PRIMARY AND β-SECONDARY DEUTERIUM ISOTOPE EFFECTS IN THE O-DEETHYLATION OF PHENACETIN

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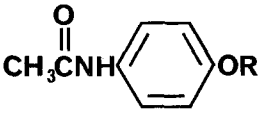
SUMMARY: Phenacetin (p-ethoxy acetanilide) specifically labelled with deuterium in the α-methylene or β-methyl groups of the p-ethoxy function was used to study the oxidative deethylation of phenacetin by rabbit liver microsomes. The slower deethylation of p-[1,1-^{2}H]ethoxy acetanilide compared with phenacetin [k_H/k^2_H = 1.61 ± 0.19] supports hemiacetal formation as the rate determining step in the in vitro conversion of phenacetin to p-hydroxy acetanilide. Essentially no β-secondary deuterium isotope effect (k_H/k^2_H = 1.03 ± 0.09) was found as the result of deuterium substitution in the methyl group of the p-ethoxy function. The Km values of phenacetin (56 ± 2 μM), p-[1,1-^{2}H]ethoxy acetanilide (56 ± 5 μM), and p-[2,2,2-^{2}H]ethoxy acetanilide (50 ± 2 μM) were all similar.

INTRODUCTION: The analgesic and antipyretic agent phenacetin (p-ethoxy acetanilide I) when administered to man (1), rat (2), cat (3), rabbit (2,4), guinea pig (2), and dog (3,4) is rapidly deethylated to form p-hydroxy acetanilide as its principal metabolite. This communication reports the results of a deuterium kinetic isotope study of this deethylation reaction.

Phenacetin analogs, p-[1,1-^{2}H]ethoxy acetanilide II and p-[2,2,2-^{2}H]ethoxy acetanilide III, were synthesized and their relative rates of deethylation by rabbit liver microsomes were compared with that of phenacetin.



I: R = OCH_2CH_3
II: R = $OC^2H_2CH_3$
III: R = $OCH_2C^2H_3$

A substantial decrease in rate (primary isotope effect, $k_H/k^2_H > 1.44$), when deuterium is substituted for hydrogen in the substance undergoing reaction, would suggest cleavage of the substituted bond as the rate-determining step in the overall reaction sequence (5-8). Changes in rate due to deuterium substitution in a position proximate to the bond undergoing rate determining breakage (secondary isotope effect) can give information concerning the transition state of the reaction (5,7-9).

MATERIALS AND METHODS:

A. Chemicals. Phenacetin and p-hydroxy acetanilide were purchased from Eastman Organic Chemicals and recrystallized from methanol-water. All deuterated reagents were purchased from Stohler Isotopes Inc. p-[1,1-^{2}H]Ethoxy acetanilide II and p-[2,2,2-^{2}H]ethoxy acetanilide III were prepared by refluxing potassium carbonate, p-hydroxy acetanilide and either $CH_3C^2H_2I$ or $C^2H_3CH_2I$ in acetone (10). The isotopic purity, as determined from the M+ ions in the electron impact mass spectra of I, II, III (70 ev, Varian CH-7, direct insertion probe) was 100% $^2H_2$ for II and 3.5% $^2H_2$, 95.8% $^2H_3$ for III.

N-[3,3,3-^{2}H]Acetyl-p-hydroxy aniline was prepared by the reaction of fully deuterated acetic anhydride with p-hydroxy aniline (3). Complete details of the synthesis of these compounds will be published (11).

NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co.

B. Tissue Preparation. Two New Zealand white rabbits, unexposed to any metabolic inducing agent and weighing 1,500 and 2,000 g respectively, were decapitated and exsanguinated. The rabbit livers were homogenized at 4°C in 3 volumes of 1.15% KCl Tris buffer using a Teflon Potter-Elvehjem homogenizer. The homogenate was centrifuged (Sorvall) at 0-4°C for twenty minutes at 9,000 x g.

The resulting supernatant was further centrifuged at 100,000 x g (Spinco L) at 0-4°C for sixty minutes. Using the homogenizer, the microsomal pellets were resuspended at 4°C in a volume of 1.15% KCl Tris buffer equivalent to the weight of the pellets. The pellets, obtained following additional centrifugation of this suspension at 0-4°C for sixty minutes at 100,000 x g, were resuspended in 0.05M phosphate buffer (pH 7.4). The microsomal protein concentration was determined (12) and the suspension was diluted with sufficient 1.15% KCl Tris buffer to give a final protein concentration of 1 mg/ml.

C. Incubation Procedure. The microsomal reaction was initiated

by the addition of the NADPH generating system (1 μmole NADP, 10 μmole glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase) to an incubation mixture containing 1.5 mg microsomal protein, 1.5 μmole EDTA, 4.5 μmole $MgCl_2$, and various concentrations of phenacetin in 0.05M phosphate buffer (pH 7.4) to give a final volume of 1.5 ml. The reaction was terminated after ten minutes by the addition of 2 ml ether.

D. p-Hydroxy Acetanilide. The p-hydroxy acetanilide formed was determined by a gas chromatography-mass spectrometry assay originally developed to measure phenacetin and p-hydroxy acetanilide in human plasma and urine (11). N-[3,3,3-^{2}H] Acetyl-p-hydroxy aniline (0.5 μg), ether (4 ml) and sodium chloride (500 mg) were added to the incubation mixture. The mixture was shaken, the ether layer was isolated, and the ether was removed. The residue was dissolved in methanol (0.5 ml), and treated for two hours with sodium sulfate-dried diazomethane (0.5 ml). The residue remaining after removal of the methanol and ether was reconstituted in 500 μl of ethyl acetate, and 2 μl were injected into a Finnigan Model 1015D gas chromatograph-mass spectrometer. Isobutane was used both as gc carrier gas and as chemical ionization reagent gas. The column, 1.2 m x 2 mm i.d., was packed with 3% OV-17 on Gas Chrom Q (100-120 mesh, Applied Science Laboratories). The mass spectrometer was set by the Finnigan Model 6000 Data system to monitor ions at m/e 166 (MH^+ ion of p-methoxy acetanilide) and m/e 169 (MH^+ ion of N-[2,2,2-^{2}H]acetyl-p-methoxy aniline) in the gc effluent. p-Hydroxy acetanilide concentrations were determined from a comparison of the relative peak heights of m/e 166 and m/e 169.

E. Calculations. The deuterium kinetic isotope effect ($k_H/k^2_H \pm$ S.D.) was calculated from the individual k_H/k^2_H values obtained from separate incubations with microsomes from two different rabbits at phenacetin concentrations of 12.5, 16.6, 25, 50 and 100 μM.

Apparent Km and Vmax values were determined from the amounts of p-hydroxy acetanilide generated from the two incubations at each phenacetin concentration. The data were fitted to $S/V = S/Vm + Km/Vm$ by a least squares analysis of S/V versus S (13).

RESULTS AND DISCUSSIONS: An initial determination was made of amount of p-hydroxy acetanilide evolved at 0, 3, 6, 9, 12 and 15 min following separate 50 μM incubations of I, II, III. A linear rate of deethylation for all three compounds was observed for 3 to 12 minutes after initiation of the incubation ($r>0.98$).

The apparent Km and Vmax values obtained for all three substrates are listed in Table I, and the mass spectral data from a typical analysis are shown in Figure 1. The kinetic deuterium

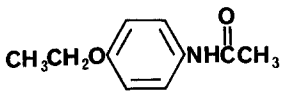
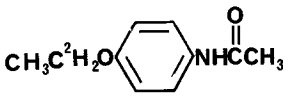
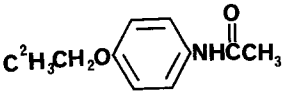
Compounds	K_m (μM)	Vmax (nmoles/mg of protein/min)
 Phenacetin	55.6 ± 2.1	7.55 ± 0.31
 p-[1,1-^{2}H]Ethoxy acetanilide	55.8 ± 5.2	4.29 ± 0.49
 p-[2,2,2-^{2}H]Ethoxy acetanilide	49.6 ± 2.2	7.33 ± 0.35

Table I. The apparent Km and Vmax values obtained from the 10 minute incubation of 12.5, 16.6, 25, 50 and 100 μM concentration of phenacetin, p-[1,1-^{2}H]ethoxy acetanilide, and p-[2,2,2-^{2}H]ethoxy acetanilide with rabbit liver microsomes.

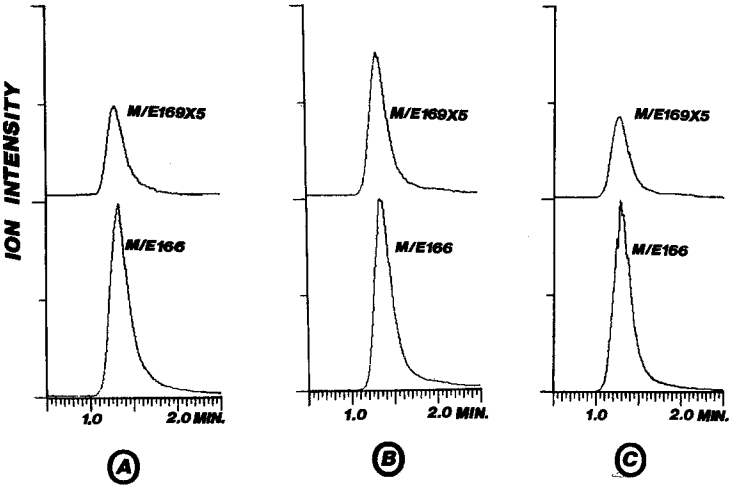
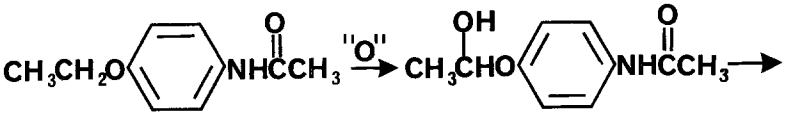
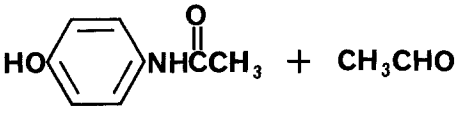


Figure 1. Specific ion monitoring tracing of p-hydroxy acetanilide generated during the incubation of 25 μM of phenacetin (A), p-[1,1-^{2}H]ethoxy acetanilide (B), and p-[2,2,2-^{2}H]ethoxy acetanilide (C). The ion at m/e 169 is the O-methyl derivative of the N-[3,3,3-^{2}H]acetyl-p-hydroxy aniline (500 ng) added as internal standard. The p-hydroxy acetanilide generated was 5.42 μg, 3.35 μg, and 5.82 μg for incubations A, B. and C, respectively.

isotope effects (k_H/k^2_H) calculated from the individual rates at each concentration were 1.61 ± 0.19 for II and 1.03 ± 0.09 for III. The value for II is consistent with a primary kinetic isotope effect at the methylene carbon. Essentially no β-secondary isotope effect is observed as the result of deuterium substitution at the adjacent methyl position.

A primary isotope effect at the methylene carbon supports the following generally accepted (14) deethylation mechanism:

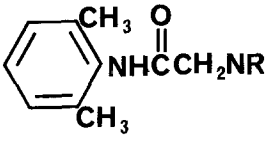




The hemiacetal intermediate generated is analogous to the carbinolamine believed to be formed as an intermediate in the metabolic N-dealkylation of secondary and tertiary amines (15-18). In addition, these data support the contention of Renson et al (19) that the demethylation of p-methoxy acetanilide proceeds by oxidation of the methoxy carbon and not via methoxy displacement.

Although of larger magnitude, similar primary isotope effects have been previously observed in studies by Mitoma et al (20) on the O-demethylation of o-[1,1,1-^{2}H]methoxy nitrobenzene (average k_H/k^2_H = 1.98, values ranged from 1.46 to 2.78) and by Al-Gailany et al (21) who studied the O-deethylation of p-[1,1-^{2}H]ethoxy nitrobenzene (k_H/k^2_H = 3).

The lack of a β-secondary deuterium isotope effect is somewhat surprising. In a recent deuterium isotope effect study of the N-deethylation of N,N-diethyl-glycinexylidide (lidocaine IV), similar isotope effects were observed for N,N-[1,1-^{2}H]diethyl-glycinexylidide V, $k_H/k^2_H = 1.49 \pm 0.11$, and N,N-[2,2,2-^{2}H] diethyl-glycinexylidide VI, $k_H/k^2_H = 1.52 \pm 0.10$ (22).



IV: R = $(CH_2CH_3)_2$
V: R = $(C^2H_2CH_3)_2$
VI: R = $(CH_2C^2H_3)_2$

The large β-secondary effect for VI was interpreted in terms of considerable sp^2 character in the transition state for the dealkylation reaction. The lack of a β-secondary deuterium isotope effect for the O-deethylation of phenacetin is consistent, based on those chemical reactions whose β-deuterium isotope effects have been studied, with a free radical (8) or Sn2 reaction mechanism (7). This difference between the O-deethylation of phenacetin and the N-deethylation of lidocaine could be due to different enzymes (23,24) and/or the difference between having an oxygen or a nitrogen adjacent to the carbon undergoing biotransformation (25).

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