reactions shown in Scheme II, coordinatively unsaturated Rh(III) electrophilically attacks a C-H bond and competitive migration of either proton or the alkyl fragment to a ligand in the coordination sphere of the metal generates the mixture of products observed (see Scheme III).[8,10] Elucidation of this catalytic alkane activation is now in progress.

**Acknowledgment.** We acknowledge support for this work given by the National Science Foundation

(6) Conversion of alkanes to alkyl chlorides has been accomplished using electrophilic Pt(II) as a catalyst and Pt(IV) salts as reoxidants. Gol'dschleyer, N. F.; Eskova, V. V.; Shilov, A. E.; Shteinman, A. A. *Russ. J. Phys. Chem. (Engl. Transl.)* **1972**, *46*, 785.

(7) Several iridium complexes have been proposed to activate alkanes by an electrophilic mechanism: Crabtree, R. H.; Mellea, M. I.; Mihelcic, J. M.; Quirk, J. M. *J. Am. Chem. Soc.* **1982**, *104*, 107.

(8) Relative rates for chlorination of $CH_4$, $CH_3Cl$, $CH_2Cl_2$, and $CH_3Cl$ were determined to be 1.0, 1.6, 0.5, 0.4. For free-radical chlorination, these relative rates are reported[9] as 1.0, 1.8, 1.3, 0.6. These relative rate observations, however, cannot conclusively prove a non-free-radical pathway.

(9) Goldfinger, P.; Huybrechts, G.; Martens, G. *Trans. Faraday Soc.* **1961**, *57*, 2210.

(10) Stoichiometric incorporation of hydrocarbons into an organolutetium complex has recently been noted and may proceed according to a pathway such as the one shown in Scheme I: Watson, P. L. *J. Am. Chem. Soc.* **1983**, *105*, 6491.

(11) These frequencies were measured by FT/IR and are slightly different from those obtained[3] by using conventional IR techniques for the same materials.

## Acyl-Enzyme Exchange Detection by Intermolecular Oxygen Scrambling: An Application of the $^{18}O$-Isotope Effect in $^{13}C$ NMR

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We report herein the use of $^{18}O$-induced shifts on $^{13}C$ NMR signals[3] as a general method for detecting enzymic acyl-exchange processes by intermolecular oxygen scrambling. A key feature of this technique is the preparation of substrate that is doubly labeled in the carboxylate group involved in the putative exchange mechanism: $^{13}C$ enrichment to increase sensitivity and $^{18}O$ monolabeling to detect the scrambling phenomenon.

Bacterial citrate lyase (citrate oxaloacetate-lyase; EC 4.1.3.6) from *Enterobacter aerogenes* provides an ideal illustration. The "resting" enzyme has been shown to be a covalent acetyl-enzyme thioester via the sulfhydryl of the 4-phosphopantetheine cofactor bound to the enzyme.[4] The catalytic process may be described in two steps (Scheme I), an acyl exchange yielding free acetate and a citryl-enzyme complex and a Claisen cleavage releasing oxaloacetate with concomitant regeneration of the acetyl-enzyme.[5] The acetyl group has been shown to originate from the *pro-S* carboxymethylene "arm" of citrate.[6] Evidence for this mechanism has rested on the detection and regeneration of the acetyl-enzyme, approaches that require reasonable amounts of pure enzyme and the fortuitous reactivation of nonfunctional enzyme by acetic anhydride.[7] While the existence of the acetyl-enzyme strongly implies acyl exchange, little has been accomplished toward the direct observation of this process independent of intermediate detection.[8]

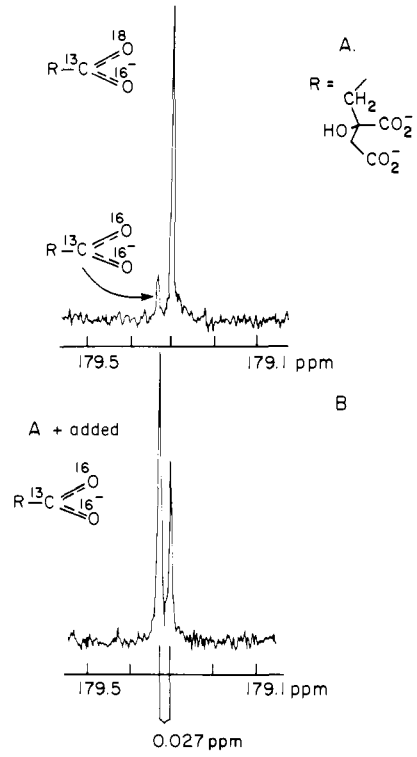
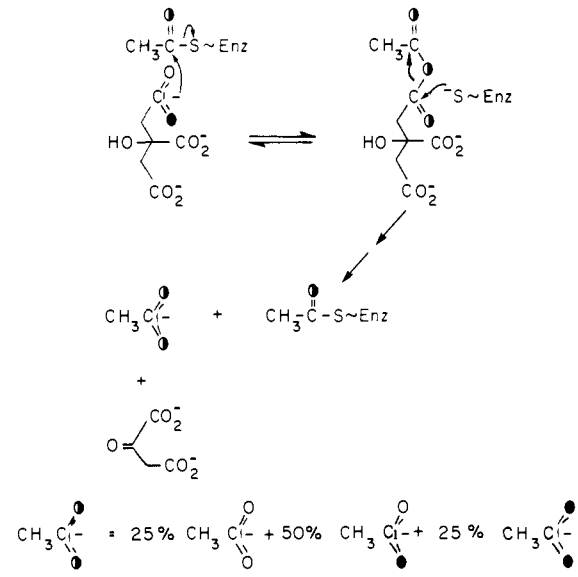



**Figure 1.** 125.8-MHz $^{13}C$ NMR spectra of [5-$^{13}C$,$^{18}O$]-(3*S*)-citrate (20 mM): (A) prepared by citrate synthase as discussed in text, (B) with added [5-$^{13}C$]-(3*S*)-citrate. Spectra were obtained on a Bruker WM 500 spectrometer by using broad-band proton decoupling. Chemical shifts are referenced relative to tetramethylsilane. Samples required Chelex chromatography and the presence of EDTA (40 mM) to throughly remove citrate-metal complexes.

Scheme I




The basis of our analysis is outlined in Scheme I. If citrate labeled with a single $^{18}O$ in the *pro-S* carboxylate is acted upon by the lyase, the acetyl-enzyme generated after one turnover has

(1) National Research Service Postdoctoral Awardee (1983-1985).

(2) American Cancer Society Faculty Research Awardee (1983-1988).

(3) (a) Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 252. (b) Vederas, J. C. *Ibid.* **1980**, *102*, 374. (c) Risley, J. M.; Van Etten, R. L. *Ibid.* **1980**, *102*, 4609; (d) *Ibid.* **1980**, *102*, 6699.

(4) Srere, P. A. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 57 and references cited therein.

(5) Buckel, W.; Buschmeier, V.; Eggerer, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1971**, *352*, 1195.

(6) Wheat, R. W.; Ajl. S. L. *J. Biol. Chem.* **1955**, *217*, 909.

(7) Buckel, W.; Buschmeier, V.; Eggerer, H. *Hoppe-Seyler's S. Physiol. Chem.* **1969**, *350*, 1367.

(8) A mass spectral approach to this problem utilizing a mixture of individually $^{13}C$- and $^{18}O$-labeled substrate has been briefly described for citramalate lyase from *Clostridium tetanomorphum* (Martinoni, B.; Arigoni, D. *Chimia* **1975**, *29*, 26).

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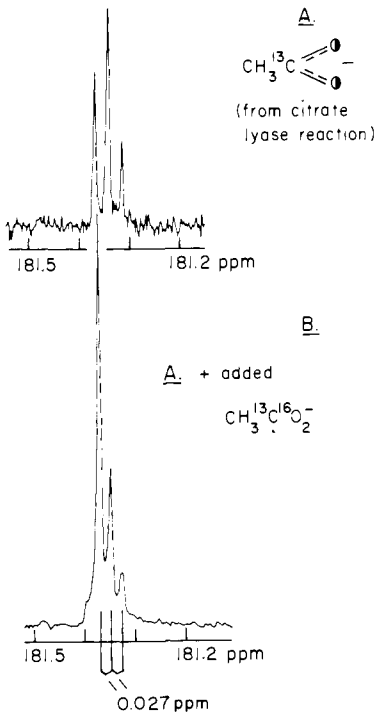


**Figure 2.** 125.8-MHz $^{13}C$ NMR spectra of [1-$^{13}C$,$^{18}O$]acetate (20 mM): (A) prepared from [5-$^{13}C$,$^{18}O$]-(3*S*)-citrate by the action of citrate lyase, (B) with added [1-$^{13}C$]acetate.

a 50% probability of containing $^{18}O$ at the thioester carbonyl.[9] Since a mixed anhydride intermediate or four-center reaction is the most plausible hypothesis for acetyl-exchange, the simple binary statistics of $^{18}O$ occurrence in the thioester and of $^{18}O$ attack by the incoming carboxylate will result in a randomization of $^{18}O$ throughout the acetate oxygen pool—oxygen scrambling. The additional incorporation of $^{13}C$ into the carboxylate permits the direct measurement of $^{18}O$ distribution by virtue of the additive upfield shift on the $^{13}C$ NMR signal.[3]

[5-$^{13}C$,$^{18}O$]-(3*S*)-citrate[10] was prepared by the action of citrate (*si*)-synthase [citrate oxaloacetate-lyase (CoA acetylating); EC 4.1.3.7] (Sigma) on [1-$^{13}C$]acetyl-CoA and oxaloacetate (both generated in situ) in $H_2{}^{18}O$.[11] This enzyme catalyzes the hydrolysis of the initially formed citryl-CoA to incorporate the $^{18}O$ label from solvent. The 125-MHz $^{13}C$ NMR spectrum (Figure 1A) of the purified citrate reveals two resonances (85:15 relative intensity). Addition of [5-$^{13}C$]-(3*S*)-citrate to the sample (Figure 1B) establishes that the minor peak in Figure 1A is due to citrate lacking the $^{18}O$ label. The upfield shift of the major peak (0.027 ppm) is consistent with a singly $^{18}O$-labeled carboxylate.[12]

Processing of the doubly labeled citrate by citrate lyase[13] afforded [1-$^{13}C$]acetate whose $^{13}C$ NMR spectrum revealed a triplet (33:49:18 relative intensity; Figure 2A). Addition of authentic [1-$^{13}C$]acetate (Figure 2B) and the chemical shift difference between peaks (0.027 ppm) establish that the resonances are due to acetates containing 0, 1, and 2 $^{18}O$'s. On the basis of the $^{18}O$ content of the citrate, the relative intensities of the acetate resonances constitute a statistical scrambling of the label without any observable washout.

These results directly verify an acyl-exchange process for citrate lyase and establish $^{13}C$ NMR as the method of choice for the analysis. While mass spectral techniques are feasible, the simplicity of execution and interpretation of the NMR analysis should be readily apparent. In addition, the method permits a distinction among possible mechanisms. For instance, a noncovalent mechanism would yield no scrambling and no washout and de novo thioester formation would result in washout of label but no scrambling. Furthermore, the method should be of use with enzymes where the evidence for acyl exchange is less secure. Experiments along these lines are in progress.

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(9) This assumes that there is no observable isotope effect against $^{18}O$ attack. In reality, even a full isotope effect will have only a small effect on the statistics.

(10) The numbering system used here is that of Glusker and Srere. The *pro-R* arm is given priority (Glusker, J. P.; Srere, P. A. *Bioorg. Chem.* **1973**, *2*, 301.

(11) A typical reaction contained (in 3.0 mL 99% $H_2{}^{18}O$) 0.25 M triethanolamine, pH 8.0, 16 mM [1-$^{13}C$]sodium acetate, 2 mM CoA, 30 mM ATP, 40 mM $MgCl_2$, 20 mM L-malic acid, 30 mM NAD, 4 units of (*S*)-acetyl-CoA synthetase, 20 units of malate dehydrogenase, and 8 units of citrate (*si*)-synthase. The reaction, monitored by NADH production, was allowed to proceed (37 °C) to 80–90% conversion. $H_2{}^{18}O$ was recovered by bulb-to-bulb distillation, and the citrate was purified by DEAE Sephadex chromatography.

(12) Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1981**, *103*, 4389.

(13) The reaction mixture (1.0 mL with 20% $^2H_2O$ for NMR locking) contained 0.4 M triethanolamine, pH 7.6, 20 mM $ZnCl_2$, 20 mM labeled citrate, 25 mM NADH, 50 units of malate dehydrogenase, 40 units of L-lactate dehydrogenase, and 10 units of citrate lyase. NADH, malate dehydrogenase, and lactate dehydrogenase are added to drive the reaction toward acetate by trapping oxaloacetate and pyruvate (formed by spontaneous decarboxylation of the former). After 10 min the mixture is heated to 100 °C for 3 min, centrifuged, filtered, and subjected to NMR analysis.

## Contribution of Tunneling to Secondary Isotope Effects in Proton-Transfer Reactions[1]

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Calculations show that tunneling can substantially increase the magnitude of secondary hydrogen isotope effects, alter the dependence of the effects on the extent of progress along the reaction coordinate, and cause the effects to show a much steeper temperature dependence than in the absence of tunneling.

Huskey and Schowen[2] recently reported calculations showing that tunneling in hydride-transfer reactions can cause kinetic secondary deuterium isotope effects to exceed the corresponding equilibrium effects, thereby explaining experimental observations to this effect.[3,4] We have found analogous anomalies with secondary tritium isotope effects in E2 reactions.

Although equilibrium isotope effects cannot be measured for E2 reactions, they can be reliably estimated from the fractionation factors of Hartshorn and Shiner.[5] From their data, the equilibrium secondary isotope effect for complete conversion of a C—CHD—C to a C=CD—C moiety at 25 °C is 1.124. Assuming exponential temperature dependence, $(K_H/K_D)_{sec}$ becomes 1.115 at 45 °C, corresponding to $(K_H/K_T)_{sec} = 1.170$ (employing the usual relation between tritium and deuterium isotope effects[6]).

We have, however, observed kinetic isotope effects for reaction 1 substantially larger than the above values. When $X=NMe_3^+$

$$PhCHTCH_2X + OR^- \rightarrow PhCT{=}CH_2 + ROH + X^- \quad (1)$$

and $RO^- = EtO^-$, $(k_H/k_T)_{sec}$ at 40 °C is 1.31 (corresponding to $(k_H/k_D)_{sec} = 1.21$[6]), and we have since found additional examples

(1) This work was supported by the National Science Foundation.

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(3) Kurz, L. C.; Frieden, C. *J. Am. Chem. Soc.* **1980**, *102*, 4198–4203.

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(5) Buddenbaum, W. E.; Shiner, V. J., Jr. In "Isotope Effects on Enzyme-Catalyzed Reactions"; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, 1977; p 11. Hartshorn, S. R.; Shiner, V. J., Jr. *J. Am. Chem. Soc.* **1972**, *94*, 9002–9012.

(6) Swain, C. G.; Stivers, E. C.; Reuwer, J. F., Jr.; Schaad, L. J. *J. Am. Chem. Soc.* **1958**, *80*, 5885–5893.

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