Kinetic Isotope Effect in the Metabolic Demethylation of Temazepam

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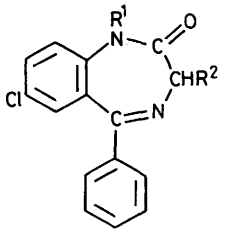
The kinetics of the metabolic demethylation of temazepam were examined. Oxidative cleavage of the 1-methyl C–H bonds is rate-determining in the 13 000 g supernatant of mouse liver homogenate, as shown by the primary deuterium isotope effect (3.6) for demethylation of 1-CD_3-labelled temazepam. With a reduced supply of NADPH, reduction of the cytochrome P-450–substrate complex becomes rate-limiting. Decomposition of the 1-*N*-hydroxymethyl intermediate is fast enough not to influence the rate of demethylation.

1-Dealkylation and 3-hydroxylation are the most important metabolic routes for several 1,4-benzodiazepine anxiolytics[1] related to diazepam (**1**). These processes frequently lead to (more) active metabolites, thus the rate of these metabolic steps may significantly influence the time dependence of pharmacological activity.[2] Isotopic substitution at the site of metabolic transformation may not only modify the pharmacological activity of the compound but kinetic isotope effects may also help to elucidate the mechanism of the metabolic process.[3,4] Deuterium substitution at position 3 of demethyldiazepam (**3**)



(**1**) $R^1 = CH_3$, $R^2 = H$
(**2**) $R^1 = CH_3$, $R^2 = OH$
(**3**) $R^1 = R^2 = H$

was demonstrated to reduce hepatic microsomal 3-hydroxylation and anticonvulsant activity in mice.[5] Isotopic substitution in position 1 has not yet been reported. We have studied the kinetic deuterium isotope effect for the 1-dealkylation of temazepam (**2**), an important anxiolytic and antiaggressive drug whose biotransformation is predominantly restricted to 1-demethylation to oxazepam.[1] Brain accumulation of oxazepam formed *in vivo* is mainly responsible for the anticonvulsant activity of temazepam in mice.[1] Similarly, 1-dealkylated active metabolites are responsible for prolonged pharmacological activity in several 1,4-benzodiazepines.[2,6]

Results and Discussion

Rate of Demethylation and its Isotope Effect.—The demethylation rates of 1-CH_3- and 1-CD_3-labelled [2-^{14}C]temazepam isotopomers were compared. The rate of hepatic microsomal demethylation was very slow and without an isotope effect. However, the demethylation activity of the 13 000 g, 15 min supernatant containing microsomes was much higher and displayed a deuterium isotope effect. After checking the linear dependence of the conversion into oxazepam on time and on the amount of the hepatic supernatant, the effect of the substrate concentration on the rate of demethylation was evaluated by means of an Eadie–Hofstee plot. Figure 1 demonstrates a characteristic pair of experiments. It shows that deuterium substitution decreased both the ordinate intercept and the slope of the line which characterize the maximal rate of demethylation and its apparent Michaelis constant, respectively.

Table 1. Kinetic parameters and their deuterium isotope effects for the demethylation of [2-^{14}C]temazepam in the 13 000 g supernatant of the mouse liver homogenate

	$V_{max.}\left(\frac{\text{pmol oxazepam}}{\text{mg protein} \times \text{min}}\right)$	K_M/μmol l^{-1}	$V_{max.}/K_M$
H	45.7 ± 13.7	10.6 ± 4.2	4.3 ± 2.0
D	12.7 ± 4.1	6.1 ± 1.5	2.1 ± 0.9
H/D[a]	3.6 ± 0.4	1.7 ± 0.3	2.0 ± 0.3

Data are averages (± s.d.) of three pairs of experiments each containing six concentrations in the range 1—50 μM. A fourth experiment was carried out for eight concentrations (1—170 μM) of 1-CD_3-temazepam to confirm the linearity of the Eadie–Hofstee plot.
[a] Isotope effects were determined independently in each pair of experiments.

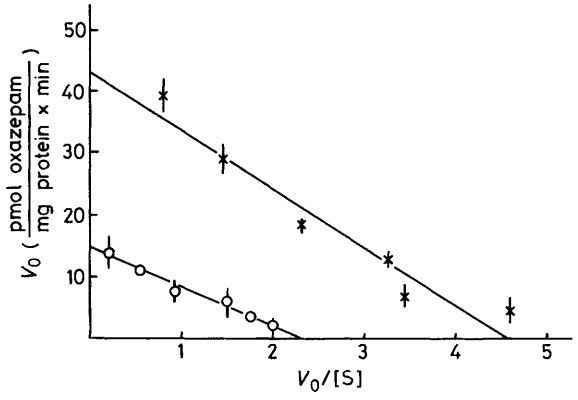


Figure 1. Eadie–Hofstee plot of a typical pair of demethylation experiments for 1-CH_3- (×) and 1-CD_3-temazepam (○) in the 13 000 g supernatant of the mouse liver homogenate. The initial rate of oxazepam formation is plotted against its ratio to the substrate concentration [S] (in μM). Data are averages (± s.d.) of three determinations. Linear regression resulted in $V_{max.}$ values of 43.1 and 14.9 (pmol oxazepam/mg protein × min) and K_M values of 9.5 and 6.5 μmol l^{-1} for 1-CH_3- and 1-CD_3-temazepam, respectively

Table 1 summarizes the kinetic parameters and their isotope effects. It is remarkable that day-to-day differences in the demethylation activity of the hepatic supernatant resulted in large standard deviations in the kinetic parameters. However,

the ratios for simultaneous experiments give more reproducible isotope effects.

A large value for the kinetic isotope effect is generally considered to be a primary effect[3] and as an indication that cleavage of the corresponding bond is rate limiting. Since the deuterium isotope effect is 3.6 for the maximal rate of demethylation, $V_{max.}$ (Table 1), we suppose that the rate-determining step of demethylation is, at least partly, cleavage of a 1-methyl C–H bond.

The linearity of the Hofstee plots between 10^{-6}—10^{-4}M-temazepam shows that a kinetically homogeneous enzyme population is responsible for demethylation. The isotope effect of the apparent Michaelis constants K_M is quite high (1.7). If K_M properly represented the dissociation constant of the substrate, K_D, its isotope effect, could hardly exceed 1.1, *i.e.* a secondary isotope effect reflecting changes only in the binding affinity of the substrate. The higher isotope effect suggests steady-state kinetics according to Briggs and Holdane, *i.e.* K_M is substantially influenced by the bond-breaking step.

The isotope effect was also analysed by mass spectrometry. A 1:1 mixture of 1-CH_3- and 1-CD_3-labelled temazepam was metabolized, conversion was stopped, and unmetabolized temazepam was isolated. The decreased H/D ratio allowed the ratio of the conversions of the temazepam isotopomers to be determined as 1.98 ± 0.09 (mean ± s.d. of three experiments). Since conversion of the isotopomers occurred simultaneously, this isotopic effect corresponds to the reactivity parameter, *i.e.* to $V_{max.}/K_M$. Its value agreed excellently with the isotopic effect of $V_{max.}/K_M$ (2.0 ± 0.3) determined from the formation of oxazepam (Table 1). This agreement also demonstrates that the disappearance of temazepam is equivalent to the formation of oxazepam.

The rate of demethylation is dependent on the supply of the reduced coenzyme NADPH (triphosphopyridine nucleotide). The decreased addition of NADP, in the presence of an NADPH-regenerating system, reduced not only the rate of demethylation (Figure 2), but also its isotope effect. Without any added NADP, the slow rate of demethylation corresponds to the remaining endogenous NADP of the supernatant. Consequently, at a nearly saturated concentration of the coenzyme, NADPH–cytochrome P-450 reductases work fast enough, so that the C–H bond-breaking step is rate determining. However, since the isotope effect on $V_{max.}$ in Table 1 (3.6) did not approach the theoretical limit for primary deuterium isotope effects, reduction of the cytochrome P-450–substrate complex might also contribute to the demethylation rate, even with a saturated supply of NADPH. But when the reducing capacity decreases below a certain level (Figure 2), the reduction of the cytochrome P-450–substrate complex becomes rate determining.[3]

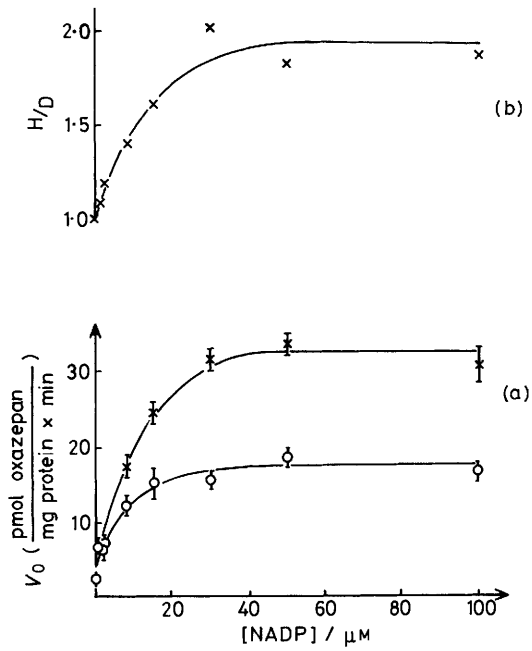


Figure 2. Effect of NADP added to the 13 000 g supernatant of the liver homogenate on the rate (a) and deuterium isotope effect (b) for demethylation of 1-CH_3- (×) and 1-CD_3-tempazepam (○). (a) Data are averages (± s.d.) of three determinations. Substrate concentration 60 μM. (b) The ratios of the average rate data. The curves were fitted manually to the data

Life-time of the Hydroxylated Intermediate.—Metabolic *N*-demethylation is considered to proceed through the enzymatic formation of an *N*-hydroxymethyl intermediate which spontaneously decomposes.[7]

Since data have been reported on the relative stability of the *N*-hydroxymethyl derivatives of some amides,[7–9] we also examined the stability of the metabolic intermediate of temazepam. For some amides with similar structural elements, glucuronide formation was reported to stabilize the intermediate.[10] However, with the application of a glucuronidating system, in concert with hydroxylation, we could not trap any *N*-hydroxymethyl intermediates conjugated with β-glucuronide (data not shown).

The kinetics of the equilibrium between the demethylation products oxazepam and formaldehyde was studied spectrophotometrically. Addition of the hydroxymethylene group to oxazepam results in a hypochromic shift of the absorption peak of the amide group. As with the kinetics of the addition of formaldehyde to uracils[8] or some amides, both the association (k_a) and dissociation (k_d) rate constants increased with increasing pH (Table 2) and could be experimentally determined up to pH 6.0. Both log k_a and k_d values linearly correlated with pH values. The slope of the log k_d–pH correlation did not significantly differ from unity which is characteristic for specific base catalysis [equation (1)].

$$\log k_d = -6.43\,(\pm 0.36) + 1.03\,(\pm 0.07)\,\text{pH} \quad r^2\ 0.99 \quad (1)$$

Linear extrapolation to pH 7.4 allows an estimate of the rate of decomposition of the metabolic intermediate (k_d 14.9 min^{-1}). Its half-life is so short ($t_{1/2}$ 2.8 s) that it cannot accumulate and its decomposition cannot be rate determining.

A common feature of the *N*-hydroxymethyl derivatives which were reported to be relatively stable[7–9] is that they have an amide group attached to a conjugated π-electron system as for 1,4-benzodiazepin-2-ones. But the quasi-boat conformation prevents the benzodiazepine ring system[11] from reaching coplanarity and full conjugation. Therefore the conjugated system might not properly contribute to the stabilization of the hydroxylated intermediate.

In conclusion, the deuterium isotope effect observed might be large enough to modify the pharmacological activity of

Table 2. Association (k_a) and dissociation (k_d) rate constants for the reaction of oxazepam with formaldehyde at 37 °C for different pH values

pH	k_a/min^{-1} l mol^{-1}	k_d/min^{-1}	log/k_d
3.8	6.0×10^{-3}	2.5×10^{-3}	−2.60
4.8	2.5×10^{-2}	4.4×10^{-2}	−1.36
5.75	1.9×10^{-1}	3.0×10^{-1}	−0.52
6.0	2.3×10^{-1}	4.7×10^{-1}	−0.33

1-CD_3-temazepam if the supply of NADPH is sufficient *in vivo*. It also suggests that the rate of demethylation of temazepam is determined by the slow cleavage of a C–H bond of the 1-methyl group. The *N*-hydroxymethyl intermediate subsequently formed decomposes rapidly and spontaneously. Furthermore, if the kinetic isotope effects also apply to the 1-demethylation of other 1,4-benzodiazepines, the tritium isotope effect in the retardation of the demethylation of [1-^{3}H]diazepam and [1-^{3}H]flunitrazepam can contribute to the suitability of these compounds for an investigation of the *in vivo* occupation of the benzodiazepine receptors.[12,13]

Experimental

Synthesis.—1-[*Me*-$^2H_3$,2-^{14}C]temazepam (4.25 mCi mmol^{-1}) was obtained from its acetic ester[14] prepared by standard methods[15,16] *via* diazepam using trideuteriomethyl iodide as alkylating agent.[17] N.m.r. and mass spectrometric investigations proved that the deuterium content of the 1-CD_3 group was > 99%.

Measurement of the Demethylation Rate and its Isotope Effect.—The livers of male albino mice (CFLP) were homogenized in 4 volumes of ice-cold 1.15% KCl solution with a Teflon glass homogenizer. The homogenate was centrifuged at 13 000 g for 15 min (Janetzky K 26 D-type centrifuge) and the supernatant was immediately used for incubations. The microsomal fraction was pelleted at 120 000 g for 60 min (Janetzky, VAC 601) and resuspended in 1.15% KCl solution.

The protein content was determined according to Lowry *et al.*[18] and calibrated with bovine serum albumin.

Microsomal demethylation of [2-^{14}C]temazepam and its 1-CD_3 isotopomer was carried out in a medium similar to that described by Marcucci *et al.*[5] It contained 100mM-phosphate buffer (pH 7.4), 100μM-NADP (Aldrich), 25mM-sodium D-glucose-6-phosphate (P-L Biochemical), 0.5 units ml^{-1} of glucose-6-phosphate dehydrogenase (Boehringer), 4mM-$MgCl_2$, and 46mM-KCl, typically containing 15 mg ml^{-1} microsomal protein. Experimental conditions for the investigation of the 13 000 g, 15 min hepatic supernatant were similar except for the inclusion of glucose-6-phosphate dehydrogenase. The suspensions were preincubated at 37 °C for 3 min and then 1—50μM-[2-^{14}C]temazepan was added in dimethyl sulphoxide, the final concentration of which did not exceed 0.1%. Standard incubations were carried out for 10 min, the samples were extracted three times with 7 volumes of cold (−20 °C) diethyl ether. Blank samples were immediately extracted after the addition of the substrate. Portions of the concentrated extracts were spotted on thin silica plates (Merck; HF_{254}) and chromatographed in benzene–diethyl ether–methanol (18:12:1). The spots corresponding to temazepam and oxazepam were scraped off and their radioactivity determined in a liquid scintillation spectrometer (Packard; type 2650).

For mass spectrometric examination of the deuterium isotope effect, 1-CH_3- and 1-CD_3-labelled temazepam isotopomers were used in equal amounts for the incubations. The conversion of [^{14}C]temazepam (Cv_H 10—20%) was determined after extraction and the shift of the isotopic ratio from 1 was measured at the molecular peaks of temazepam (*m/z* 300 and 303) in a Micromass 12F1A spectrometer. It is the ratio for unconverted temazepam $= (1 - Cv_H)/(1 - Cv_D)$. The unknown conversion Cv_D can be calculated from this relation. The isotopic effect equals Cv_H/Cv_D.

Spectrophotometric Analysis of the Kinetics of the N-*Hydroxymethyl Intermediate.*—The kinetics of the equilibrium reaction between 10μM-oxazepam and formaldehyde (0.2—3M) was studied in acetate (pH 3.8—5.6) and phosphate (pH 5.6—6.0) buffers. The absorbance change was measured at 320 nm and 37 °C on a Specord M 40 spectrophotometer (Carl Zeiss). A large molar excess of formaldehyde assured pseudo-first-order conditions. The change of the rate constants (k_{obs}) as a function of the formaldehyde concentration allowed the separation of the association (k_a) and dissociation (k_d) rate constants according to the equation: $k_{obs} = k_a[CH_2O] + k_d$.

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