEA, were apparent in the ^{1}H NMR profiles of media collected following exposure of cells to FEA. These signals, centered at δ 4.53, were assigned as being from the *N*-carbamate of FEA (Figure 1D; Table 1). OX resonances were absent from spectra of media collected following incubation of MDCK cells with PIEA (data not shown). Moreover, HOX resonances were not detected in the ^{1}H NMR profiles of media generated following incubation of cells with this group of haloalkylamines (Figure 1). The NMR observations indicated that the production of OX following incubation of BEA, CEA, and FEA with renal cell cultures could be associated with the bicarbonate component of the growth media. This was consistent with the hypothesis generated in vivo, regarding an interaction of BEA with HCO_3^- leading to the production of the *N*-carbamate and OX.

Table 1. ^{1}H NMR Spectral Data for Haloalkylamine-Related Species

compound	chemical shift (δ), signal multiplicity and assignment	structure
2-oxazolidone	3.70 (t)[a] (4) **4.50** (t) (5)	
2-bromoethanamine	3.45 (t) (1) **3.68** (t) (2)	^{2} ^{1} $BrCH_2CH_2NH_2$
2-bromoethanamine carbamate	3.40 (m) (1) **3.50** (m) (2)	^{2} ^{1} $BrCH_2CH_2NHCOO^-$
2-chloroethanamine	3.48 (t) (1) **3.85** (t) (2)	^{2} ^{1} $ClCH_2CH_2NH_2$
2-chloroethanamine carbamate	3.33 (m) (1) **3.60** (m) (2)	^{2} ^{1} $ClCH_2CH_2NHCOO^-$
2-fluoroethanamine	3.30 (dt)[b] (1) 4.71 (dt) (2)	^{2} ^{1} $FCH_2CH_2NH_2$
2-fluoroethanamine carbamate	3.31 (dt) (1) 4.49 (dt) (2)	^{2} ^{1} $FCH_2CH_2NHCOO^-$

[a] t indicates pseudo triplets arising from the following second-order spin systems: OX, BEA and CEA, AA′XX′. [b] dt indicates doublets of triplets (FEA spin system, AA′MM′X); mean δ position only given. NMR peaks used for kinetic analyses are represented in bold type.

Formation of *N*-Carbamates and 2-Oxazolidone from Haloalkylamines and HCO_3^- in Model Solutions. To verify the origin of OX, NMR experiments were performed on a series of buffer solutions in which BEA and its three halide-substituted analogues were reacted with an excess of HCO_3^-. The pH of each solution was adjusted to between 7.4 and 7.9, as a preliminary experiment conducted on a solution of BEA in HCO_3^- had

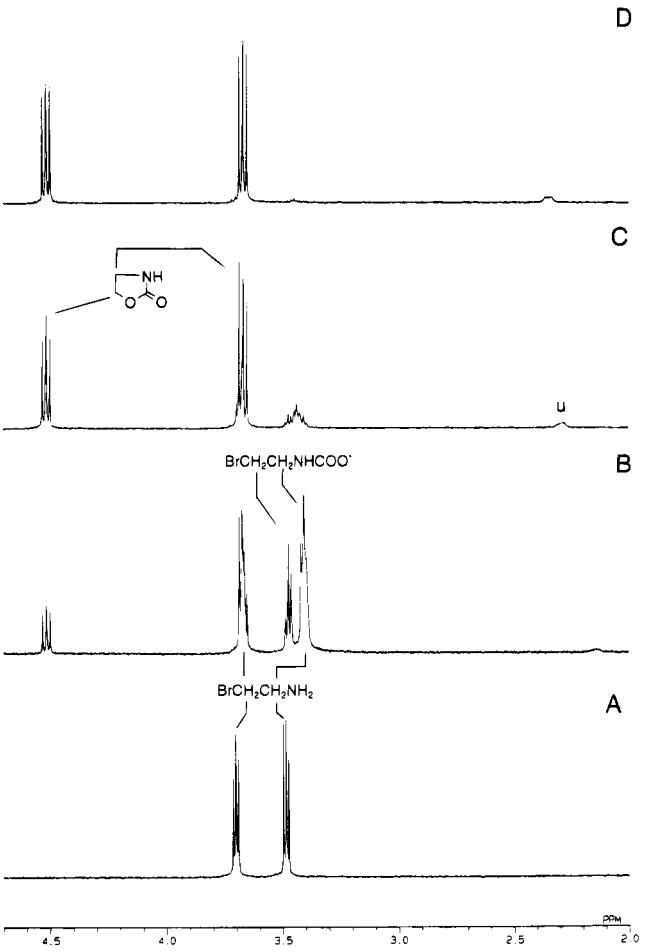


Figure 2. Partial 500 MHz ^1H NMR spectra (δ 2.0–4.7) of a standard solution (pH 7.2) of BEA in ^2H_2O (A) and a model solution of BEA in the presence of 20-fold excess of HCO_3^- in ^2H_2O (pH 7.6). Spectra illustrated were acquired 15 min (B), 1 h (C), and 3 h (D) following reaction initiation. Note pH shift of position of BEA on changing to HCO_3^- solution. Abbreviation: u, unknown species. Experimental procedures are described in Materials and Methods.

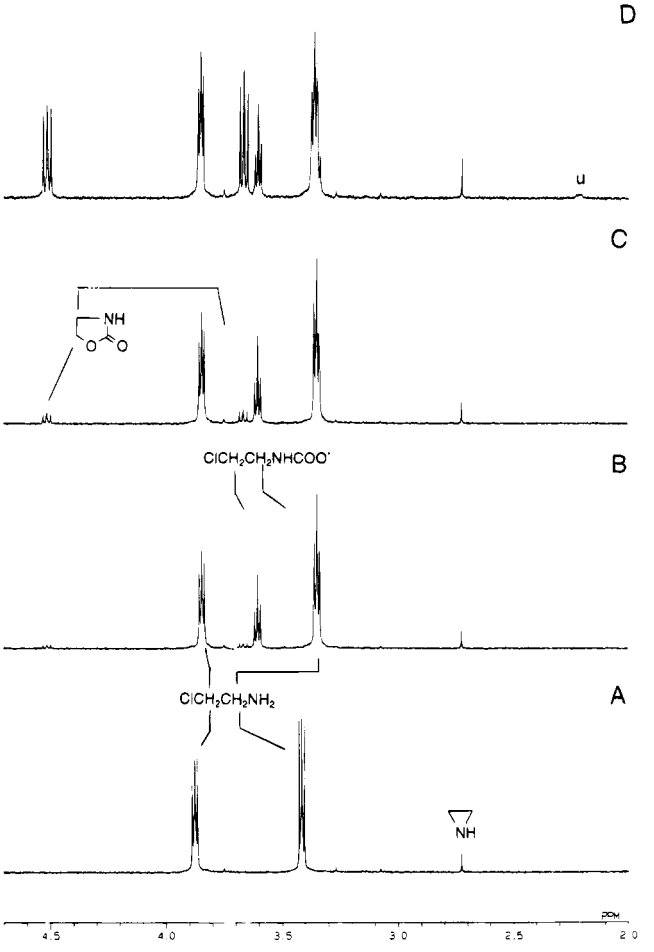


Figure 3. Partial 500 MHz ^1H NMR spectra (δ 2.0–4.7) of a standard solution of CEA in ^2H_2O (A) and a model solution of CEA in the presence of 20-fold excess of HCO_3^- in ^2H_2O. Spectra illustrated were acquired 30 min (B), 1 h (C), and 7 h (D) following reaction initiation. Note pH shift of position of CEA on changing to HCO_3^- solution. Experimental procedures are described in Materials and Methods.

revealed that OX formed readily under these conditions. OX was not produced under strongly acidic conditions (pH 1.8 and 3.9) or when the pH of BEA was adjusted to >7 with NaOH (data not shown). In the current experiments, the time dependence of the appearance of OX was investigated by reacting BEA (5 mM) with a solution containing a 20-fold excess of HCO_3^- (pH 7.6). The ^1H NMR spectra acquired at various intervals over approximately 6 h (Figure 2) contained a pair of multiplet resonances that were assigned to BEA, accompanied by the characteristic OX signals at high frequency to BEA and resonances assigned to BEA carbamate (Table 1). There was a gradual disappearance, over approximately 3 h, of the BEA signals with a concurrent increase in the intensity of signals from OX (observed within 15 min of initiating the reaction; Figure 2B). Signals from BEA carbamate appeared immediately upon mixing BEA with HCO_3^-; however, like BEA, these signals had largely disappeared within 3 h, concomitant with the increase in OX levels (Figure 2D). This may explain their absence in spectra of media collected 9 h after incubation with BEA (Figure 1B). A broad resonance from an unidentified species was also detected, whose chemical shift altered from δ 2.15 in the ^1H NMR spectrum measured at 15 min to δ 2.30 and 2.35 at 1 and 3 h, respectively (Figure 2).

Equivalent experiments were conducted to examine the occurrence and/or time course of appearance of the *N*-carbamates of CEA, FEA, and PIEA. Signals from CEA and FEA decreased in intensity throughout the duration of the reaction, a considerable proportion of both haloalkylamines immediately undergoing a transition to the corresponding *N*-carbamate (Figures 3 and 4; Table 1). However, the intensity of the FEA CH_2 resonances close in chemical shift to water were severely attenuated with the application of a water-suppressing secondary irradiation field, the insert demonstrating amelioration of the intensity distortion in the absence of water suppression (Figure 4A). These signals also appeared broader than the FEA CH_2 signals to low frequency, suggesting different T_2 values for the CH_2 protons and slightly distorting the relative intensities of the FEA carbamate signals centered at δ 4.49 (Figure 4). OX multiplets were observed within 30 min of reacting CEA with HCO_3^- (Figure 3B) and progressively increased in intensity over the next 7 h (Figure 3C,D) accompanied by a singlet resonance (δ 2.71) characteristic of AZ (also present in the solution of CEA in ^2H_2O; Figure 3A). In the case of CEA, a small, unidentified broad singlet resonance (at δ 2.22) was also observed from approximately 7 h following reaction initiation (Figure 3D). OX was not detected in buffer solution until approximately 6 h following addition of FEA (Figure 4B) and remained

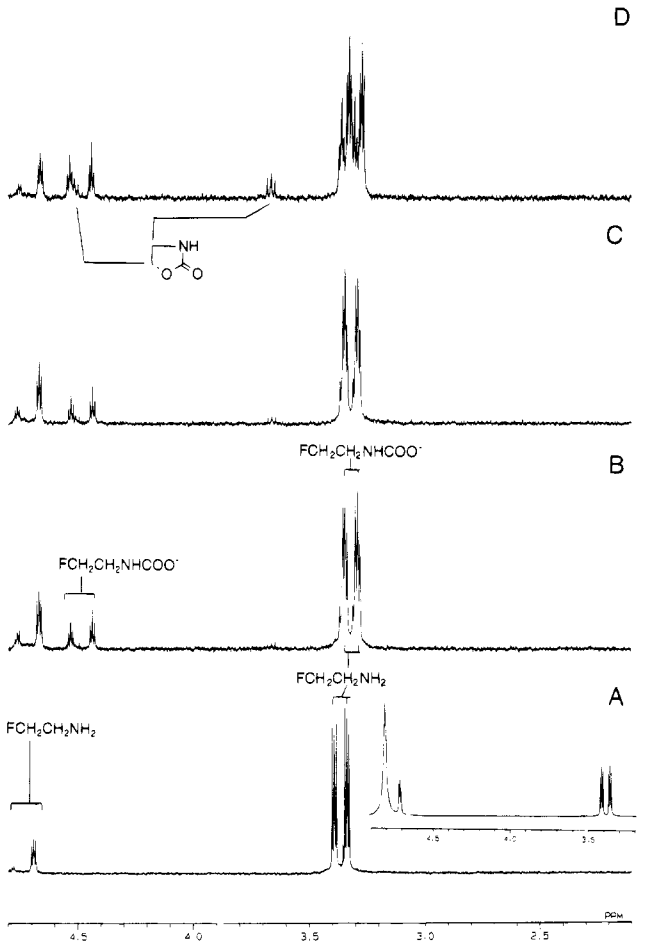


Figure 4. Partial 500 MHz ^1H NMR spectra (δ 2.1–4.8) of a standard solution of FEA in ^2H_2O (A) and a model solution of FEA in the presence of 20-fold excess of HCO_3^- in ^2H_2O. Spectra illustrated were acquired 5 h 40 min (B), 8 h 20 min (C), and 24 h (D) following reaction initiation. The insert in (A) illustrates a spectrum of a standard solution of FEA in ^2H_2O, measured without water suppression, showing additional signal splitting. Note pH shift of position of FEA on changing to HCO_3^- solution. Experimental procedures are described in Materials and Methods.

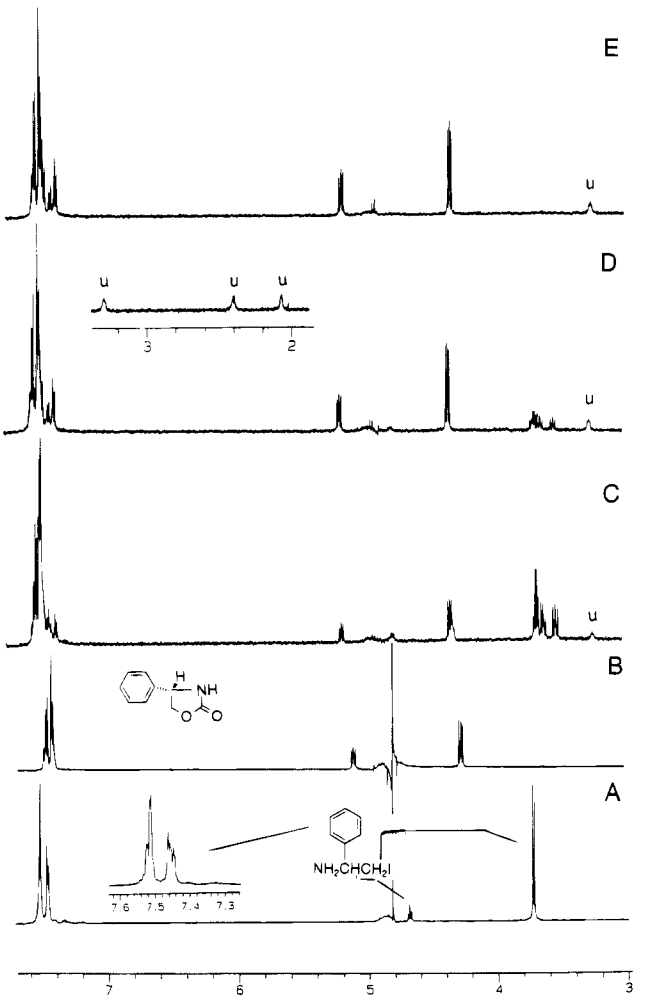


Figure 5. Partial 500 MHz spectra (δ 3.0–7.7) of standard solutions of PIEA (A) and PHOX (B) in ^2H_2O and a model solution of PIEA in the presence of 20-fold excess of HCO_3^- in ^2H_2O. Spectra illustrated were acquired 10 min (C), 34 min (D), and 2 h (E) following reaction initiation. The insert (D) expands the unidentified broad resonances (between δ 1.9 and 3.4). Experimental procedures are described in Materials and Methods.

at low levels in spectra recorded 2 and 24 h later (Figure 4C,D). The AZ singlet was not detected following reaction initiation with FEA. Similarly, AZ was not observed after PIEA was reacted with HCO_3^- (Figure 5), consistent with their absence in cell incubation media. However, PIEA (Figure 5A) was converted to a novel species, assigned as PHOX (Figure 5B), within 10 min of mixing (Figure 5C). Moreover, PIEA resonances (at δ 3.65, 4.62, and 7.44–7.54) had virtually disappeared within 2 h of reaction initiation (Figure 5E). The spectra also suggest the formation of *N*-carbamates of PIEA and further small, as yet unidentified, broad resonances (δ 1.90–3.20) following reaction initiation, analogous to the situation with BEA and CEA (Figures 2 and 3).

Kinetics of the Reactions of Haloalkylamines with HCO_3^-. As the starting haloalkylamine concentration was known (5 mM), it was possible to calculate the concentrations of each haloalkylamine and the corresponding *N*-carbamate and OX over time by a comparison of the peak intensities of the appropriate ^1H NMR signals (Table 1). Representative plots of the time dependence of the disappearance of BEA, CEA, and their respective *N*-carbamates and the rate of formation of OX are illustrated in Figure 6. The plots show that, in these solutions and with this initial excess of HCO_3^-, signals from BEA had disappeared after approximately 3 h, signals from BEA carbamate disappearing approximately 1 h later (Figure 6A). The formation of OX reached a plateau (at a concentration of 4.0 mM) within 6 h of reaction initiation with BEA (Figure 6A), OX being present at an equilibrium concentration of 4.06 mM at 24 h. The equivalent reaction with CEA proceeded relatively slowly, with CEA, CEA carbamate, and OX being present at 0.96, 0.76, and 2.29 mM, respectively, after 24 h (Figure 6B). However, the reaction reached completion after a few days, with OX attaining a concentration of 5.40 mM. As the formation of OX from FEA carbamate clearly proceeded at a lower rate than with the other haloalkylamines (Figures 2–4), kinetic analysis was restricted to the interaction of HCO_3^- with BEA and CEA. In these cases, pseudo-first-order rate constants were estimated from the ^1H NMR-derived concentration data obtained within the first 1 h following reaction initiation. Regression lines were fitted to the first 10 concentrations of OX recorded (see Figure 6C,D), and it was demonstrated that the initial rate of OX formation was over 200-fold faster for BEA than for CEA, with k values of 0.66×10^{-4} s^{-1} and 2.59×10^{-6} s^{-1}, respectively.

Reactions of BEA in Rat Plasma. Additional supporting evidence that the formation of OX was related

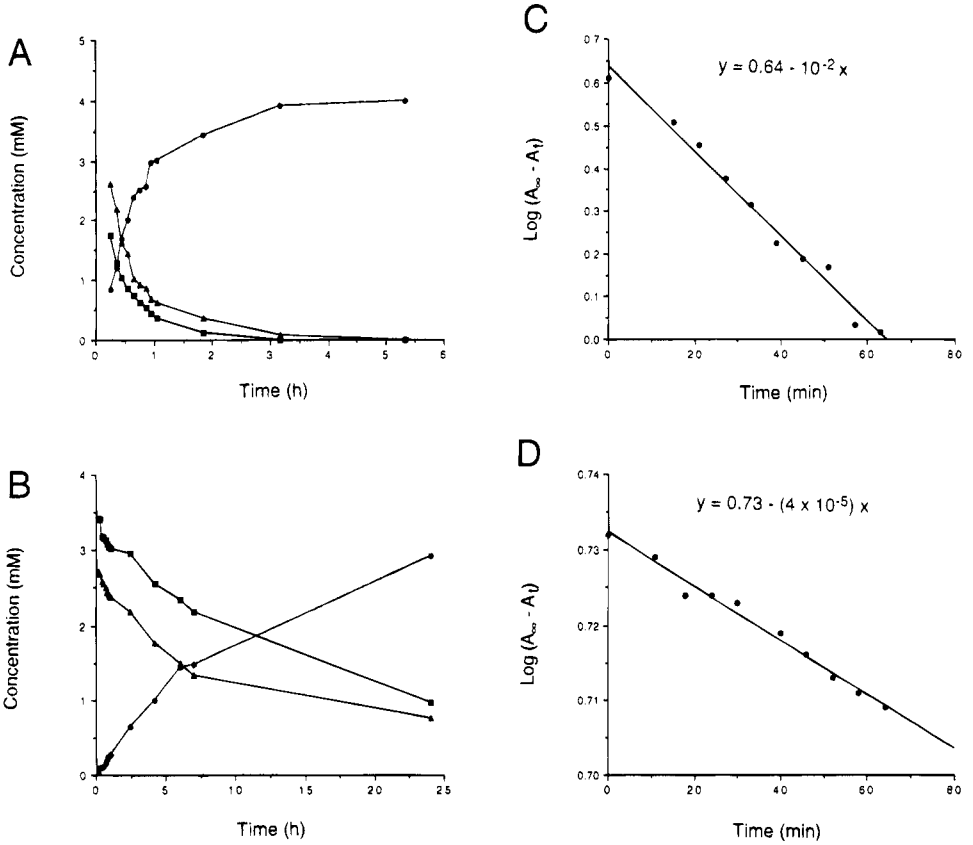


Figure 6. Representative plots of the time dependence (A, B) and pseudo-first-order kinetics (C, D) of OX formation from BEA and CEA (each 5 mM) in the presence of a 20-fold initial excess of HCO_3^-. Experimental procedures are described in Materials and Methods. Key to plots: (A) ■ BEA, ▲ BEA carbamate, ● OX; (B) ■ CEA, ▲ CEA carbamate, ● OX.

to the presence of HCO_3^- and, further, that this reaction was feasible under in vivo conditions was provided by conducting an experiment on rat plasma (pH 7.4) to which 3 mg of BEA had been added. ^1H NMR spectra measured from plasma collected from control F344 rats revealed characteristic broad resonances from macromolecular plasma constituents with short T_2 relaxation times, such as lipids and lipoproteins (Figure 7A). In contrast, spectra acquired from plasma samples that had been spiked with BEA for 16 h clearly displayed multiplets from OX and a singlet resonance of AZ (Figure 7B).

Discussion

***N*-Carbamate and OX Formation from Haloalkylamines.** These ^1H NMR studies show that BEA is chemically transformed to *N*-carbamate intermediates and OX in the presence of excess HCO_3^- and have demonstrated that the reaction occurs in a variety of in vitro systems including whole blood plasma (Figures 1, 2, and 7). Of more widespread significance, however, was the evidence demonstrating the spontaneous degradation in vitro of both CEA and FEA to OX (Figures 1, 3, and 4), showing that the *N*-carbamoylation reaction and subsequent cyclization to OX share the same mechanism for these compounds. The equivalent interaction of HCO_3^- with PIEA, the phenyl-substituted derivative of the iodo analogue of BEA, led to the ^1H NMR detection of PHOX (Figure 5). Interestingly, ^1H NMR analysis of culture media collected following incubation of MDCK cells with BEA, CEA, and FEA revealed alterations in the proportions of *N*-carbamates and OX formed, relative to the parent haloalkylamines (Figure 1). Initially, this observation was related to the different incubation

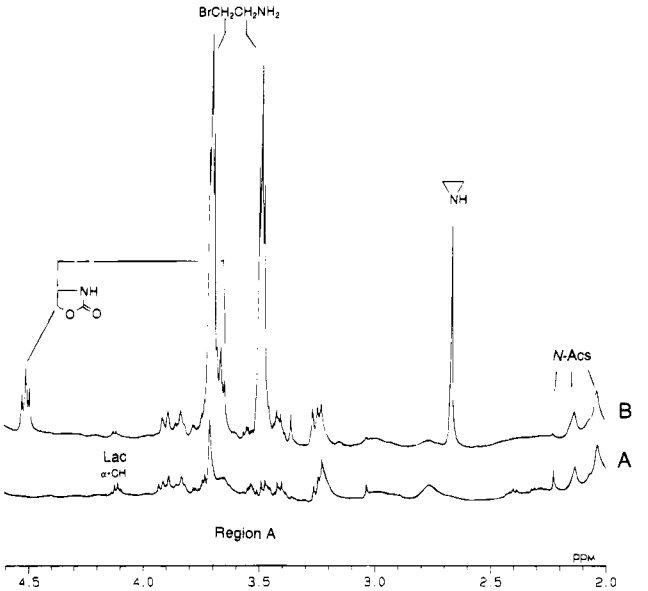


Figure 7. Partial 500 MHz ^1H NMR spectra (δ 2.0–4.6) of control F344 rat plasma (A) and control F344 rat plasma to which BEA had been added (B). Experimental procedures are described in Materials and Methods.

concentrations, as ^1H NMR spectra of media obtained following exposure of cells with FEA, the compound administered at the highest concentration (20 mM), contained high levels of the parent amine and *N*-carbamate intermediates and significantly lower levels of OX, relative to the findings with BEA and CEA (Figure 1). However, kinetic analysis of the haloalkylamine transformation experiments conducted in HCO_3^--containing buffer solutions indicated that the degree of

N-carbamate formation was related to the different basicities of the haloalkylamines and can be explained in terms of the inductive effects of the halides. The subsequent degree of OX formation from *N*-carbamates was influenced by the variation in the electrophilicity of the halide. The fastest formation of OX in buffer solution occurred with BEA, where the initial rate constant was >200 times more than the rate of the equivalent reaction between CEA and HCO_3^-. The slowest formation of OX in buffer solution (and cell culture media) was observed with FEA, F being the strongest electrophile. In the case of PIEA, the OX analogue PHOX was formed in buffer solution at a rate higher than that of OX formation by BEA (complete within 2 h of reaction initiation; Figure 5). This may be explained in terms of iodine being more weakly electrophilic than Br, but the analogy is incomplete due to the difficulty in obtaining comparable kinetic data.

Toxicological and Metabolic Significance of Haloalkylamine Carbamate Formation. The formation of OX and HOX in vivo has been reported in F344 rats 4–12 h after BEA administration (*16*). Similarly, a related study where Sprague-Dawley rats were dosed with 100 mg/kg OX showed that 43% of the dose was recovered as HOX (*16*). In the present studies on cell lines HOX was not observed, indicating that its formation in vivo may be dependent on extrarenal metabolism, possibly via a P450-mediated conversion. This apparent disparity in results might, therefore, reflect important differences in the metabolic handling of BEA (and OX) in vitro compared to in vivo. In vivo NMR studies demonstrated that OX was recovered in the urine of F344 rats at 9% of the delivered BEA dose and that AZ was the favored urinary product with a recovery of 31% of the dose (*16*). This may be consistent with the current observation of higher levels of AZ, relative to OX, in control F344 rat plasma to which BEA was added (Figure 7). Further, the detection of OX resonances in plasma indicates that the OX observed in the in vivo toxicity studies was likely produced by reaction of BEA with HCO_3^- in the blood, as has been reported for a novel bromoethylamino prodrug (*18*). Significantly, the presence of both AZ and OX in blood plasma incubated with BEA indicates that both species are likely to exist under physiological conditions, although it appears likely that they are formed via different pathways of BEA decomposition, an excess of HCO_3^- favoring OX formation (Scheme 1). Interestingly, NMR analysis of growth media and buffer solutions showed that OX was the major BEA (CEA and FEA)-related species formed in vitro, AZ being present in minor amounts (or not at all in the case of FEA). It appears, therefore, that the in vitro studies might overestimate the contribution of OX to the overall transformation of this series of haloalkylamines. This indicates that a more detailed investigation of the in vivo pharmacokinetic processes regarding the formation, disposition, and elimination of AZ and OX following administration of BEA, CEA, and FEA is required. Preliminary studies have demonstrated the presence of OX in urine from F344 rats within 8 h of ip administration of 500 and 600 mg/kg CEA.[2] Thus, the observed in vitro formation of *N*-carbamate intermediates and OX from BEA, CEA, and FEA may be predictive of the metabolic fate of these haloalkylamines in vivo.

[2] M. L. Anthony, E. Holmes, and J. K. Nicholson, unpublished observations.

The identification of a causative agent for BEA nephrotoxicity remains unclear, although it is generally accepted that the RPN effects induced by BEA are largely a consequence of its direct action or its dissociation to AZ (*10*). However, we have demonstrated that, under physiologically-relevant conditions, haloalkylamines show high reactivity toward HCO_3^-, leading to the formation of carbamates. In the haloalkylamine transformation studies, dosing solutions were administered within 30 min of formulation, by which time the decomposition products may have included various proportions of carbamoylated species and oxazolidones in addition to AZ, as indicated by the model solution experiments (Figures 2–5). These in vitro observations suggest that BEA and its analogues must be converted to carbamates virtually immediately in vivo and, more importantly, that the toxicity of this series of haloalkylamines may be related to (or limited by) the production of these intermediates. Carbamates are known to be toxicologically active, the use of *N*-methylcarbamate insecticides being accompanied by numerous acute poisoning episodes (*19*). The HCO_3^--dependent neurotoxicity of L-cysteine and β-(*N*-methylamino)-L-alanine is also established (*20*, *21*). Further, carbamates have been implicated in tumor initiation and promotion (*22*, *23*). In contrast, although *N*-nitrosooxazolidone decomposition products of (hydroxylalkyl)nitrosoureas have been implicated in chemical carcinogenicity (*24*), literature reports on oxazolidones generally describe their use as derivatives in organic synthesis (*25*, *26*), Thus, a relationship between nephrotoxicity and the production of *N*-carbamates and OX has not been reported previously.

The participation of OX in the mechanism of RPN may be questioned, however, as papillotoxic potential is not necessarily retained by halide substitution. In vivo, CEA and BEA both produce extensive necrosis of the papilla and mid medulla, CEA being less potent due to the decreased propensity for Cl^- dissociation (*10*). In contrast, although FEA has the greatest toxic potential in vivo, its effects have been reported to range from lethality (at doses above 10 mg/kg) to the production of corticomedullary mineralization (when administered at 5 mg/kg) in the absence of RPN (*10*). Similarly, PIEA has been reported to induce proximal tubular nephrotoxicity rather than RPN (*10*). For this reason, it appears unlikely that OX or PHOX are directly involved in the mechanism of RPN. Indeed, the absence of AZ in NMR spectra measured following FEA transformation supports the hypothesis that AZ is directly implicated in the mechanism of RPN rather than OX. Significantly, ^1H NMR profiles of urine collected up to 48 h following administration of OX to Sprague-Dawley rats did not share the same abnormal biochemical features characteristic of BEA nephrotoxicity, such as GTA-uria and the other low MW biomarkers of RPN (*16*). These observations suggest that the formation of OX (or PHOX) and *N*-carbamates may represent the conversion of the parent haloalkylamines to novel, albeit less toxic, species. The nephrotoxic effects may be strain-specific, however, so that a positive involvement of OX in the toxicity of BEA and its analogues cannot be ruled out at this stage. The contribution of *N*-carbamate intermediates and OX (or PHOX) to the mediation of haloalkylamine-induced nephrotoxicity in general, therefore, must be addressed further both in vivo and in vitro.

In summary, the present study has demonstrated the utility of NMR spectroscopy in the integration of biotrans-

formation and toxicity studies. The transformation studies and model solution experiments have confirmed the hypothesis that the OX observed in vivo following BEA administration is produced from *N*-carbamate intermediates. More importantly, these data suggest that the formation of these species is also an integral part of the metabolism of the halide-substituted analogues of BEA. However, the exact contribution of *N*-carbamates and OX to the mediation of haloalkylamine-induced nephrotoxicity remains under scrutiny.

Acknowledgment. Financial support for this and related work was provided by the Wellcome Trust (M.L.A), grant no. 035804/1:40, the Medical Research Council (E.H. and P.C.R.M.), and Sanofi Research Division (P.C.R.M.). We thank the University of London Intercollegiate Research Service Biomedical NMR Services at Birkbeck College and Queen Mary and Westfield College for NMR facilities and the University of Edinburgh and the Toxicology Department at Sanofi Research Division, U.K., for provision of isolated renal cells and cell culture facilities. We also thank Dr. D. B. Davies for helpful discussion.

References

(1) Murray, G., Wyllie, G., Hill, G. S., Ramsden, P. W., and Heptinstall, R. H. (1972) Experimental papillary necrosis of the kidney. I. Morphologic and functional data. *Am. J. Pathol.* **67,** 285–302.

(2) Bach , P. H., Grasso, P., Molland, E. A., and Bridges, J. W. (1983) Changes in the medullary glycosaminoglycan histochemistry and microvascular filling during the development of 2-bromoethanamine hydrobromide-induced renal papillary necrosis. *Toxicol. Appl. Pharmacol.* **69**, 333–344.

(3) Nanra, R. S. (1983) Renal effects of analgesics. *Am. J. Med.* **November**, 70–79.

(4) Sabatini, S. (1988) Analgesic-induced papillary necrosis. *Semin. in Nephrol.* **8,** 41–52.

(5) Arruda, J. A. L., Sabatini, S., Mehta, P. K., Sodhi, B., and Baranowski, R. (1979) Functional characterisation of drug-induced experimental papillary necrosis. *Kidney Int.* **15,** 264–275.

(6) Bach, P. H., and Bridges, J. W. (1982) Chemically associated renal papillary necrosis. In *Nephrotoxicity, Assessment and Pathogenesis* (Bach, P. H., Bonner, F. W., Bridges, J. W., and Lock, E. A., Eds.) pp 437–459, John Wiley, Chichester.

(7) Ross, W. C. J. (1962) *Biological alkylating agents*, p 232, Butterworths, London.

(8) Davies, D. J. (1969) The structural changes in the kidney and urinary tract caused by ethyleneimine ("vinylamine"). *J. Pathol.* **97,** 695–703.

(9) Powell, C. J., Tyobeka, T., Bach, P. H., and Bridges, J. W. (1985) The nephrotoxicity of structural analogues of 2-bromoethanamine. In *Renal Heterogeneity and Target Cell Toxicity* (Bach, P. H., and Lock, E. A., Eds.) pp 195–198, John Wiley, Chichester.

(10) Powell, C. J., Grasso, P., Ioannides, C., Wilson, J., and Bridges, J. W. (1991) Haloalkylamine-induced renal papillary necrosis: a histopathological study of structure–activity relationships. *Int. J. Exp. Pathol.* **72,** 631–646.

(11) Powell, C. J., and Ioannides, C. (1992) Nephrotoxicity is closely correlated with mutagenicity for a series of analogues of 2-bromoethanamine, suggesting a mechanistic relationship. *Hum. Exp. Toxicol.* **11,** 588–589.

(12) Nicholson, J. K., and Wilson, I. D. (1989) High resolution proton nuclear magnetic resonance spectroscopy of biological fluids. *Prog. Nucl. Magn. Reson. Spectrosc.* **21,** 444–501.

(13) Gartland, K. P. R., Bonner, F. W., and Nicholson, J. K. (1989) Investigations into the biochemical effects of region-specific nephrotoxins. *Mol. Pharmacol.* **35,** 242–250.

(14) Holmes, E., Bonner, F. W., Gartland, K. P. R., and Nicholson, J. K. (1990) Proton NMR monitoring of the onset and recovery of experimental renal damage. *J. Pharm. Biomed. Anal.* **8,** 959–962.

(15) Holmes, E., Bonner, F. W., Sweatman, B. C., Lindon, J. C., Beddell, C. R., Rahr, E., and Nicholson, J. K. (1992) Nuclear magnetic resonance spectroscopy and pattern recognition analysis of the biochemical processes associated with the progression and recovery from nephrotoxic lesions in the rat induced by mercury II chloride and 2-bromoethanamine. *Mol. Pharmacol.* **42,** 922–930.

(16) Holmes, E., Caddick, S., Lindon, J. C., Wilson, I. D., Kryvawych, S., and Nicholson, J. K. (1995) ^{1}H and ^{2}H NMR spectroscopic studies on the biochemical effects and metabolism of 2-bromoethanamine in the rat: induction of transient glutaric aciduria. *Biochem. Pharmacol.* **49,** 1349–1359.

(17) Borenfreund, E., and Puerner, J. A. (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol. Lett.* **24**, 119–124.

(18) Binger, M., and Workman, P. (1991) Pharmacokinetic contribution to the improved therapeutic selectivity of a novel bromoethylamino prodrug (RB 6145) of the mixed-function hypoxic cell sensitizer/cytotoxin α-(1-aziridinomethyl)-2-nitro-1*H*-imidazole-1-ethanol (RSU 1069). *Cancer Chemother. Pharmacol.* **29,** 37–47.

(19) Gupta, R. C., and Dettbarn, W. D. (1993) Role of carboxylesterases in the prevention and potentiation of *N*-methylcarbamate toxicity. *Chem.-Biol. Interact.* **87,** 295–303.

(20) Myers, T. G., and Nelson, S. D. (1990) Neuroactive carbamate adducts of β-N-methylamino-L-alanine and ethylenediamine. Detection and quantitation under physiological conditions by ^{13}C NMR. *J. Biol. Chem.* **265,** 10193–10195.

(21) Olney, J. W., Zorumski, C., Price, M. T., and Labruyere, J. (1990) L-Cysteine, a bicarbonate-sensitive endogenous excitotoxin. *Science* **248,** 596–599.

(22) Leithauser, M. T., Liem, A., Stewart, B. C., Miller, E. C., and Miller, J. A. (1990) 1,N^6-Ethenoadenosine formation, mutagenicity and murine tumor induction as indicators of the generation of an electrophilic epoxide metabolite of the closely related carcinogens ethyl carbamate (urethane) and vinyl carbamate. *Carcinogenesis* **11,** 463–473.

(23) Pereira, M. A., Khoury, M. M., Glauert, H. P., and Davis, R. A. (1991) Screen of five alkyl carbamates for initiating and promoting activity in rat liver. *Cancer Lett.* **57,** 37–44.

(24) Singer, S. S. (1985) Decomposition reactions of (hydroxylalkyl)-nitrosureas and related compounds: possible relationship to carcinogenicity. *J. Med. Chem.* **28,** 1088–1093.

(25) Gonzalez, F. B., Baz, J. P., and Espina, M. I. R. (1989) Synthesis of (+)-pilocarpine analogs with a 2-oxazolidone structure. *Tetrahedron Lett.* **30,** 2145–2148.

(26) Evans, D. A., and Sjogren, E. B. (1985) The asymmetric synthesis of β-lactam antibiotics—I. Application of chiral oxazolidones in the Staudinger reaction. *Tetrahedron Lett.* **26,** 3783–3786.

TX9500538