Synthesis of α-Deuterium-Labelled Cyclohexylamine and Its Deamination by Rabbit Liver Microsomes

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In order to investigate the isotope effect on the microsomal oxidative deamination of cyclohexylamine, α-deuterium(D)-labelled cyclohexylamine was synthesized. The deuterium labelling was found exclusively at the α-position with a purity of greater than 99 atom percent. Metabolic studies *in vitro* indicate that a significant deuterium isotope effect operates in the oxidative deamination of α-D-labelled cyclohexylamine. On incubation with rabbit liver microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen, the ratio of apparent deamination rate constants (k_H/k_D) was 2.0±0.2 (mean±S.D.).

Keywords synthesis; regioselectivity; ^{13}C-NMR; α-deuterium-labelled cyclohexylamine; isotope effect; microsomal deamination; rabbit liver

Introduction

Cyclohexylamine is one of the urinary metabolites of cyclamate, an artificial sweetening agent.[1-3] In a report on the fate of cyclohexylamine in man and other species,[4] deamination appeared to be the major metabolic reaction in rabbits, guinea pigs and humans.

Microsomal deamination was first reported by Axelrod for amphetamine,[5] and Brodie *et al.* proposed the reaction to proceed *via* the α-hydroxylated carbinolamine intermediate.[6] This pathway seemed to be confirmed by the partial incorporation of molecular oxygen-18 into phenylacetone[7] and alicyclic ketones.[8] Further, the deamination was suggested to be catalyzed by cytochrome P-450 dependent monooxygenase.[9] Therefore, we consider that the C–H bond at the α-position of primary amines may be broken during the oxidative deamination and that a deuterium isotope effect can be anticipated if this event is involved in the rate-limiting process. However, few isotope effects have been reported in the deamination of primary amines except for amphetamine.[10]

This paper deals with the synthesis of α-deuterium(D)-labelled cyclohexylamine and its isotope effect in rabbit liver microsomal deamination under specified conditions with reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen, $NaIO_4$ or $NaClO_2$.

Experimental

Materials NADPH, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were obtained from Boehringer–Mannheim GmbH. Lithium aluminum deuteride (Min. 99 atom% D) was from Merck Sharp & Dohme, Montreal, Canada. All other chemicals were commercially available. α-D-labelled cyclohexylamine was synthesized by the reduction of cyclohexanone oxime with lithium aluminum deuteride.[10,11] From 0.94 g of cyclohexanone oxime and 0.42 g of lithium aluminum deuteride, the yield of the hydrochloride salt of D-labelled cyclohexylamine was 400 mg.

Methods Microsomal preparation, and incubating and extracting conditions were as described previously.[9] The relative deamination rates of cyclohexylamine and α-D-labelled cyclohexylamine were determined by performing simultaneous incubations of substrates (5 mM) under identical conditions. The deamination activity was expressed as the sum of cyclohexanone and cyclohexanol formed. Protein concentrations were determined by the method of Lowry *et al.*[12] using bovine serum albumin as a standard.

In the cases of $NaIO_4$ (3 mM) and $NaClO_2$ (5 mM), the incubation mixture contained 5 mM $MgCl_2$, 30 mM KCl, 30 μM ethylenediaminetetraacetic acid (EDTA), 50 mM potassium phosphate (pH 7.4), microsomal fraction (1.0—1.9 mg of protein/ml) and 5 mM substrate in a final volume of 10 ml. After a final addition of $NaIO_4$ or $NaClO_2$, the mixtures were incubated at 37 °C for 10 min according to the method of Gustafsson *et al.*[13]

Analysis Gas-liquid chromatographic analyses were performed as described previously.[9] Integration was performed with a Shimadzu C-R4A computed integrator. The samples were analyzed by gas chromatography-mass spectrometry (GC-MS) on a JEOL DX 300 with a DA 5000 data system operating in the electron impact mode (70 eV) and equipped with Hewlett Packard fused silica Methyl Silicone or Carbowax 20M column (10 m × 0.53 mm i.d.) at 80 °C. The injector, separator line, inlet, and source temperatures were set at 250, 220, 250 and 200 °C, respectively. Helium was utilized as the carrier gas, at 20 ml/min. ^{1}H- and ^{13}C-Nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were recorded on a JEOL FX-200, Fourier-transform spectrometer operating at 200 MHz and 50 MHz, respectively, at 27 °C, in $CDCl_3$ solution.

Results and Discussion

Isotopic purity at the α-hydrogen position was greater than 99 atom percent in α-D-labelled cyclohexylamine based on mass and NMR spectra: cyclohexylamine was converted to the acetylated derivative for clear determination of the incorporation ratio of deuterium by mass spectroscopy. The mass spectrum (MS) of acetyl α-D-labelled cyclohexylamine (Fig. 1(B)) shows peaks one mass unit bigger than those of acetyl cyclohexylamine (Fig. 1(A)), with fragment ions of m/z 56 (C_3H_6N), 67 (C_4H_5N), 70 (C_4H_8N), 82 (C_6H_{10}), 98 (M^+-43), and 141 (M^+), respectively. In the ^{1}H-NMR spectra, the C_1-H signal of cyclohexylamine disappeared in D-labelled cyclohexylamine (Fig. 2). The extent of deuteration was >99% as judged from the mass spectra and the complete absence of a

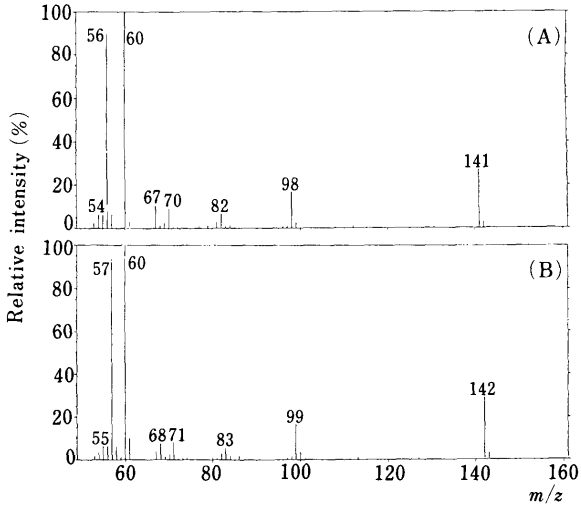

Fig. 1. MS of Acetylated Cyclohexylamine (A) and Acetylated α-D-Labelled Cyclohexylamine (B)

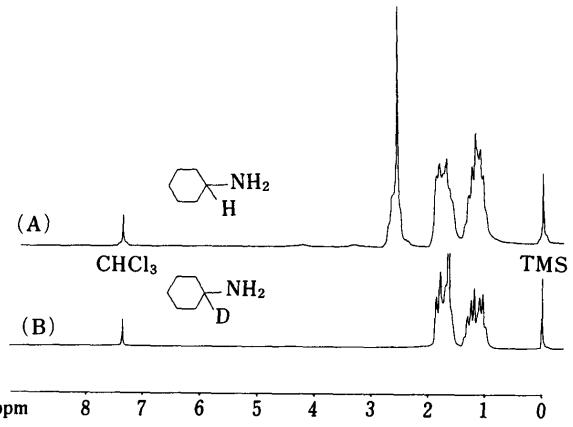

Fig. 2. ^{1}H-NMR Spectra of Cyclohexylamine (A) and α-D-Labelled Cyclohexylamine (B)

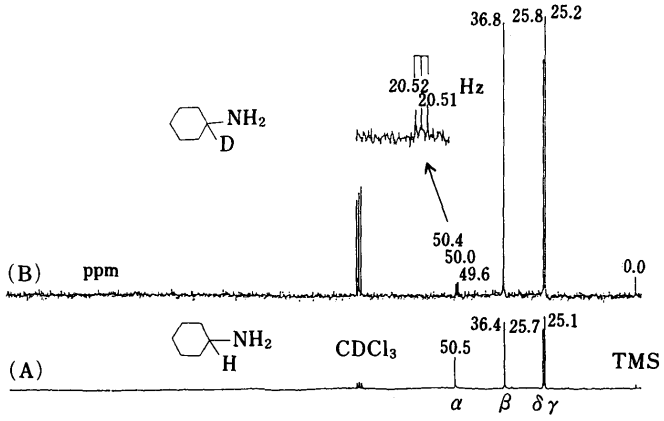

Fig. 3. ^{13}C-NMR Spectra of Cyclohexylamine (A) and α-D-Labelled Cyclohexylamine (B)

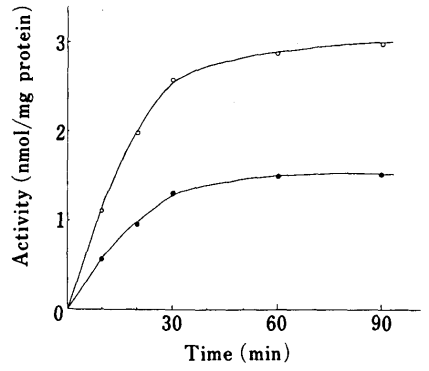

Fig. 4. Time Course of Microsomal Deamination of Cyclohexylamine (○—○) and α-D-Labelled Cyclohexylamine (●—●)

The reaction was carried out at 5 mM amine. Other conditions are described in Methods.

peak at 2.56 ppm downfield from tetramethylsilane due to the methyl protons in the ^{1}H-NMR spectrum.

It is well known that the isotope shift caused by deuterium substitution shows a small shielding effect in terms of the resonance positions of the substituted carbons[14] and that signal lines of deuterated carbons are substantially split and less intense than those of protonated ones.[15] D-Labelled cyclohexylamine showed the ^{13}C-NMR signal of the C_1-carbon at 50.0 ppm ($J^{C-D}=20.5$ Hz), indicating shielding and splitting when compared with the signal at 50.5 ppm of cyclohexylamine (Fig. 3). Thus, the deuterium was established to be at the α-position of cyclohexylamine.

The deamination of cyclohexylamine and α-D-labelled cyclohexylamine was studied *in vitro*, using rabbit liver microsomal fractions with an NADPH-generating system (Fig. 4). The microsomal deamination proceeded almost linearly over 30 min, after which the deamination rates of both amines decreased slowly. A significant deuterium-isotope effect operates in the oxidative deamination of α-D-labelled cyclohexylamine. The ratio of apparent rate constants (k_H/k_D) was 2.0±0.2 (mean±S.D; $N=5$) at a substrate concentration of 5 mM. The result is consistent with that of Foreman *et al.* who used the 9000×*g* supernatant fractions of rabbit liver at 0.15 mM amphetamine ($k_H/k_D=2.0$).[10] These data suggest that this ratio might be unaffected by the change of substrate amine.

In the cases of $NaIO_4$ and $NaClO_2$, a low deuterium-isotope effect also operates in the oxidative deamination of α-D-labelled cyclohexylamine. With incubation of the samples at 37 °C for 10 min, the ratios of apparent rate constants (k_H/k_D) were 2.3 and 1.6, respectively (means of two determinations). These values showed that these oxidizing agents might act similarly to the NADPH and oxygen system in microsomal deamination of cyclohexylamine, as they were found to be potent hydroxylating agents in hydroxylation reactions catalyzed by liver microsomes[16,17] and purified cytochrome P-450.[18,19]

Whether the oxidizing agents were NADPH and oxygen, $NaIO_4$ or $NaClO_2$, cyclohexanone and cyclohexanol produced from α-D-labelled cyclohexylamine gave unlabelled peaks in the MS. The fact that deuterium did not remain in the products is consistent with the suggestion that cyclohexanone is a precursor of cyclohexanol in the deamination.[9] Therefore, the C–H bond at the α-position of the primary amine was broken during the oxidative deamination and the deuterium-isotope effect showed that this event was involved in the rate-limiting process.

References

1) S. Kojima and H. Ichibagase, *Chem. Pharm. Bull.*, **14**, 971 (1966); *idem, ibid.*, **16**, 1851 (1968); *idem, ibid.*, **17**, 2620 (1969).
2) M. Asahina, T. Yamaha, K. Watanabe, and G. Sarrazin, *Chem. Pharm. Bull.*, **19**, 628 (1971); *idem, ibid.*, **20**, 102 (1972).
3) B. S. Drasar, A. G. Renwick, and R. T. Williams, *Biochem. J.*, **129**, 869 (1972).
4) A. G. Renwick and R. T. Williams, *Biochem. J.*, **129**, 857 (1972).
5) J. Axelrod, *J. Biol. Chem.*, **214**, 753 (1955).
6) B. B. Brodie, J. R. Gillette, and B. N. La Du, *Annu. Rev. Biochem.*, **27**, 427 (1958).
7) C. J. Parli, N. Wang, and R. E. McMahon, *Biochem. Biophys. Res. Commun.*, **43**, 1204 (1971).
8) H. Kurebayashi, A. Tanaka, and T. Yamaha, *Arch. Biochem. Biophys.*, **215**, 433 (1982).
9) H. Kurebayashi, A. Tanaka, and T. Yamaha, *Biochem. Pharmacol.*, **28**, 1719 (1979).
10) R. L. Foreman, F. P. Siegel, and R. G. Mrtek, *J. Pharm. Sci.*, **58**, 189 (1969).
11) Z. Pelah, M. A. Kielczewski, J. M. Wilson, M. Ohashi, H. Budzikiewicz, and C. Djerassi, *J. Am. Chem. Soc.*, **85**, 2470 (1963); H. J. Gordon, J. C. Martin, and H. McNab, *J. Chem. Soc., Perkin Trans. 1*, **1984**, 2129.
12) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
13) J.-A. Gustafsson, E. G. Hrycay, and L. Ernster, *Arch. Biochem. Biophys.*, **174**, 440 (1976).

14) H. Batiz-Hernandez and R. A. Bernheim, *Prog. Nucl. Magn. Reson. Spectrosc.*, **3**, 63 (1967).
15) J. H. Brewster, *J. Am. Chem. Soc.*, **76**, 6361 (1954).
16) E. G. Hrycay and P. J. O'Brien, *Arch. Biochem. Biophys.*, **153**, 480 (1972).
17) F. F. Kadlubar, K. C. Morton, and D. M. Ziegler, *Biochem. Biophys. Res. Commun.*, **54**, 1255 (1973).
18) E. G. Hrycay, J.-A. Gustafsson, M. Ingelman-Sundberg, and L. Ernster, *Biochem. Biophys. Res. Commun.*, **66**, 209 (1975).
19) G. D. Nordblem, R. E. White, and M. J. Coon, *Arch. Biochem. Biophys.*, **175**, 524 (1976).