β-Deuterium and β-Tritium Isotope Effects on the Distribution Coefficient of Carbonyl Compounds for Transfer from Water to Cyclohexane or Chlorocyclohexane[1]

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Abstract: β-Deuterium and β-tritium isotope effects on phase transfer equilibria between water and cyclohexane or chlorocyclohexane were determined for acetone and *p*-nitroacetanilide spectrophotometrically and for acetone and ethyl acetate by a dual-label radioactive isotopic technique. Results of all measurements yield 1–2% normal (protium favored in the organic phase) β secondary isotope effects per deuterium, which implies tighter binding of the β-CH bonds in the aqueous phase than in the organic phase. Since the β-isotope effects on distribution coefficients of carbonyl compounds in the above systems exceed isotopic discrimination reported in the literature for phase transfer involving compounds with other functional groups, it appears that the effects reported here are specific for carbonyl compounds and originate from smaller hyperconjugative release of β-CH electrons into the carbonyl center in water than in organic solvents. Such a factor might contribute to isotope effects observed with enzymes.

β-Deuterium secondary isotope effects (DIE) are commonly used in the elucidation of transition state (TS) structures for acyl transfer mechanisms.[2-8] The maximal isotope effect k_{3H}/k_{3D} predicted for sp^2 to sp^3 conversion at carbonyl carbon from equilibrium-addition models is 0.87 ± 0.04,[3,4] i.e., 13% per CD_3. Many experimental kinetic observations have been reported in the range between 2% and 10% per CD_3, with some reactions of aryl acetates and oxy anions in protic solvents[4-7] and some enzymic acyl transfers[8] showing only very small inverse effects. Other enzymic reactions have been found to exhibit *normal* secondary isotope effects (e.g., $k_H/k_D \sim 1.00$–1.04 for L-asparaginase action on asparagine and related substrates;[9,10] $k_H/k_D = 1.02$–1.27 for dipeptidyl peptidase IV with alanylalanyl-*p*-nitroanilide[11]). These could arise in whole or in part from "phase transfer" out of an aqueous environment into a less aqueous, protein region. Thus, there is a potential complication, particularly in the enzymic cases, in comparing to a simple, aqueous equilibrium as a standard, or model reaction, because in formation of the activated complex, or binding into the active site of an enzyme, there may be a change in environment. Thus, we perceived a need to examine the dependence of isotopic discrimination on solvent attachment and removal around carbonyl reaction centers.

A portrayal of how nucleophilic solvation in water of the incipient carbonyl center might diminish hyperconjugation between the β-CH bonds and the π orbital of the carbonyl (and thus produce a secondary isotope effect) is offered in Scheme I. The upper part (a) shows the expected loss of hyperconjugation[12-14] and attendant increase of the β-CH force constants upon nucleophilic interaction at carbonyl. The lower scheme (b) symbolizes interference of nucleophilic solvent with hyperconjugation and how removal of such interference would restore hyperconjugation. This latter process would then be accompanied by a decrease in force constants of the β-CH bonds, i.e., by a normal β-DIE. Phase transfer equilibria of labeled carbonyl compounds, distributed between water and a nonnucleophilic medium such as cyclohexane or chlorocyclohexane, seemed to be good systems in which to estimate the possible magnitude of such contributions to β-DIEs of some acyl transfer reactions.

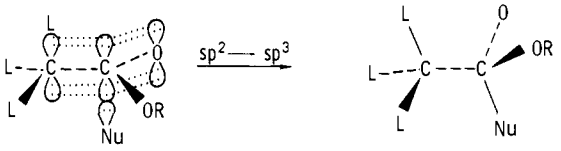
We report herein isotopic discrimination in the phase transfer equilibria for acetone and its β-deuterated or tritiated isomer, for ethyl acetate and its β-tritiated isomer, and for *p*-nitroacetanilide and its β-deuterated isomer.

Results

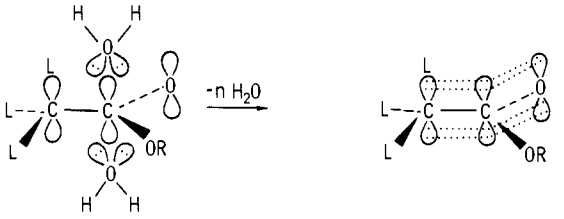
Spectrophotometric Measurements. Distribution coefficients (K_{xL}) were determined spectrophotometrically at 25 °C for two

Scheme I

a. NUCLEOPHILIC INTERACTION AT CARBONYL hyperconjugation decreases, force constant increases.



b. NUCLEOPHILIC SOLVATION OF CARBONYL; interferes with hyperconjugation. Removal of solvent, force constant decreases.



compounds, acetone-L_6 and *p*-nitroacetanilide-L_3, L = H, D (eq 1, where A = absorbance, ϵ = molar absorptivity; o = organic

$$K_{xL} = \frac{(A_o/\epsilon_o)_{xL}}{(A_w/\epsilon_w)_{xL}} = \left(\frac{A_o}{A_w}\right)_{xL}\left(\frac{\epsilon_w}{\epsilon_o}\right)_{xL} \quad (1)$$

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Table I. Distribution Coefficients and Their β-Deuterium Isotope Effects for the Transfer of Acetone-L_6 from Cyclohexane to Water, at 25 °C

$(A_o/A_w)_6H$[a] ([CH_3COCH_3], M)	$(A_o/A_w)_6D$[a,b] ([CD_3COCD_3], M)	$(A_o/A_w)_6D$[a,c] ([CD_3COCD_3], M)	$(A_o/A_w)_6H/(A_o/A_w)_6D$ sample 1[b]	$(A_o/A_w)_6H/(A_o/A_w)_6D$ sample 2[c]
0.1293 ± 0.0027 (0.0302)	0.1206 ± 0.0035 (0.0224)	0.1153 ± 0.0014 (0.0225)	1.072 ± 0.038	1.121 ± 0.027
0.1221 ± 0.026 (0.0326)	0.1104 ± 0.0012 (0.0355)	0.1114 ± 0.0033 (0.0301)	1.106 ± 0.027	1.096 ± 0.041
0.1226 ± 0.0020 (0.0489)	0.1123 ± 0.0017 (0.0533)	0.1144 ± 0.0024 (0.0451)	1.092 ± 0.024	1.072 ± 0.029
0.1190 ± 0.0009 (0.0495)	01.096 ± 0.0043 (0.0465)	0.1103 ± 0.0012 (0.0458)	1.086 ± 0.043	1.079 ± 0.014
0.1268 ± 0.0079 (0.066)	0.1151 ± 0.0063 (0.0620)	0.1157 ± 0.0178 (0.0610)	1.102 ± 0.091	1.096 ± 0.182
0.1184 ± 0.0007 (0.0991)	0.1091 ± 0.0004 (0.0931)	0.1087 ± 0.0018 (0.0917)	1.085 ± 0.008	1.089 ± 0.019
		Averages		
0.1230 ± 0.0043	0.1129 ± 0.0044	0.1126 ± 0.0029	1.090 ± 0.012	1.092 ± 0.017

K_{6H}	K_{6D}		K_{6H}/K_{6D}
0.1455 ± 0.0051	0.1375 ± 0.0054	0.1371 ± 0.0036	1.060 ± 0.013

[a] Averages of three determinations. Initial concentration in cyclohexane is indicated in parentheses below each $(A_o/A_w)_{xL}$ value. [b] From Diaprep. [c] From SIC.

Table II. Distribution Coefficients and Their β-Deuterium Isotope Effects for the Phase Transfer of *p*-Nitroacetanilide Acetyl-L_3 between Chlorocyclohexane and Water, at 25 °C

$(A_o/A_w)_3H$[a] (10^5[$CH_3CONHC_6H_4NO_2$], M)	$(A_o/A_w)_3D$[a] (10^5[$CD_3CONHC_6H_4NO_2$], M)	$\frac{(A_o/A_w)_3H}{(A_o/A_w)_3D}$[b]
2.142 (3.44)	1.958 (3.79)	1.094
2.124 (4.91)	1.950 (5.46)	1.089
2.099 (6.62)	1.974 (7.34)	1.063
2.069 (7.93)	1.953 (9.02)	1.059
av 2.109 ± 0.032	av 1.958 ± 0.010	av 1.076 ± 0.018

K_{3H}	K_{3H}	K_{3H}/K_{3D}
1.767 ± 0.028	1.685 ± 0.012	1.049 ± 0.018

[a] Determined from one experiment; estimate error in absorbance readings is 0.1%. The initial concentration of anilide is indicated in parentheses next to each $(A_o/A_w)_{3L}$ value. [b] Estimated errors are ±0.1%.

phase (cyclohexane or chlorocyclohexane), w = water phase, and *x* indicates the number of isotopic labels present in each molecule). The ratio of isotopic partition coefficients is given by eq 2. Ratios

$$\frac{K_{xH}}{K_{xD}} = \frac{(A_o/A_w)_{xH}}{(A_o/A_w)_{xD}} \frac{(\epsilon_w/\epsilon_o)_{xH}}{(\epsilon_w/\epsilon_o)_{xD}} \quad (2)$$

of the absorbance reading in the organic phase to that in the water phase at increasing concentrations of the isotopic carbonyl compounds are presented in Tables I and II for acetone and *p*-nitroacetanilide, respectively. To establish confidence in the technique, acetone was studied extensively by using two different samples of acetone-d_6, and the ratios at each concentration were calculated from three parallel determinations. To reduce the possibility of error from possible impurities in the commercial samples of acetone-d_6, a successive-partitioning approach was used. Solutions of acetone and of acetone-d_6 in water were extracted in succession with three aliquots of cyclohexane. For each extraction, an isotope effect was calculated from eq 2 and these were found to be equal within the experimental error as the extractions proceeded. The last column of each table lists the ratios of the ratios of absorbance readings from the two phases (first factor in eq 2). These quantities, in principle, should give the isotope effects on the distribution coefficients, since the molar absorptivity is not anticipated to depend on isotopic substitution. This is essentially true, although the molar absorptivity coefficients of acetone from both sources and that of *p*-nitroacetanilide show a slight phase-dependent β-DIE (details in the Experimental Section). The last lines in Tables I and II show the mean distribution coefficients with their standard deviations, calculated from the mean values of absorbance ratios and molar absorptivities, and the corresponding β-DIEs.

Radiometric Measurements. The results of Tables I and II were confirmed by measurement of tritium isotope effects by a dual-label procedure. Two precautions had to be observed in using this technique: (1) To avoid inhomogenieties in the scintillation vials during counting, a toluene-base scintillation cocktail (see Experimental Section) was employed and was mixed with 20% methanol before use for solubilization of small, aqueous samples; (2) To eliminate complications due to radioactive trace impurities present in a commercial sample of acetone-*1,3*-$^{14}C_2$, successive extractions were performed from an aqueous solution of the mixture of ^3H- and ^{14}C-labeled isomers. Extensive purification of acetone-*t*, synthesized from ^3H_2O had previously included extraction of a cyclohexane solution of acetone-*t* with water to remove traces of ^3H_2O. This was repeated until stable partitioning ratios were obtained. However, surprisingly large, apparently normal isotope effects in the initial double-label experiments seemed to suggest that trace impurities with ^{14}C label might still be present, partitioning with a preference for the cyclohexane phase. If a radioactive impurity were indeed being distributed with a different distribution coefficient from that of acetone, the apparent isotope effect should change as the ratio of impurity to acetone changes. Accordingly, we observed that if the first cyclohexane extract was discarded, much lower and essentially constant values of the isotope effects were observed. This was true for two samples of different origin in three successive distributions, even when counts became quite low and the precision began to decrease. Table III demonstrates decomposition-per-minute counts and the corresponding $^3H/^{14}C$ ratios in some typical dual-label experiments. Table IV gives a summary of tritium and β-deuterium isotope effects obtained in this study. Equivalent

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Table III. Representative Data, Decomposition per Minute Ratios (DPM_T:DPM_C) of Double-Label Experiments, at 25 °C[a]

$^3HCH_2COCH_3$:$^{14}CH_3COCH_3$ sample 1, second distribution
water phase: 136223:110926 = 1.228; 138218:114008 = 1.212; 131999:112809 = 1.170; 132905:109506 = 1.214; 127718:108388 = 1.178; 123823:103842 = 1.192
mean: 1.199 ± 0.023
cyclohexane phase: 92396:80468 = 1.148; 90267:79400 = 1.137; 88466:76385 = 1.158; 88451:77431 = 1.142; 84180:74140 = 1.135
mean: 1.144 ± 0.009
K_H/K_T = 1.048 ± 0.022
$^3HCH_2COCH_3$:$^{14}CH_3COCH_3$ sample 1, fourth distribution
water phase: 100593:83670 = 1.202; 92696:76246 = 1.216; 94610:77992 = 1.213; 96620:79718 = 1.212; 94213:79369 = 1.187
mean: 1.206 ± 0.012
cyclohexane phase: 65054:55709 = 1.168; 59681:50837 = 1.174; 64009:54608 = 1.172; 56485:48555 = 1.163; 57956:50108 = 1.157; 55037:47483 = 1.159
mean: 1.166 ± 0.007
K_H/K_T = 1.035 ± 0.012
$^3HCH_2COOCH_2CH_3$:$CH_3COO^{14}CH_2CH_3$
water phase: 12660:11724 = 1.080; 13599:12656 = 1.075; 13940:12772 = 1.091; 13343:12959 = 1.0297; 13071:11738 = 1.113
mean: 1.078 ± 0.031
cyclohexane phase: 10385:9818 = 1.058; 14153:13747 = 1.0295; 13554:13069 = 1.037; 14536:13906 = 1.045; 13778:13191 = 1.045
mean: 1.043 ± 0.031

[a] More details of the experiments and calculations are given in the Experimental Section.

Table IV. β-Deuterium (K_{xH}/K_{xD}) and β-Tritium (K_H/K_T) Isotope Effects on the Distribution Coefficients[a] of Carbonyl Compounds in Cyclohexane or Chlorocyclohexane–Water Systems

compd	K_{xH}/K_{xD}[b]	x[c]	$(K_{xH}/K_{xD})^{1/x} = K_H/K_D$
acetone	1.060 ± 0.013	6	1.010 ± 0.002
p-nitroacetanilide (*)	1.049 ± 0.018	3	1.016 ± 0.006

compd	K_H/K_T[d]	$(K_H/K_T)^{1/1.443} = K_H/K_D$
acetone	1.035 ± 0.012	1.024 ± 0.010
ethyl acetate	1.034 ± 0.031	1.023 ± 0.021

[a] $K_{xL} = [C]_{organic}/[C]_{H_2O}$. [b] Spectroscopic measurements; correction is applied for the isotopic discrimination on the molar absorptivity as given in Table V. [c] Number of protium or deuterium. [d] Dual-labeled radioactive isotopic measurements.

deuterium isotope effects were generated from tritium effects through the Swain–Schaad relationship[15]

$$K_H/K_T = (K_H/K_D)^{1.443} \quad (3)$$

for purposes of comparison with β-DIEs per deuterium. These were calculated from

$$K_H/K_D = (K_{xH}/K_{xD})^{1/x} \quad (4)$$

Discussion

β-Isotope effects per deuterium on the partition coefficients for the three types of carbonyl compounds of this study are between 1.0 ± 0.2% and 2.4 ± 1.0%,[16] favoring protium in the organic phase. This agrees with a model in which aqueous solvation impedes hyperconjugation of the β-CH electrons into the carbonyl group. Loss of this solvation upon extraction into the organic phase would then increase the hyperconjugation, leading to weaker β-CH bonding and a normal isotope effect.

Phase transfer isotope effects, as a general phenomenon, are well-known. Jancso and Van Hook[17] have discussed the extensive work on vapor pressure isotope effects and isotopic separation of compounds by gas chromatographic techniques. Results of such experiments have been analyzed within the framework of the statistical-mechanical theory of isotope effects in condensed systems, as advanced by Bigeleisen.[18] Vapor pressure isotope effects on hydrocarbons and functionalized organic compounds[19] are generally small (0.5% per D) and inverse (protium favoring water). The direction of isotopic separation of such compounds in adsorption systems seems to depend heavily on the details of the solute–solvent interactions involved. Tanaka and Thornton[20] found 0.2–0.5% per deuterium isotope effects for high-pressure liquid chromatography of various organic compounds on μ-Bondpak-C_{18} columns. The protium isomer was preferred in the hydrophobic phase. Significantly larger normal β-DIEs have been reported[21] lately, however, on the adsorption equilibrium of aqueous acetic acid on charcoal. The ratios of the Freundlich adsorption parameters were K_{3H}/K_{3D} = 1.3 ± 0.2 and $(1/n)_{3H}/(1/n)_{3D}$ = 1.2 ± 0.1. It seems likely that these large effects and those of 1–2% per deuterium that we report here originate from some specific molecular interaction, such as hyperconjugation, as distinct from the factors that give rise to the effects in vapor pressure and chromatographic experiments. Not only hyperconjugation but various other models might be suggested for the specific interactions involved.

A conceivably important application of this experimental observation is to kinetic β-DIEs in enzymic acyl transfer reactions, such as those mentioned in the introduction. Such acyl transfer reactions involve binding of the carbonyl substrate into the active site of the enzyme, a process similar in some senses to extraction of the substrate from water into an organic solvent. This process alone might give rise to a normal isotope effect of 1–2% per deuterium, according to our findings. Such considerations will have to be taken into account when isotope effects of this type are interpreted.

Experimental Section

Materials. Water used in phase transfer experiments was distilled from a copper-bottom still and passed through a Barnstead mixed-bed ion-exchange column. Reagent grade cyclohexane and chlorocyclohexane were purified by washing 3 times with each of the following: concentrated H_2SO_4 (half equal volume), followed by distilled water, then by saturated Na_2CO_3, and finally by distilled water to neutrality. The solvents thus purified were dried again over $CaCl_2$ for a day, refluxed for several hours with activated charcoal, and finally fractionally distilled. Reagent grade acetone was distilled before use. Acetone-d_6 was purchased from Stohler Isotope Chemicals (99.5% deuterated, lot 3062) and from Diaprep (99+ atom %; lot 072947). Acetone-*1,3*-$^{14}C_2$ 250 μCi (50 mCi/mmol) and ^3H_2O (50 Ci/mL) were purchased from ICN. Toluene-*t* and toluene-^{14}C standards were acquired from New England Nuclear. Scintillation grade fluors and suppliers were 1,4-bis[2-(5-phenyloxazolyl)]benzene from Packard Instrument Co., Inc., 2,5-diphenyloxazole from RPI, and toluene and scintillation cocktail 3a70B from RPI. Absolute methanol used in scintillation cocktails was from Fisher Scientific Co.

Synthesis. *p*-$NO_2C_6H_4XHCOCH_3$ and *p*-$NO_2C_6H_4NHCOCD_3$ were prepared by the reaction of *p*-nitroaniline with CH_3COCl or CD_3COCl (Merck; 99% deuterated) in either pyridine at room temperature or benzene containing dimethylaniline at 10 °C. Each amide was recrystallized 3 times from CH_3OH, yielding yellow crystals melting at 214 °C (lit. mp 215–216 °C).

Acetone-*1,3*-t_2 was prepared by mixing 25 μL of ^3H_2O (1 Ci/mL) 0.01 g of K_2CO_3, and 5 mL of purified acetone and refluxing the mixture for 6 h at 81 °C. Acetone-*1,3*-t_2 was distilled off (4 mL) and dried over

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Table V. Molar Absorptivity Constants of Isotopic Carbonyl Compounds in H_2O and Cyclohexane or Chlorocyclohexane, at 25 °C

compound	solvent	λ, nm	ε, abs M^{-1} cm^{-1} [a] H	ε, abs M^{-1} cm^{-1} [a] D
acetone	H_2O	265.5	17.94 ± 0.029	17.13 ± 0.027
acetone	cyclohexane	279	15.16 ± 0.074	14.07 ± 0.041
p-nitroacetanilide	H_2O	313	10324 ± 20	9950 ± 32
p-nitroacetanilide	chlorocyclohexane	313	12323 ± 48	11563 ± 37

compound	$(\epsilon_w/\epsilon_o)_{xH}$	$(\epsilon_w/\epsilon_o)_{xD}$	$(\epsilon_w/\epsilon_o)_{xH}/(\epsilon_w/\epsilon_o)_{xD}$	x [b]
acetone	1.1834 ± 0.0060	1.2175 ± 0.0040	0.9720 ± 0.0059	6
p-nitroacetanilide	0.8378 ± 0.0036	0.8605 ± 0.0039	0.9736 ± 0.0061	3

[a] Error limits of absorptivity constants are standard deviations of linear least-squares slopes of Beer–Lambert law plots. [b] Number of protium or deuterium.

anhydrous $CaCl_2$ for a day and then kept over P_2O_5 for 2 days. The acetone-*1,3*-t_2 was distilled off again and the drying cycle was repeated. A sample of the final pure acetone-*1,3*-t_2 was tested for trace contamination of ^3H_2O by distribution between 1 mL of cyclohexane and successive 1-mL aliquots of water. The distribution coefficient thus determined by scintillation counting of aliquots drawn from both layers was 0.126 ± 0.003 and remained constant during three successive distributions.

Radioactivity-labeled ethyl acetates were synthesized as described earlier.[22]

Analytical Procedures. Spectroscopic measurements were performed in a Cary 118 spectrophotometer. Phase transfer equilibria for acetone-L_6 were established by combining 3 mL of cyclohexane solutions of acetone in concentrations indicated in Table I and 3 mL of water in well-sealed, Teflon-lined screwcap test tubes that were immersed into a 25.00 ± 0.01 °C water bath for 2–3 days and were shaken gently several times. Solutions (1 mL) from each phase were withdrawn with syringes, placed into 1-cm UV cells, and read against the appropriate solvent at the λ_{max} given in Table V. Maximal absorbance values in the aqueous phase indicated complete equilibrium. To test interference from possible impurities present in the commercial acetone-d_6 samples, a series of successive distributions between 3-mL cyclohexane solutions of ~0.05 M of each acetone sample and 3 mL of water were carried out. The cyclohexane phase was discarded after each distribution and replaced by fresh cyclohexane. One-third of the aqueous layer was also replaced by fresh water once. Successive ratios of the absorbance readings were as follows: $(A_o/A_w)_{6H}$ = 0.1226 ± 0.0020, 0.1221 ± 0.0027, 0.1214 ± 0.0010; $(A_o/A_H)_{6D}$ (Diaprep) = 0.1123 ± 0.0017, 0.1104 ± 0.0012, 0.1131 ± 0.0027; $(A_o/A_w)_{6D}$ (SIC) = 0.1144 ± 0.0024, 0.1114 ± 0.0033, 0.1114 ± 0.0028. Biphasic systems containing the isotopic *p*-nitroacetonilides were set up by placing 2.00 mL of H_2O and 1.00 mL of chlorocyclohexane in a set of five UV cells. Into the organic phase of the five cells were injected aliquots of 9 × 10^{-4} M anilide in chlorocyclohexane to give total concentrations as indicated in Table II. The five cells were kept at 25.00 ± 0.05 °C and gently agitated from time to time for 2 days until absorbance in the H_2O phase remained stable. These absorbance readings on the aqueous phase were determined directly in each cell against a reference cell that contained the same biphasic system, but without anilide solute. Approximately 0.8 mL from the top of the chlorocyclohexane phase of each cell was then drawn into 1.7 mL capacity UV cells. Absorbance in these cells was thus recorded vs. chlorocyclohexane in the reference cell.

Molar absorptivity constants are reported in Table V. They were measured from serial 10-μL injections of concentrated stock solutions of the substrate (CH_3CN solutions of the anilides) into 3.00 mL of each solvent with subsequent absorbance readings taken at λ_{max}. As a check on the results, dilutions of a stock solution into volumetric flasks for acetone-L_6 were sampled; linear least-squares treatment of absorbance readings from these reproduced the molar absorptivities within experimental error.

For dual-labeled radioactive isotopic measurements, 1 mL of 10–12% stock solutions of acetone and ethyl acetate, respectively, were prepared in cyclohexane so that ^3H and ^{14}C activities were about equal (~10^5 DPM). In the case of ethyl acetate, this solution was equilibrated with an equal amount of water (pH adjusted to 4 with HCl) that was discarded and the extraction repeated twice to remove all traces of possible labeled acid contaminants. The 1-mL cyclohexane solutions were equilibrated with 1 mL of H_2O (which was acidified to pH 4 for ethyl acetate to retard any hydrolysis) in well-sealed vials in a shaker–constant-temperature bath at 25 °C for 48 h. Samples were then centrifuged and kept at 25 °C for a new more hours. The organic layer was sampled first by drawing 10 μL of ethyl acetate sample and 50 μL of acetone sample with Hamilton syringes and injecting them into 3 mL of the appropriate cocktail. The bottom of the aqueous layers were sampled without delay by drawing 30-μL and 10-μL aliquots from the ethyl acetate and acetone samples, respectively. Unreasonably large isotope effects of 1.156 and 1.158 from two different samples of acetone raised the suspicion of ^{14}C trace contamination present in the commercial acetone-*1,3*-$^{14}C_2$ that partitions preferentially to cyclohexane. The cyclohexane layer, therefore, was totally removed after the experiment from each acetone sample, and the aqueous solutions of dual-labeled acetone each were equilibriated with 1 mL of fresh cyclohexane and then sampled again. Four such successive distributions were carried out. After the second distribution the tritium isotope effects were 1.048 ± 0.021 and 1.010 ± 0.030, and these values did not change within experimental error through the third and fourth distribution, although radioactivity in the samples fell to a ~10^4 DPM in the process. Results of the fourth distribution were 1.035 ± 0.010 and 1.016 ± 0.030.

Standards for counting efficiency were made by dilution of toluene-*t* and -^{14}C with the respective cocktail to yield activities in the range of those of the samples. Portions (3 mL) of these standards were pipetted into scintillation vials and mixed with 10–50 μL of cyclohexane or 10–30 μL of H_2O. Counts per minute (CPM) and the external standard-channel ratio (ESCR) both remained constant, indicative of no quenching. Thus, counting efficiencies were calculated from averages of CPMs of the toluene-*t* and -^{14}C standards, respectively, with each solvent in each channel divided by the decomposition per minute (DPM). The corresponding efficiencies with the two solvents as quenchers were identical within experimental error (±1.0%).

Scintillation cocktail 3a70B was used for ethyl acetate samples; for acetone samples a solution of 6 g of 2,5-diphenyloxazole and 0.8 g of *p*-bis[2-(5-phenyloxazolyl)]benzene/L of toluene was mixed with 20% absolute methanol before use. Samples were counted in all glass vials in the tritium channel and then in the ^{14}C channel of a three-channel Beckman LS-3155 liquid scintillation counter with automatic quench calibration. Samples of later experiments were counted without ESCR when this was found to increase precision.

The following equations were used to calculate DPMs for ^3H- and ^{14}C-labeled compounds (subscripts T and C, respectively) where subscripts 1 and 2 refer to the tritium and ^{14}C channels, respectively, E_{ij} refers to the efficiency of the isomer indicated by the letter subscript, in the channel indicated by the number subscript, and B_j is the background activity.

$$DPM_T = \frac{(CPM_2 - B_2)E_{C_1} - (CPM_1 - B_1)E_{C_2}}{(E_{C_1}E_{T_2} - E_{C_2}E_{T_1})}$$

$$DPM_C = \frac{(CPM_2 - B_2) - E_{T_1}DPM_T}{E_{C_2}}$$

Isotope effects were calculated as follows:

$$\frac{K_H}{K_T} = \frac{[[DPM_T]/[DPM_C]]_w}{[[DPM_T]/[DPM_C]]_o}$$

where w = water phase and o = organic phase.

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Registry No. *p*-$NO_2C_6H_4NHCOCH_3$, 104-04-1; deuterium, 7782-39-0; tritium, 10028-17-8; cyclohexane, 110-82-7; chlorocyclohexane, 542-18-7; acetone, 67-64-1; ethyl acetate, 141-78-6.

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