α-Deuterium and Carbon-13 Isotope Effects for Methyl Transfer Catalyzed by Catechol O-Methyltransferase. S_N2-Like Transition State[1]

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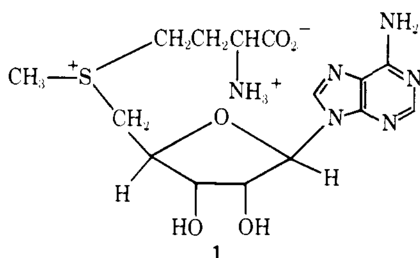
Abstract: The rate at near saturation of methylation of 3,4-dihydroxyacetophenone by S-adenosyl-L-methionine, catalyzed by rat-liver catechol O-methyltransferase at pH 7.58 and 37.00 ± 0.05 °C, is increased by deuteration of the methyl group (V_{CH_3}/V_{CD_3} = 0.83 ± 0.05) and decreased by introduction of ^{13}C to the methyl group (V_{12}/V_{13} = 1.09 ± 0.05). This shows the rate-determining step to be transfer of the methyl group, with an S_N2-like transition state in which the methyl is located "symmetrically" and "tightly" between leaving group and nucleophile.

The transfer of an intact methyl group to an acceptor from a donor, with enzyme catalysis, is a reaction of substantial biological significance.[2] The methyl donor is in general S-adenosylmethionine (1, AdoMet).[3] Enzymic transmethylation from 1 is a major route of inactivation of circulating norepi-



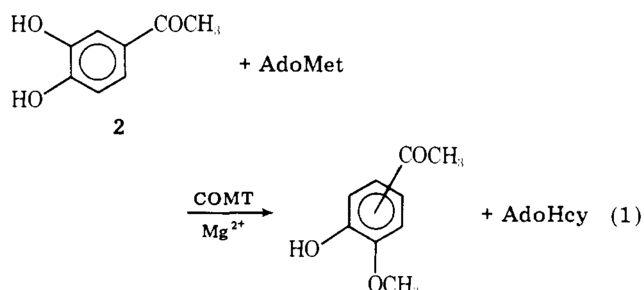
nephrine and a step in the hepatic metabolism of various exogenous compounds including drugs.[4] It is important in bacterial chemotaxis[5] and in various other biological phenomena, including brain function.[6] The origins of the catalytic power of transmethylation enzymes are unknown and would be illuminated by information about the catalytic transition states.[7]

Enzymic methyl transfer is an attractive target for transition-state characterization by means of kinetic isotope effects.[8] Deuteration at methyl should produce an α-deuterium isotope effect, about which a substantial body of experimental and theoretical information from model systems currently exists.[9-12] Carbon-13 substitution at methyl ought to yield a primary carbon isotope effect (for which again experimental and theoretical calibrations are available)[13-16] if the transfer of the methyl group occurs in the enzymic rate-determining step. On the other hand, only small effects would be anticipated if another event, such as substrate binding, product release, or a conformation change, were rate limiting.

The measurement of these two effects should thus permit some deductions about the identity of the enzymic rate-determining step, and, with luck, some conclusions about the structure of its transition state, in particular the bond lengths from the methyl group to the entering and leaving groups. This is because the carbon-13 effect should be maximal when these two bonds are of similar strength[17] ("symmetrical transition state") and smaller otherwise, while the α-deuterium effect should be normal (CH_3 reaction faster) when the two bonds are rather long (loose transition state) and inverse (CD_3 reaction faster) when the two bonds are rather short (tight transition state). Indeed, the combination of such measurements with model calculations of the Wolfsberg–Stern type,[10] which establish a semiquantitative relationship of transition-state structure to kinetic isotope effects, could lead to a substantial geometric definition of the structure of the enzymic transition state. This structure would then represent a reasonable goal for the synthesis of transition-state analogue (TSA) inhibitors,[18] and the effectiveness of an inhibitor of this structure would constitute a good test of the calculated relationship of model structure and isotope effects.

We have undertaken a program of transition-state structural elucidation, with rat-liver catechol O-methyltransferase (S-adenosyl-L-methionine:catechol O-methyltransferase, EC 2.1.1.6, COMT)[19,20] as our initial target enzyme. Recent studies[21] have shown this single-subunit, Mg^{2+}-requiring enzyme (mol wt 23 000) to exhibit only minor differences in substrate and inhibitor specificities from the COMTs of rat brain and rat heart and to be similar to them in molecular weight and immunological properties. Deductions from experiments on rat-liver COMT, about transition-state structure and its implications for the origins of methyltransferase catalytic power and for the design of TSA inhibitors, thus stand a reasonable chance of being applicable to these other enzymes as well.

We report here the measurement and qualitative interpretation of the α-deuterium and carbon-13 kinetic isotope effects for COMT-catalyzed methylation of 3,4-dihydroxyacetophenone (2) by AdoMet to yield S-adenosylhomocysteine (AdoHcy) and a mixture of meta- and para-methylated products (eq 1). Measurements of these isotope effects might



have been carried out by a competitive method, in which reactant or product isotopic ratios are followed as a function of time, either radiometrically (with tritium and carbon-14) or by use of a mass spectrometer. These techniques are capable of high precision but we have chosen not to use them. Instead, we measured both effects by direct determination of the isotopic rates. This was done because of the inherent potential of the direct method for determining isotope effects on all enzyme kinetic parameters (e.g., V_m and V_m/K_m of the Michaelis–Menten law) and of observing directly the effects for the rate-determining step at the steady state. The competitive techniques, being relative-rate measurements, are limited to isotope effects on V_m/K_m and thus to effects on steps up to and

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Table I.[a] α-Deuterium Isotope Effects for Methylation of 3,4-Dihydroxyacetophenone by AdoHcy-CH_3 and AdoHcy-CD_3 Catalyzed by Rat-Liver Catechol *O*-Methyltransferase at pH 7.58 and 37.00 ± 0.05 °C

V,[b,c] pmol s^{-1} (mg of protein)$^{-1}$	V_{CH_3}/V_{CD_3}
Day A	
396 (CD_3-I); 326 (CH_3-I); 407 (CD_3-I); 343 (CH_3-I); 399 (CD_3-I); 345 (CH_3-I); 397 (CD_3-I); 313 (CH_3-I); 392 (CD_3-I); 343 (CH_3-I)	0.82, 0.80, 0.84, 0.86, 0.87, 0.87, 0.79, 0.80, 0.88 (adjacent runs)
$\bar{V}_{CD_3}$ = 398, R = 15, AD = 4, N = 5; $\bar{V}_{CH_3}$ = 334, R = 32, AD = 12, N = 5	0.84 ± 0.03
Day B	
367 (CH_3-II); 282 (CH_3-II); 356 (CD_3-I); 362 (CD_3-I); 285 (CH_3-II); 367 (CD_3-I)	0.77, 0.80, 0.79, 0.78 (adjacent runs)
$\bar{V}_{CD_3}$ = 363, R = 11, AD = 4, N = 4; $\bar{V}_{CH_3}$ = 284, R = 3, AD = 2, N = 2	0.78 ± 0.01
Day C	
373 (CD_3-II); 343 (CH_3-I); 374 (CD_3-II); 310 (CH_3-I); 371 (CD_3-II); 301 (CH_3-I)	0.92, 0.92, 0.83, 0.84, 0.81 (adjacent runs)
$\bar{V}_{CD_3}$ = 373, R = 3, AD = 1, N = 3; $\bar{V}_{CH_3}$ = 318, R = 42, AD = 17, N = 3	0.85 ± 0.04
Mean Values	
all runs:	$\bar{V}_{CD_3}$ = 380, R = 51, AD = 15, N = 13
	$\bar{V}_{CH_3}$ = 326, R = 60, AD = 8, N = 10
	$\bar{V}_{CD_3}/\bar{V}_{CH_3}$ = 0.86, SD = 0.04
adjacent runs:	$(\overline{V_{CD_3}/V_{CH_3}})$ = 0.83, SD = 0.05

[a] Spectrophotometric velocities at 360 nm ($\Delta\epsilon$ = 2877); 0.125 M phosphate buffer, pH 7.58; $[Mg^{2+}] = 2 \times 10^{-3}$ M; [3,4-dihydroxyacetophenone] = 2×10^{-4} M; [AdoMet] = 10^{-3} M; [dithiothreitol] = 4.5×10^{-3} M. [b] The codes in parentheses identify the isotopic cofactor (AdoHcy-CH_3 or AdoHcy-CD_3); the Roman numerals specify independent biological preparations of the materials. [c] $\bar{V}$ means the arithmetic average velocity, R means the range of velocities (highest-lowest), AD means average deviation and SD standard deviation from the arithmetic average, and N means the number of determinations.

including the first irreversible step.[22] We believe the direct methods described here can eventually be developed to the level of a powerful and general tool of enzyme-mechanism study.

Besides a technique for reproducible measurement of enzymic rates, the direct method required the preparation of the labeled AdoMet derivatives (AdoHcy-CD_3 and AdoHcy-$^{13}CH_3$, AdoHcy meaning *S*-adenosylhomocysteine) in high isotopic enrichment and with the correct chirality at the sulfonium pole (*S* configuration).[23] This was accomplished in the present work by chemical synthesis of the labeled methionines (where no problem of chirality at sulfur arises) and biological adenosylation of these in cultures of the yeast *Saccharomyces cerevisiae*.[24,25] In order to control for accidental contamination of AdoMet samples by materials from the yeast, both labeled and unlabeled AdoMet samples were repeatedly prepared. The various samples were then used in a known but indiscriminate order in the experiments so as approximately to randomize effects of contamination. No such contamination could be detected nor were any effects attributable to it.

Results

α-Deuterium Isotope Effects. Table I shows velocities of methylation of **2** by the isotopic cofactors AdoHcy-CH_3 and AdoHcy-CD_3, catalyzed by COMT. The concentration of the isotopic AdoMet in each experiment was 1 mM (about 22 times the K_m value of 45 μM), while the level of **2** was kept at 0.2 mM or about 10 times its K_m value[21] of 20 μM. The velocities thus approach but do not equal the maximum velocity, and the isotope effects should correspondingly approach but not necessarily equal the V_m isotope effects. The experiments were conducted on three separate days (called A, B, and C in Table I). On each day, a series of experiments was conducted in which first a rate with AdoHcy-CD_3 was measured, then a rate with AdoHcy-CH_3, then again with AdoHcy-CD_3, and so on, with the labeled cofactors being alternated. Throughout the series, the same enzyme stock solution was used. The isotope effects can then be calculated in several ways.

First, to minimize the effect of uncontrolled variations in enzyme activity, an isotope effect can be calculated from each pair of adjacent isotopic rates. Enzyme activity should vary minimally from one run to the next one conducted. These isotopic ratios are shown at the right of Table I. The largest ratio V_{CH_3}/V_{CD_3} calculated for any pair of adjacent runs is 0.92 and the smallest such ratio is 0.77, suggesting that the isotope effect lies in this range. The mean of all isotope effects thus calculated is 0.83 ± 0.05 (standard deviation).

A second calculation, assuming enzyme activity to vary negligibly in the course of a single day, is one in which the daily means of V_{CH_3} and V_{CD_3} are computed. The isotope effects thus obtained are $\bar{V}_{CH_3}/\bar{V}_{CD_3}$ = 0.84 ± 0.03 (day A), 0.78 ± 0.01 (day B), and 0.85 ± 0.04 (day C).

Finally, one can simply average the V_{CH_3} for all three days and the V_{CD_3} for all three days. This yields an isotope effect of 0.86 ± 0.04.

All these calculations produce in effect the same result: V_{CH_3}/V_{CD_3} = 0.83 ± 0.05. It is this value that we will therefore adopt.

Carbon-13 Isotope Effects. An exactly similar study with AdoHcy-$^{12}CH_3$ and AdoHcy-$^{13}CH_3$ is reported in Table II. Here again all methods of calculation lead to the same finding: V_{12}/V_{13} = 1.09 ± 0.05.

Variation of Cofactor Concentration. Tables III and IV show how the velocities depend on cofactor concentration. The results are not of high quality because (a) the analytical method is not very good at low AdoMet concentrations and (b) the Michaelis–Menten law is not properly followed by these systems at low concentrations. The departures from Michaelis–Menten kinetics, which have been observed before[26] and are not well exemplified by the sparse data collected in the present study, are such that rates at low AdoMet levels are slower than predicted by a hyperbolic fit to rates at high AdoMet concentrations. This gives the V (AdoMet) curve a sigmoid appearance. It seems unlikely that this arises from cooperative effects with this low molecular weight, single-subunit enzyme. Another hypothesis is that the rapid-equilibrium assumption is incorrect for one or both of the substrates,[27] but the isotope effects reported here make this improbable (see below). This matter is currently under study but its relevance in the interpretation of these data is that a fit to the Michaelis–Menten law can be achieved only poorly and over a limited concentration range. Nevertheless, such fits were made in order: (1) to check for qualitative (or semiquantitative) consistency between the isotope effects obtained in the near-saturation region, and those obtained by extrapolation to complete saturation, and (2) to gain a rough idea as to whether the isotope effects on V_m and V_m/K_m were different.

The data of Tables III and IV were fitted to the Michaelis–Menten law by the nonlinear least-squares procedure of Cleland.[28] For our purposes here, the Michaelis–Menten law can be written as in eq 2, where V_m is the velocity at saturation of the enzyme-**2** complex by AdoMet and V_m/K_m is the pseudo-first-order rate constant for bimolecular reaction of AdoMet with the enzyme-**2** complex. V_m/e_0, e_0 being the total enzyme concentration, would then be the first-order rate constant for reaction from the enzyme-**2**-Adomet complex and $V_m/K_m e_0$ the second-order rate constant for reaction of

Table II.[a] Carbon-13 Isotope Effects for Methylation of 3,4-Dihydroxyacetophenone by AdoHcy-$^{12}CH_3$ and AdoHcy-$^{13}CH_3$ Catalyzed by Rat-Liver Catechol *O*-Methyltransferase at pH 7.58 and 37.00 ± 0.05 °C

V,[b,c] pmol s^{-1} (mg of protein)$^{-1}$	$V_{12}V_{13}$
Day A	
380 (12-III); 345 (13-I); 378 (12-III) 351 (13-I); 345 (13-I); 381 (12-I); 357 (13-I); 386 (12-I); 344 (13-I); 371 (12-III)	1.10, 1.09, 1.08, 1.11, 1.07, 1.08, 1.12, 1.08 (adjacent runs)
$\bar{V}_{12}$ = 379, R = 10, AD = 4, N = 5; $\bar{V}_{13}$ = 348, R = 13, AD = 4, N = 5	1.09 ± 0.02
Day B	
418 (13-II); 441 (12-IV); 412 (13-II); 440 (12-IV); 407 (13-II); 433 (12-IV); 384 (13-II)	1.06, 1.07, 1.06, 1.07, 1.08, 1.15, 1.13 (adjacent runs)
$\bar{V}_{12}$ = 438, R = 8, AD = 3, N = 4; $\bar{V}_{13}$ = 405, R = 34, AD = 11, N = 4	1.08 ± 0.03
Day C	
407 (12-V); 372 (13-III); 420 (12-V); 379 (13-III); 433 (12-V); 386 (13-III); 418 (12-IV); 386 (13-III); 422 (12-V); 396 (13-III)	1.09, 1.13, 1.11, 1.14, 1.12, 1.08, 1.08, 1.09, 1.06 (adjacent runs)
$\bar{V}_{12}$ = 420, R = 26, AD = 6, N = 5; $\bar{V}_{13}$ = 384, R = 24, AD = 7, N = 5	1.09 ± 0.02
Mean Values	
all runs:	$\bar{V}_{12}$ = 411, R = 70, AD = 22, N = 14
	$\bar{V}_{13}$ = 377, R = 74, AD = 21, N = 14
	$\bar{V}_{12}/\bar{V}_{13}$ = 1.09, SD = 0.08
adjacent runs:	$\overline{(V_{12}/V_{13})}$ = 1.09, SD = 0.05

[a] Conditions same as for Table I. [b] The codes in parentheses identify the isotopic cofactor (AdoHcy-$^{12}CH_3$ or AdoHcy-$^{13}CH_3$); the Roman numerals specify independent biological preparations of the materials. [c] See footnote *c* to Table I.

AdoMet with the enzyme–**2** complex. All these statements must be qualified by the fact that the enzyme is saturated with **2** under our conditions only to the extent of 90–95%.

$$[\text{AdoMet}]/V = \{(V_m/K_m)^{-1} + [\text{AdoMet}](V_m)^{-1}\} \quad (2)$$

The velocities in Table IV generate in this way eq 3 and 4. The last five entries (data for 48.2 μM and above) of Table IV generate eq 5 and 6. Equations 4 and 5, barring differences in enzyme activity, ought to be identical since they are both for the natural-abundance isotopic form of AdoMet. Indeed, the values of both V_m and V_m/K_m are the same in the two equations.

$$[\text{AdoHcy-CD}_3]/V_{CD_3} = \{(97 \pm 9 \times 10^{-4}\ (\text{g/L})^{-1}\ \text{s}^{-1})^{-1} + [\text{AdoHcy-CD}_3](419 \pm 8\ \text{pmol s}^{-1}\ \text{mg}^{-1})^{-1}\} \quad (3)$$

$$[\text{AdoHcy-CH}_3]/V_{CH_3} = \{(88 \pm 13 \times 10^{-4}\ (\text{g/L})^{-1}\ \text{s}^{-1})^{-1} + [\text{AdoHcy-CH}_3](359 \pm 12\ \text{pmol s}^{-1}\ \text{mg}^{-1})^{-1}\} \quad (4)$$

$$[\text{AdoHcy-}^{12}\text{CH}_3]/V_{12} = \{(71 \pm 16 \times 10^{-4}\ (\text{g/L})^{-1}\ \text{s}^{-1})^{-1} + [\text{AdoHcy-}^{12}\text{CH}_3](345 \pm 27\ \text{pmol s}^{-1}\ \text{mg}^{-1})^{-1}\} \quad (5)$$

$$[\text{AdoHcy-}^{13}\text{CH}_3]/V_{13} = \{(63 \pm 7 \times 10^{-4}\ (\text{g/L})^{-1}\ \text{s}^{-1})^{-1} + [\text{AdoHcy-}^{13}\text{CH}_3](304 \pm 28\ \text{pmol s}^{-1}\ \text{mg}^{-1})\} \quad (6)$$

Summary of Isotope Effects. The values of the α-deuterium and carbon-13 isotope effects obtained in various ways are summarized in Table V. The value of $V_m^{CH_3}/V_m^{CD_3}$ from the Michaelis–Menten fit is equal to the isotope effect from adjacent-run ratios at near saturation, and the error in both measurements is similar. The value of V_m^{12}/V_m^{13} is also equal within a large experimental error to the isotope effect from adjacent-run ratios. The error in V_m^{12}/V_m^{13} is quite large because data were usable only over a narrow range of AdoMet concentration (48.2–120 μM) somewhat above the K_m value (45 μM), and, as explained above, the Michaelis–Menten law describes the data only approximately in this range. Nevertheless, the mean value can be considered to confirm the direction and rough magnitude of the near-saturation isotope effect.

The value of $(V_m/K_m)^{CH_3}/(V_m/K_m)^{CD_3}$ also has a large error but seems essentially equal to $V_m^{CH_3}/V_m^{CD_3}$, suggesting (1) no α-deuterium isotope effect on binding of AdoMet, and (2) that the rate-determining steps at high and low concentrations of AdoMet are either identical or coincidentally generate the same α-deuterium isotope effect.

The largest error of all arises in $(V_m/K_m)^{12}/(V_m/K_m)$,[13] but here again the mean value of 1.13 is in agreement with that of V_m^{12}/V_m^{13} (1.14) and is within one standard deviation of

Table III.[a] Velocities of Methylation of 3,4-Dihydroxyacetophenone by Various Concentrations of AdoHcy-CH_3 and of AdoHcy-CD_3 Catalyzed by Rat-Liver Catechol *O*-Methyltransferase at pH 7.58 and 37.00 ± 0.05 °C

	V, pmol s^{-1} (mg of protein)$^{-1}$	
10^5[cofactor], M	AdoHcy-CH_3	AdoHcy-CD_3
3.86	174, 127	
7.73	250, 241, 237	267, 263, 255
11.6	262, 264	311, 298
15.5	297, 300, 297	346, 338, 338
38.6	317, 331	366, 370
103	355, 307	394, 411

[a] Except for cofactor concentration, all other conditions are the same as for Table I.

Table IV.[a] Velocities of Methylation of 3,4-Dihydroxyacetophenone by Various Concentrations of AdoHcy-$^{12}CH_3$ and of AdoHcy-$^{13}CH_3$ Catalyzed by Rat-Liver Catechol *O*-Methyltransferase at pH 7.58 and 37.00 ± 0.05 °C

	V, pmol s^{-1} (mg of protein)$^{-1}$	
10^5[cofactor], M	AdoHcy-$^{12}CH_3$	AdoHcy-$^{13}CH_3$
3.61	110, 112	91, 81
4.13	143, 137	111, 112
4.82	174, 155	145, 152
6.08	196, 185	158, 161
7.29	217, 222	199, 192
9.72	230, 235	214, 203
12.0	243, 237	212, 206

[a] Except for cofactor concentration, all other conditions are the same as for Table I.

Table V. Isotope Effects in the Methylation of 3,4-Dihydroxyacetophenone by *S*-Adenosylmethionine Catalyzed by Rat-Liver Catechol *O*-Methyltransferase at pH 7.58 and 37.00 ± 0.05 °C

isotope effect	magnitude (± SD)
V_{CH_3}/V_{CD_3}, near saturation, mean of adjacent-run ratios	0.83 ± 0.05
V_{12}/V_{13}, near saturation, mean of adjacent-run ratios	1.09 ± 0.04
$V_m^{CH_3}/V_m^{CD_3}$, least-squares fit to Michaelis–Menten equation	0.86 ± 0.03
$(V_m/K_m)^{CH_3}/(V_m/K_m)^{CD_3}$, least-squares fit to Michaelis–Menten equation	0.90 ± 0.10
V_m^{12}/V_m^{13}, least-squares fit to Michaelis–Menten equation	1.14 ± 0.14
$(V_m/K_m)^{12}/(V_m/K_m)^{13}$, least-squares fit to Michaelis–Menten equation	1.13 ± 0.27

Table VI. Estimated Maximal Velocity α-Deuterium and Carbon-13 Isotope Effects for Various Rate-Determining Steps in COMT-Catalyzed Methyl Transfer

rate-determining step	estimated V_{CH_3}/V_{CD_3}	estimated V_{12}/V_{13}	remarks
enzyme conformation change or other process preceding methyl transfer	~0.9–1.00	~1.00	no change from reactant vibrational force constants expected except for "solvent extraction"[32]
transfer of methyl group from AdoMet to **2**	~0.85 to ~1.25 (or 1.45[d])		inverse effects expected for "tight" transition states, normal effects for "loose" transition states; magnitudes from Seltzer and Zavitsas[a]
		~0.98 to ~1.08	large effects expected for "symmetric" transition states, small effects for "asymmetric" transition states, magnitudes from Willi[b] and below
enzyme conformation change, product release, or other process succeeding methyl transfer	~0.88 (to 1.02[d])	~0.98	equilibrium isotope effect for S-to-O transfer, estimated from Hartshorn–Shiner fractionation factors for CH_3Cl and CH_3F[c]

[a] The "most inverse" effect cited for a methyl-transfer reaction by Seltzer and Zavitsas[30] is 0.87 for reaction of water with methyl iodide in water at 70 °C, and the "most normal" effect is 1.21 for reaction of $(CL_3)_3S^+$ with phenoxide ion in ethanol at 76 °C. [b] The largest value cited for methyl transfer by Willi[15] is 1.0815 ± 0.0068 for reaction of methyl bromide with cyanide ion in water at 31 °C. [c] The fractionation factors of Hartshorn and Shiner,[31] calculated relative to ethane are (for CH_3Cl and CH_3F, respectively) 1.033 and 1.077 (D/H, per D) and 0.9885 and 1.0083 ($^{13}C/^{12}C$). [d] Maximum correction for electrostatic contribution.

the mean isotope effect at near saturation (1.09 ± 0.04). The provisional indication is thus that there is no carbon-13 isotope effect on AdoMet binding and that the rate-limiting transition states at high and low AdoMet concentration are the same.

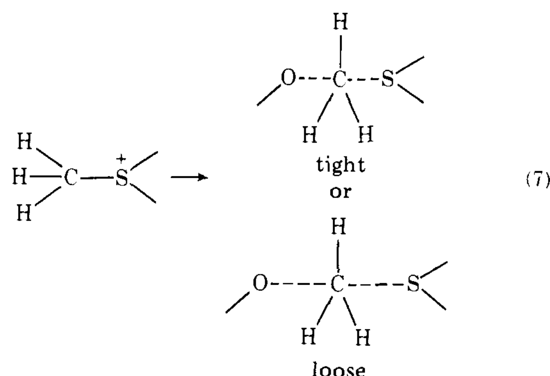
Discussion

In the enzymic catalysis of methyl transfer from AdoMet to **2**, three possible kinds of events[29] might limit the rate: (1) binding of either substrate to the enzyme, or conformation changes in the enzyme–substrate complexes preceding the methyl-transfer step; (2) transfer of the methyl group (the so-called "chemical step"); (3) conformation changes in complexes succeeding the methyl transfer, or release of a product from one of these complexes.

If we limit our attention to maximal-velocity isotope effects, then substrate binding is excluded from (1). Table VI gives estimates of the α-deuterium and carbon-13 effects to be expected for each of the three possible rate-limiting processes.

If conformation changes preceding transfer of the methyl group are rate determining, then no sizable effect of carbon isotopic substitution on the rate should be seen and either small or modest effects of deuterium substitution. Three reasonable sources for the small possible effects would be: (a) changes in force constant upon removal of AdoMet from an aqueous environment on the surface of the enzyme into a possibly nonaqueous region within the enzyme active site; (b) changes in force constant from distortion at the sulfonium center, perhaps induced by the enzyme in aid of catalysis; (c) changes in force constant from compression of the methyl group of AdoMet against the nucleophilic center of **2**, in preparation for transfer. The first kind of effect has been modeled by Tanaka and Thornton,[32] who found that solvent extraction from water to the hydrophobic medium μ-Bondapak C_{18} is favored for deuterated materials by factors of 0.2–0.8% per deuterium. Such a process should then lead to inverse effects of less than 3% (for CH_3 vs. CD_3). The carbon-13 effect would surely be considerably smaller. Distortion at the neighboring sulfur can be modeled from the fractionation-factor calculations of Hartshorn and Shiner. For conversion of one methyl group in ethane to a cyano group (a much larger distortion than is imaginable here), they calculated isotope effects of 1.009 (D/H per D) and 0.994 (13/12). Here again then, an effect of less than about 3% for CH_3 vs. CD_3 is expected, as is a much smaller carbon-13 effect. Compression of the AdoMet methyl group against a nucleophilic center in **2**, in a complex preceding methyl transfer, might produce larger deuterium effects than the solvent-extraction or sulfonium-distortion phenomena. Carter and Melander[33] have reviewed isotope effects arising from molecular compression. The closest examples to the process of interest here seem to be the racemization of 9,10-dihydro-4,5-dimethylphenanthrene (for which Mislow et al.[34] found k_{6H}/k_{6D} = 0.85 at 23 °C) and the solvolysis of 8-methylnaphthoyl chloride (for which Karabatsos et al.[35] found k_{3H}/k_{3D} = 1.03 at 25 °C). In the former case, compression is increased in the transition state: the corresponding CH_3/CD_3 effect is $(0.85)^{1/2}$ = 0.92. In the latter case, compression is relieved in the transition state, so that the corresponding effect for an equal *increase* of compression would be $(1.03)^{-1}$ = 0.97. Thus, compression of the substrates together might give rise to inverse α-deuterium isotope effects of 3–8% for CH_3 vs. CD_3. The carbon-13 effects arising from the same force constant changes would be negligible (the same force-constant changes that produce a deuterium isotope effect of 8% will produce a carbon-13 effect of ~0.9997).

If transfer of the methyl group is itself occurring in the transition state for the enzyme reaction, then quite important force-constant alterations will have been made at the isotopic centers and large isotope effects are anticipated. A thorough analysis of α-deuterium effects in S_N2 reactions is given in an accompanying paper.[36] In the majority of cases previously studied, the α-deuterium effect arises from changes in bending force constant[9] and its magnitude and direction should depend on the "tight-loose" character of the transition state[37] (eq 7).

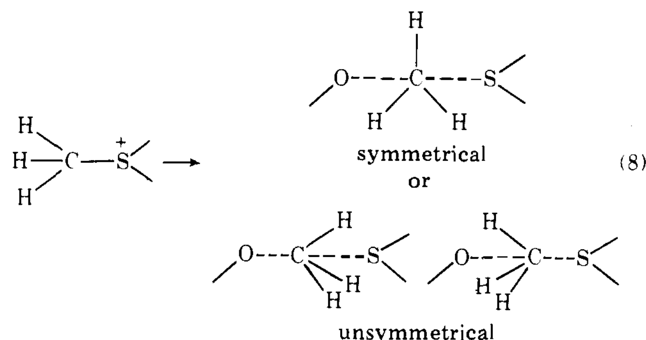


(7)

In a "tight" transition state, the methyl out-of-plane bending force constant will exceed the corresponding force constant in the tetrahedral reactant methyl group and an inverse isotope effect will be observed. In a loose transition state, the out-

of-plane force constant will be smaller than in the reactant and a normal effect will result. A compilation of such effects by Seltzer and Zavitsas[30] shows a range of k_{3H}/k_{3D} of about 0.85–1.25, although either more inverse or more normal effects might be seen if transition states were found that had greater degrees of tightness or looseness. If other factors than steric effects on the out-of-plane bending force constant (such as electrostatic charges) contribute to the magnitude of the α-deuterium isotope effect, then the relationship between the magnitude of the isotope effect and loose–tight character in the transition state may be altered. Indeed, the positive charge on sulfur in the reactant AdoMet may perturb the methyl-group force constants. If the nucleophilic oxygen of **2** is negatively charged in the transition state (from full or partial removal of its proton in the course of nucleophilic attack), then an overall decrease in positive charge near the methyl group will be occurring. An indication of the expected effect can be derived from the observations of Northcott and Robertson[38] and of van der Linde and Robertson,[39] who found that ionization of methylammonium, dimethylammonium, and trimethylammonium ions generated K_H/K_D of 1:042–1.048 per deuterium for isotopic substitution in the methyl groups. A kinetic effect in the corresponding direction was observed by Kaplan and Thornton,[40] who found that reaction in nitrobenzene at 51 °C with methyl tosylate of $C_6H_5N(CL_3)_2$, L = H or D, produced k_D/k_H of 1.021 per deuterium. These effects are likely to be strongly dependent on details of molecular structure and solvation, since ionization of carboxylic acids with deuterium substitution at the α position, two bonds from the site of charge alteration, frequently gives rise to no isotope effect or very small ones.[41] Thus, an effect of 1.05 for deuterium or about 1.16 for CH_3 vs. CD_3 seems likely to constitute a maximum effect for complete loss of adjacent charge. The true correction should be substantially smaller because of the longer distance of the carbon–sulfur bond than the carbon–nitrogen bond in the examples, the greater polarizability of the sulfur than the nitrogen, possible partial positive charge on the nucleophilic oxygen in the transition state, and the fact that the sulfur charge will probably still be somewhat positive in the transition state. The effect of such a correction will be to "slide the scale" of the Seltzer–Zavitsas compilation ($k_{3H}/k_{3D} \simeq 0.85$–1.25) toward larger (more normal) isotope effects by a factor between 1.00 and 1.16, thus generating a range of 0.85–1.45.

The carbon isotope effect for the "chemical step" should be a typical group-transfer isotope effect (analogous to a hydrogen isotope effect in proton transfer), being large for "symmetrical" and small for "unsymmetrical" transition states (eq 8).[17] If



the entire isotope effect came from only the isotope zero-point energy difference of a C–S stretching vibration of 700 cm^{-1} frequency, a maximum k_{12}/k_{13} of about 1.05 would be expected, tending toward 1.00 for the unsymmetrical reactant-like transition-state structure and toward the equilibrium isotope effect $k_{12}/k_{13} = 0.98$ (see below) for the product-like structure. Willi, in a recent review,[15] cites values as large as 1.071 for displacements on methyl chloride (where the reactant stretching zero-point energy difference should actually be *smaller*). Buddenbaum and Shiner[10] have calculated effects as large as 1.075 for the reaction of iodide ion with methyl chloride (experimental value 1.072). Yamataka and Ando[42] have found a carbon-14 effect of 1.160 (corresponding to $k_{12}/k_{13} = 1.084$) for reaction of benzyl benzenesulfonate with *N,N*-dimethyltoluidine in acetone. These results suggest that a maximum value, expected for the symmetrical transition state, might be taken as around 1.08.

Finally, if a conformation change of some complex formed after transfer of the methyl group has been accomplished or the release of a product molecule from an enzymic complex determines the rate, only quite small equilibrium isotope effects should be seen. The carbon–oxygen bond which carries the product methyl group is shorter and stronger than the carbon–sulfur bond of the reactant AdoMet. This should increase both the bending force constants of the C–H bonds and the stretching force constant to the carbon from the oxygen (vs. the sulfur), leading to inverse α-deuterium and inverse carbon-13 equilibrium isotope effects. These can be modeled from the fractionation factors of Hartshorn and Shiner[31] on the assumption that fluorine is a rough simulacrum for oxygen and chlorine for sulfur. Their deuterium fractionation factors for CH_3Cl, simulating AdoMet, and CH_3F, simulating the product, are 1.003 and 1.077, respectively (D/H, per D, relative to ethane). The equilibrium α-deuterium effect for CH_3 vs. CD_3 should thus be around $(1.033/1.077)^3 = 0.88$. As above, a maximum electrostatic contribution of 1.16 would move this value to 1.02. A more exact estimate of appropriate fractionation factors[36] suggests a range of possible values of 0.84–0.97. The carbon-13 fractionation factors are 0.9885 and 1.0083 ($^{13}C/^{12}C$, relative to ethane), suggesting an equilibrium carbon-13 effect of $(0.9885/1.0083) = 0.98$.

Conclusions

The measured maximal velocity isotope effects are $V_{CH_3}/V_{CD_3} = 0.83 \pm 0.05$ and $V_{12}/V_{13} = 1.09 \pm 0.05$. These are consistent only with rate-limiting transfer of the methyl group. The structure of the transition state appears to be at least as tight as any observed for an S_N2 reaction previously (since the α-deuterium effect is more strongly inverse). The structure is also symmetrical, the methyl group having similar bonding to the departing sulfur and incoming oxygen, as shown by the near-maximum value of the carbon-13 effect. The methyl is thus centrally located and roughly planar. These conclusions will be converted to quantitative structural form by means of model calculations now in progress.

Experimental Section

Materials. Water was deionized (Barnstead Ultrapure mixed-bed resin), distilled in glass, and, when necessary, deaerated by bubbling with nitrogen gas from which traces of oxygen had been removed using a series of gas scrubbers containing chromous perchlorate solution and several amalgamated zinc rods. Magnesium chloride hexahydrate (Mallinckrodt Chemical Works) was dried at 100 °C for 11–12 h before use. Dithiothreitol (Sigma Chemical Co.), sodium dihydrogen phosphate monohydrate (Analytical Reagent, Mallinckrodt Chemical Works), and sodium monohydrogen phosphate (secondary anhydrous powder, Fisher Certified Reagent of Fisher Scientific Co.) were used as supplied. 3,4-Dihydroxyacetophenone was obtained from Dr. Borchardt's group, who had prepared it by the Fries rearrangement of pyrocatechol diacetate with aluminum chloride in chlorobenzene solvent.

Labeled *S*-Adenosylmethionines. All samples of AdoMet (AdoHcy-$^{12}CH_3$, AdoHcy-$^{12}CD_3$, and AdoHcy-$^{13}CH_3$) used in the isotope-effect measurements were prepared and purified by the method of Hegazi et al.[25] *S*-Benzyl-L-homocysteine was allowed to react with the appropriate methyl iodide ($^{12}CH_3I$, $^{12}CD_3I$, or $^{13}CH_3I$, Merck, Sharp and Dohme Isotopes, Kirkland, Quebec) in sodium–liquid ammonia to form the corresponding L-methionine. This was then fed,

along with other nutrients, to a culture of commercial activated dry yeast (Fleischmann-Standard Brands, Inc.). Extraction and purification by ion-exchange chromatography produced the labeled AdoMet chloride. The isotopic purity of the deuterium-labeled AdoMet was 90 ± 5% (NMR), from L-methionine that contained 95% label. Mass spectrometry showed the ^{13}C-labeled L-methionine to contain at least 90% label and it was thus assumed that the corresponding AdoMet was at least 85% labeled.

Catechol *O*-Methyltransferase. The enzyme was obtained by the methods of Nikodejevic et al.[43] and of Borchardt et al.[20] The livers of male Sprague-Dawley rats (180-200 g) were homogenized in 10 mM phosphate buffer (pH 7.0, 0.25 M sucrose) and the homogenate was subjected to differential centrifugation, ammonium sulfate fractionation, Sephadex G-25 chromatography, and negative calcium phosphate absorption to produce material with a 50-fold increased COMT activity. This was then chromatographed on an affinity column of 3,4-dimethoxy-5-hydroxyphenylethylamine-agarose conjugate to yield 500-fold purified enzyme. This was employed in the isotope effect determinations.

Kinetics Procedure. Initiation. Two stock solutions, identical except that one contained the first isotopic modification of AdoMet (say AdoHcy-$^{12}CH_3$) and the other the second modification (AdoHcy-$^{12}CD_3$ or AdoHcy-$^{13}CH_3$), consisting of enough material for 4-5 runs each were prepared and stored on ice. Each stock solution contained all materials for a kinetics experiment except for **2**. To initiate an experiment, 0.900 mL of stock solution was brought to 37.00 °C in a 1-mL cuvette in the spectrophotometer cell compartment. Then 0.100 mL of a solution of **2** was added to begin the reaction and data were acquired as described below. Experiments with the two isotopic modifications of AdoMet were conducted in alternation.

Kinetics Procedure. Data Acquisition. The data consisted of the time dependence of the absorbance of the solution at 360 nm. The voltage output of the Cary-16 photomultiplier, consisting of a 60-Hz pulse train with alternating pulses representing the light intensity transmitted by the sample cell and the reference cell, was conveyed to a synchronizer constructed by Dr. Wesley White of the University of Kansas Electronics Design Laboratory. This device employed the electrical line frequency (which drives the chopper in the spectrophotometer) to identify the pulses and to initiate digitization of the voltage signal by an Analogic AN 5800 analog-to-digital converter. The digital voltage from the sample-cell pulse was then stored in one memory location of a Hewlett-Packard 2100A computer; 15 such measurements from the top of the pulse were loaded into the same location. Then 15 measurements of the dark current following the pulse were loaded with opposite algebraic sign, still into the same location. Thus, at the end of a half-cycle, the location contained a number proportional to the height of the pulse, averaged 15-fold. Then the same method was used to generate in a second location a 15-fold averaged value of the height of the reference-cell pulse occurring in the second half-cycle. The data-acquisition program ASDQ0 (automated spectrophotometric data acquisition mark zero) then caused the ratio of these two voltages to be calculated and its logarithm taken to yield a value of the absorbance. This value and the time at the center of the cycle were then stored by the computer. After the elapse of 1 s, the absorbances were averaged and this average value was assigned to the central time of the 1-s interval. Kinetic runs were conducted over a 1000-s period, so that at the end of the computer contained a table of 1000 absorbance values, each averaged from 60 absorbances, and 1000 corresponding time values.

Kinetics Procedures. Data Reduction. The first 299 points of each kinetic run were discarded to eliminate effects from initiation of the experiment, and the last 399 points were discarded to eliminate effects of product inhibition. Points 300 to 600 were fitted to the function $A = A_0 + V_A t$ by a linear least-squares program. The velocity in absorbance units s^{-1}, V_A, typically showed a within-run standard deviation of around 0.2 to 0.5%. Although the isotope effects can be calculated directly from the V_A values, the velocities in V_c in M s^{-1} were calculated for comparison with other experiments. Since AdoMet and AdoHcy do not absorb at 360 nm, V_c is given by this formula:

$$V_c = V_A \Big/ \left\{\epsilon_s - \frac{\epsilon_m r + \epsilon_p}{r + 1}\right\}$$

where the ϵ's are extinction coefficients at 360 nm of **2** (ϵ_s) and of the meta-methylated product (ϵ_m) and the para-methylated product (ϵ_p), and r is the ratio of meta methylation to para methylation. The extinction coefficients were determined directly under the experimental conditions ($\epsilon_s = 6750$, $\epsilon_m = 6850$, $\epsilon_p = 297$, all in M^{-1} cm^{-1}). The value of r is known[44] to be 1.2. Thus, $V_c = V_A/2879$. This value was used for conversion of V_A values measured for *all isotopic species* to the values of V_c shown in the Results section. The isotope effects are therefore independent of the ϵ and r measurements. It is not expected that either the ϵ values or the r values will be sensitive to isotopic composition. Since ϵ for the meta-methylated product (6850) is scarcely different from that for DHA (6750)—in other words, ϵ is almost invariant to meta methylation—it should also be invariant to the isotopic composition of the methyl group. On the other hand, ϵ for the para-methylated product (297) is quite different from that of **2**. Fortunately, even in the unlikely event of a 10% isotopic shift (say to 267), the correction factor will change only from 2879 to 2892 (0.5%). Thus, no large error in the isotope effects will come from this source. Furthermore, the calculated isotope effect is mathematically insensitive to the value chosen for r. Trial calculations show that a shift in the para/meta ratio of 20% produces only a 0.4% change in the isotope effect.

References and Notes

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(29) The implicit assumption is that methyl transfer between AdoMet and DHA occurs with both bound to COMT, and without intermediacy of a methylated enzyme. While the matter remains to be settled definitively for COMT, the demonstration of stereochemical inversion at methyl on enzymic methylation of indolepyruvate by AdoMet in preparations of *Streptomyces griseus* (L. Mascaro, Jr., R. Hörhammer, S. Eisenstein, L. K. Sellers, K. Mascaro, and H. G. Floss, *J. Am. Chem. Soc.*, **99**, 273 (1977)) suggests the assumption may be correct. The main conclusions—rate limitation by methyl transfer in a tight S_N2 transition state—would be unchanged if a methyl-enzyme intermediate were involved, although some of the numerical magnitudes used in the argument would be inappropriate.

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Stereochemistry of Intermediates in Thiamin Catalysis. 3. Crystal Structure of DL-2-(α-Hydroxybenzyl)oxythiamin Chloride Hydrochloride Trihydrate, an Inhibitor Adduct

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Abstract: The C(2) adduct of the oxythiamin inhibitor assumes a conformation that is nearly identical with that of the same derivative of thiamin, even though the unsubstituted oxythiamin was observed with a unique conformation not seen in any thiamin structure. The parameters of the oxopyrimidine ring agree well with those in oxythiamin. The crystal structure was determined using diffractometer data obtained by the θ:2θ scan technique with Mo radiation from a crystal having $P2_1/c$ space group symmetry and unit cell parameters a = 13.956 (5), b = 7.407 (3), c = 25.102 (8) Å, and β = 115.48 (2)°. The structure was solved by direct methods and refined by full-matrix least squares to an R = 0.098 for all 4520 reflections and R = 0.049 for the 2653 observed reflections.

2-(α-Hydroxybenzyl)oxythiamin, HBOT, is a C(2) adduct of the thiamin antagonist, oxythiamin. Although oxythiamin is an inhibitor of thiamin catalysis, it can react with substrate to form an intermediate in the holoenzyme system containing oxythiamin instead of thiamin as a cofactor, so that the inhibitory function appears at the step of product release.[2] Many of the intermediates of thiamin catalysis (C(2) adducts) are sufficiently stable under mildly acidic conditions to be isolated. From the crystal-structure analyses of 2-(α-hydroxyethyl)thiamin, HET,[3] and 2-(α-hydroxybenzyl)thiamin, HBT,[4] which are such intermediates, it has been shown that when there is a substituent on C(2) the molecular conformation with respect to the C(3,5′) bridge carbon atom is substantially different from that which characterizes the free thiamin molecule. Besides the change in the preferred conformation with respect to the bridge carbon, the adduct compounds display an apparent conformational stability that is imparted through an intramolecular S···O interaction with the O(2α1) oxygen and through the intramolecular ring stacking interaction between the pyrimidine and the phenyl rings in HBT. These structural features are thought to have important mechanistic properties in thiamin catalysis. The determination of the structural parameters for HBOT is of special interest, since it is known that oxythiamin assumes a conformation that differs from that of free thiamin.[5] From NMR studies, Gallo[6] finds a ring stacking interaction in HBOT similar to that which occurs in HBT, but suggests that the interaction in HBOT is weaker.

Experimental Section

Colorless tabular crystals from a sample of DL-2-(α-hydroxybenzyl)oxythiamin chloride hydrochloride trihydrate, which was kindly given to us by Dr. H. Sable at Case Western Reserve University, were grown from aqueous acetonitrile (1:6) by the addition of an equal volume of acetone at ~5 °C. The crystals are monoclinic with space group symmetry $P2_1/c$ as determined from Weissenberg photographs which indicate systematically absent reflections for $0k0$ when k is odd and $h0l$ when l is odd. The crystal used in the analysis was mounted with b approximately parallel to the φ axis and was thinly coated with epoxy as a precaution against changes in the crystal during the analysis. The cell parameters were determined from a least-squares fit of the setting angles for 12 centered reflections[7] (setting angles for each reflection were obtained from four separate measurements taken at $\pm 2\theta$, χ and $\pm 2\theta$, $180 + \chi$). The crystal data are summarized in Table I.

The intensity data were measured on a Picker FACS-I diffractometer with graphite-monochromated Mo Kα radiation ($2\theta_M$ = 11.97°) using a θ:2θ scan technique at a scan rate of 2°/min over a scan range of at least 2.4° to a sin θ/λ limit of 0.650 Å^{-1} (the scan range was adequate to accommodate the split diffraction profile which gave a maximum separation of 0.5° in ω along the C* axis). Background counts were accumulated for 20 s at the end of the scan range. Three standard reflections, which were monitored after every 50 reflections, showed gradual fluctuations throughout the analysis of less than ±3%. The data were corrected for this slow variation. Among the 6374 measured reflections, 4520 independent reflections, whose net intensities were above zero, were used for subsequent structure determination. Among 4520 reflections, 1867 reflections were treated as unobserved by the criterion of $F \leq 6\sigma(F)$.[8] Neither absorption nor extinction corrections were applied.

The structure amplitudes were converted to Es[9] and the structure was solved using MULTAN.[10] Coordinates of all 28 nonhydrogen atoms excluding the oxygen atoms of the three water molecules were determined from the E map using 306 E values ($E \geq 1.76$) with their calculated phases as coefficients. Conventional application of full-matrix least-squares refinement[9] (minimizing $\Sigma\omega(|F_o| - k|F_c|)^2$, where k is a single scale factor and $\omega = 1/\sigma^2(|F_o|)$), and difference Fourier syntheses located the remaining three oxygen atoms and all of the hydrogen atoms. In the last three cycles of refinement, parameters were separated into two blocks which consisted of (1) the thiazolium ring, the C(2), C(4), and C(5) substituents, and the two

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