^{18}O Isotope Effect in ^{13}C Nuclear Magnetic Resonance Spectroscopy. 7. Hydrolysis of 2,2-Dimethyloxirane in Dilute Acid and by Microsomal Epoxide Hydratase[1]

John M. Risley, Fengjiun Kuo, and Robert L. Van Etten*

Contribution from the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received July 28, 1982*

Abstract: The ^{18}O isotope effect in ^{13}C NMR spectroscopy affords an analytical technique that may be used to ascertain directly and simultaneously, in a continuous-assay mode, the position of bond cleavage, the rate of hydrolysis, and the extent of any accompanying oxygen exchange during the hydrolysis of oxiranes. The synthesis of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane ([1-^{13}C,^{18}O]isobutylene oxide) is described. The hydrolysis of this compound in dilute acid, by mouse liver microsomes (epoxide hydratase), and by homogeneous rat liver epoxide hydratase (EC 3.3.2.3) was studied by ^{13}C NMR spectroscopy. The ^{18}O isotope-induced shifts of the ^{13}C resonances of the primary carbon atom and of the tertiary carbon atom in the oxirane are 0.031 and 0.042 ppm upfield, respectively. The corresponding isotope shifts in the product diol (2-methylpropane-1,2-diol) and 0.019 and 0.033 ppm upfield, respectively. Dilute-acid-catalyzed hydrolysis of the oxirane results in retention of the ^{18}O label on the primary carbon atom with no accompanying oxygen exchange. The rates of nonenzymatic hydrolysis measured with this technique closely approximate the rates of hydrolysis measured previously by tedious mass spectral analyses. In contrast, when either mouse liver microsomes or homogeneous rat liver microsomal epoxide hydratase was incubated at pH 8 and 35 °C with 2,2-dimethyl[3-^{13}C,^{18}O]oxirane, the hydrolysis product was 2-methyl[1-^{13}C,2-^{18}O]propane-1,2-diol, consistent with enzyme-catalyzed hydrolytic attack at the primary carbon. Competing base-catalyzed hydrolysis of the oxirane under identical experimental conditions was much slower than the enzyme-catalyzed reactions. Although specific numerical values of k_{cat} could not be obtained due to inability to saturate the enzymes, k_{cat} for 2,2-dimethyloxirane must be significantly greater than that for styrene oxide. This study further illustrates the applicability of the ^{18}O isotope effect in ^{13}C NMR spectroscopy in simplifying the analysis of a variety of kinetic and stereochemical problems.

Upon substitution of ^{18}O for ^{16}O in organic molecules, the ^{13}C NMR signals of directly bonded carbon atoms are shifted upfield and the magnitudes of the isotope-induced shifts are easily quantitated.[2] A general understanding of this phenomenon has been obtained in studies designed to define the isotope effect in terms of basic structural properties; the structure of the carbon–oxygen functional group is the principal determinant of the magnitude of the isotope-induced shift.[3] In addition to these studies on the basic structural properties, this phenomenon provides a tool that can be used as a practical alternate method of analysis in numerous experimental problems. The technique provides a continuous, direct, and generally simplified method to obtain information about reactions involving carbon–oxygen bond-breaking and/or bond-forming processes. Recent experimental problems where the technique has been utilized are in measuring the rate of oxygen exchange at the anomeric carbon atom in sugars[1] and in delineating the site of bond cleavage in acid- and phosphatase-catalyzed hydrolysis of a phosphate monoester.[4] A reaction pathway of thermal and acid-catalyzed rearrangement of a geometrically restricted triepoxide has been elucidated with ^{18}O-labeled, ^{13}C-enriched epoxides.[5] The technique has been found to be particularly valuable in the determination of the source of oxygen atoms in the microbial syntheses of macromolecules[6a] and in a mechanistic study of formyl-group transfer.[6b] In this paper we report the application of the isotope effect in a study of the hydrolysis of 2,2-dimethyloxirane (isobutylene oxide) in dilute acid, by mouse liver microsomes (epoxide hydratase), and by homogeneous rat liver microsomal epoxide hydratase (EC 3.3.2.3, formerly EC 4.2.1.63).

In a series of papers,[7] Long and Pritchard presented the results of a study on the hydrolysis of 2,2-dimethyloxirane (isobutylene oxide) to 2-methylpropane-1,2-diol under acidic, basic, and neutral conditions. They concluded that under acidic conditions a tertiary carbonium ion was formed. Under basic conditions an S_N2 mechanism involving hydroxide ion attack at the primary carbon atom was observed. The experimental results obtained under neutral conditions were inconclusive. The rates of hydrolysis were measured by using dilatometry.[7a] Experimental evidence supporting the conclusions on the mechanisms of hydrolysis included ^{18}O isotope studies on the position of incorporation of isotopic oxygen from ^{18}O-enriched water.[7a] The extent and position of ^{18}O labeling in the diol was deduced circuitously by a difference computation from the complicated fragmentation pattern obtained upon mass spectrometric analysis. Control reactions showed that under the experimental conditions employed, no ^{18}O exchange was detected in the hydroxyl groups of the diol.

Enzymes also catalyze the hydrolysis of epoxides to diols. Microsome (epoxide hydratase)-catalyzed hydrolysis of epoxides has been studied with ^{18}O as a tracer. Rabbit liver microsomes (pH 8.0) were incubated with naphthalene 1,2-oxide, the resulting dihydrodiol was dehydrated, and the trimethylsilylated derivatives of the naphthols were analyzed for ^{18}O by mass spectrometry.[8] In studies[9a,b] designed to evaluate the regiospecificity of the hydrolysis of 2-, 2,2-, and 2,3-substituted oxiranes catalyzed by rat liver microsomes (pH 8.0), the ^{18}O contents of the substrates and products were estimated differentially by mass spectrometry as before.[7a] Substituent-group effects on the position of bond cleavage by rat liver microsomes were delineated in ^{18}O tracer studies[9c] by mass spectral analysis of the diol product and [^{18}O]water (as a benzoic acid derivative).

We have reexamined the acid-catalyzed and microsome (epoxide hydratase)-catalyzed hydrolysis of epoxides utilizing the ^{18}O

(1) Part 6: Risley, J. M.; Van Etten, R. L. *Biochemistry* **1982**, *24*, 6360–5.
(2) Part 1: Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 252–3.
(3) Part 5: Risley, J. M.; DeFrees, S. A.; Van Etten, R. L. *Org. Magn. Reson.* **1983**, *21*, 28–35 and references cited therein.
(4) Parente, J. E.; Risley, J. M.; Van Etten, R. L. *Proc. Indiana Acad. Sci.*, in press. Risley, J. M.; Parente, J. E.; Van Etten, R. L. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1982**, *41*, 906.
(5) Benner, S. A.; Maggio, J. E.; Simmons, H. E., III *J. Am. Chem. Soc.* **1981**, *103*, 1581–2.
(6) (a) Vederas, J. C. *Can. J. Chem.* **1982**, *60*, 1637–42, and references cited therein. Sankawa, U.; Shimada, H.; Kobayashi, T.; Ebizuka, Y.; Yamamoto, Y.; Noguchi, H.; Seto, H. *Heterocycles* **1982**, *19*, 1053–8. (b) Smith, G. K.; Mueller, W. T.; Slieker, L. J.; DeBrosse, C. W.; Benkovic, S. J. *Biochemistry* **1982**, *21*, 2870–4.
(7) (a) Long, F. A.; Pritchard, J. G. *J. Am. Chem. Soc.* **1956**, *78*, 2663–7. (b) Pritchard, J. G.; Long, F. A. *Ibid.* **1956**, *78*, 2667–70. (c) Long, F. A.; Pritchard, J. G.; Stafford, F. E. *Ibid.* **1957**, *79*, 2362–4.
(8) Jerina, D. M.; Daly, J. W.; Witkop, B.; Zaltzman-Nirenberg, P.; Udenfriend, S. *J. Am. Chem. Soc.* **1968**, *90*, 6525–7.
(9) (a) Hanzlik, R. P.; Edelman, M.; Michaely, W. J.; Scott, G. *J. Am. Chem. Soc.* **1976**, *98*, 1952–5. (b) Hanzlik, R. P.; Heideman, S.; Smith, D. *Biochem. Biophys. Res. Commun.* **1978**, *82*, 310–5. (c) Dansette, P. M.; Makedonska, V. B.; Jerina, D. M. *Arch. Biochem. Biophys.* **1978**, *187*, 290–8.

0002-7863/83/1505-1647$01.50/0 © 1983 American Chemical Society

isotope effect in ^{13}C NMR spectroscopy with 2,2-dimethyl[3-^{13}C,^{18}O]oxirane. The technique was used to demonstrate that in a continuous assay mode it is possible to ascertain directly and simultaneously the position of bond cleavage, the rate of hydrolysis, and whether oxygen exchange accompanies the reaction. Data analysis is shown to be greatly simplified compared to mass spectrometry. The site of bond cleavage by the mouse liver microsomal enzyme is deduced for the first time. Also, for the first time, homogeneous microsomal epoxide hydratase isolated from rat liver was studied by using the ^{18}O isotope tracer, and these results were compared with those obtained in earlier studies using microsomes. This investigation on the application of the ^{18}O isotope effect in ^{13}C NMR spectroscopy in the hydrolysis of an oxirane complements a study[10] utilizing ^{13}C NMR spectroscopy to investigate the addition of nucleophiles other than water to benzo[*a*]pyrene 4,5-oxide.

Experimental Section

Synthesis of Trimethylsulfonium Iodide.[11] Equimolar amounts of methyl iodide and dimethyl sulfide in ethanol were stirred for 24 h at room temperature. The salt was obtained in 90% yield after recrystallization from ethanol.

Synthesis of [^{13}C]Methyldiphenylsulfonium Perchlorate.[12] To a mixture of silver perchlorate (39 g, 0.14 mol) in methylene chloride (450 mL) stirred in an ice bath was added an equimolar amount of diphenyl sulfide (26 g). After the silver perchlorate dissolved, [^{13}C]methyl iodide (90 atom % excess ^{13}C, KOR, Inc.) (20 g, 0.14 mol) in methylene chloride (100 mL) was added slowly over 2 h (exothermic reaction) and the solution was stirred an additional 3 h. Silver iodide precipitated overnight and was removed by filtering. The filtrate was washed with saturated aqueous sodium chloride to remove excess silver ion. The methylene chloride solution was dried over anhydrous magnesium sulfate and concentrated under vacuum below 50 °C. The crude, white solid was dissolved in a minimal amount of chloroform, diethyl ether was added, and the product crystallized overnight at 4 °C. The product was collected and washed with ether. The yield of [^{13}C]methyldiphenylsulfonium perchlorate (90 atom % ^{13}C) was 75%, and it had a melting point of 75–77 °C (lit. 73–76 °C).

Synthesis of [^{18}O]Acetone.[13] To a stirred mixture of [^{18}O]water (97 atom % ^{18}O, KOR, Inc.) and excess 2,2-dimethoxypropane or 2,2-diethoxypropane was added a catalytic amount of *p*-toluenesulfonic acid. The immediate endothermic reaction was warmed to room temperature, and the [^{18}O]acetone was collected by distillation in nearly quantitative yield and contained a small amount of alcohol. The ^{18}O-labeled acetone was diluted with unlabeled acetone to give mixtures containing 48–70% ^{18}O-labeled ketone.

Synthesis of 2,2-Dimethyloxirane and 2,2-Dimethyl[^{18}O]oxirane.[11b,14] A three-necked flask was fitted with a magnetic stirring bar, thermometer, nitrogen gas inlet, and calcium chloride drying tube. Under nitrogen, anhydrous dimethyl sulfoxide (60 mL) was added to sodium hydride (0.2 mol; 60% in mineral oil, dispersion, washed twice with toluene) and the solution was stirred at 70 °C for 45 min. The solution was cooled to room temperature, diluted with toluene (120 mL; solvents with lower boiling points complicate isolation of the oxirane), and cooled in an ice–salt bath at 0 to −10 °C. A solution of trimethylsulfonium iodide (0.12 mol) in dimethyl sulfoxide (120 mL) was added at a rate that the temperature remained below 5 °C. After completion of the addition, the mixture was stirred for 1 min and acetone (or [^{18}O]acetone) (0.1 mol) was added as rapidly as possible while maintaining the temperature below 5 °C. The reaction mixture was stirred for 1 h in the ice–salt bath and 1 h at room temperature. Three volumes of saturated aqueous NaCl solution were added, and the toluene extract was separated and washed again with the saturated aqueous NaCl solution. To precipitate dimethyl sulfide (which boils at 37 °C and would interfere with the isolation of the oxirane), a solution of silver perchlorate (10 g in 30 mL toluene) was added in portions and the mixture was filtered. The filtrate was washed with saturated aqueous NaCl solution to remove excess silver perchlorate, and the organic solution was dried over anhydrous magnesium sulfate and distilled to give the product boiling at 50–53 °C. The yield was 50% of the oxirane in a solution of 33% (unreacted) acetone as determined by ^1H NMR and was used as a mixture in subsequent kinetic experiments.

Synthesis of 2,2-Dimethyl[3-^{13}C]oxirane and 2,2-Dimethyl[3-^{13}C,^{18}O]-oxirane. A modification of published procedures[15] was used in the synthesis of the ^{13}C-enriched oxirane. In the same apparatus as above was added diglyme (30 mL, 99.9%) and sodium hydride (2.5 g of twice washed with dry toluene). The flask was cooled to −40 to −50 °C in a dry ice–acetone bath under nitrogen. A solution of [^{13}C]methyldiphenylsulfonium perchlorate (15 g, 0.05 mol) in diglyme (100 mL) was added at −40 °C with stirring. After addition of the salt, the solution was stirred for 1 min and acetone (or [^{18}O]acetone) was added at −40 °C. The temperature of the solution was raised until foam appeared, at which time the temperature (approximately −30 °C) was maintained for 2 h and then raised gradually to 5–10 °C over 2 h. The product was collected by distillation at this temperature under vacuum. The yield was 50% of the 3-^{13}C-enriched oxirane in a solution of 33% (unreacted) acetone as determined by ^1H NMR and was used as a mixture in subsequent kinetic experiments.

Kinetics of Hydrolysis. Doubly distilled, deionized water was used to prepare all solutions. A Corning Model 130 pH meter was used to measure the solution pH, which was adjusted with 1N HCl or 1M NaOH. All solutions were preequilibrated at the probe temperature prior to initiation of the reaction. The hydrolysis reactions were followed on a Varian A60A, Varian CFT-20, or an NTC-200 spectrometer. Deuterium oxide (99.75 atom % ^2H, Baker) was used to make each solution 20% ^2H for instrumental lock.

A. Acid Catalyzed. The Varian A60A spectrometer was fitted with a 5-mm probe that was equilibrated at 40 °C. 2,2-Dimethyloxirane (8.5×10^{-4} mol) was added to a 25 mM sodium acetate buffer solution, which was adjusted to the desired pH. A total of 1.0 mL was prepared. The pH of the solution was checked after the hydrolysis reaction was complete and was not corrected for deuterium. A 130-Hz sweep width that included the proton signals of the two methyl groups in the oxide and diol was swept at a specific time interval during the reaction, and the spectrum was recorded. The Varian CFT-20 spectrometer was fitted with an 8-mm probe that was equilibrated at 32 °C. 2,2-Dimethyl[3-^{13}C,^{18}O]oxirane (90 atom % 3-^{13}C, 54 atom % ^{18}O) (8.5×10^{-4} mol) in 25 mM sodium acetate buffer was prepared as above. The instrumental parameters were previously defined.[16]

The NTC-200 spectrometer operating at 50.31 MHz was fitted with a 12-mm probe and equilibrated at 19 °C. 2,2-Dimethyl[3-^{13}C,^{18}O]oxirane (90 atom % 3-^{13}C, 48 atom % ^{18}O) (3.6×10^{-4} mol) was added to a 40 mM sodium acetate buffer solution containing 20% deuterium oxide at pH 5.22 (uncorrected) in a total volume of 5.0 mL. An 800-Hz sweep width, a 90° pulse angle, a 16K data block, and an acquisition time of 10 min per spectrum were used. Protons were broad-band decoupled, and a line-broadening factor was applied to the accumulated FID.

Two control reactions were prepared. One was made up to contain 2,2-dimethyl[^{18}O]oxirane (65 atom % ^{18}O) (8.5×10^{-4} mol) in 60% [^{18}O]water and 25 mM sodium phosphate at pH 5.2 in a total volume of 1.0 mL. The second solution was made up to contain 2,2-dimethyl-[^{18}O]oxirane (65 atom % ^{18}O) (8.5×10^{-4} mol) and sodium hydroxide (0.01 g) in a total volume of 1.0 mL (pH >10). These solutions were analyzed by ^{13}C NMR in end-point assays with the same instrument parameters as before but with considerably longer acquisition times.

B. Enzyme Catalyzed. Mouse liver microsomes were prepared from Swiss male mice by decapitating a mouse and removing the liver. The liver was minced at 4 °C and homogenized in Tris/KCl buffer (20 mM/1.15%, pH 7.4 containing 0.32 M sucrose). The solution was centrifuged at 5 °C at 9000 × *g* for 20 min. The supernatant was centrifuged at 105 000 × *g* for 60 min, and the pellet was resuspended in Tris/KCl buffer and centrifuged again at 105 000 × *g* for 50 min. The pellet was resuspended in cold microsome buffer (50 mM phosphate, pH 8.0), and aliquots of 0.5 mL were transferred to vials and frozen at −20 °C. The protein content was 3.45 mg per mL as measured by the Lowry assay[17] with bovine serum albumin as the standard.

The NTC-200 spectrometer operating at 50.31 MHz was fitted with a 12-mm probe equilibrated at 35 °C. A 12-mm NMR tube containing 1.0 mL of deuterium oxide and 1.5 mL of water was equilibrated at 37

(10) Hylarides, M. D.; Lyle, T. A.; Daub, G. H.; Vander Jagt, D. L. *J. Org. Chem.* **1979**, *44*, 4652–7.

(11) (a) Emeleus, H. J.; Heal, H. G. *J. Chem. Soc.* **1946**, 1126–31. (b) Corey, E. J.; Chaykovsky, M. *J. Am. Chem. Soc.* **1965**, *87*, 1353–64.

(12) Franzen, V.; Schmidt, H.-J.; Mertz, C. *Chem. Ber.* **1961**, *94*, 2942–50. Hashimoto, T.; Ohkubo, K.; Kitano, H.; Fukui, K. *Nippon Kagaku Kaishi* **1966**, 87, 456–9. Hinsberg, O. *Chem. Ber.* **1936**, *69*, 492–5. Stirling, C. J. M. "The Chemistry of the Sulfonium Group"; Wiley: New York, 1981; and references cited therein.

(13) Part 2: Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 4609–14.

(14) Ackermann, K.; Chapuis, J.; Horning, D. E.; Lacasse, G.; Muchowski, J. M. *Can. J. Chem.* **1969**, *47*, 4327–33.

(15) Corey, E. J.; Oppolzer, W. *J. Am. Chem. Soc.* **1964**, *86*, 1899–1900. Corey, E. J.; Jautelat, M.; Oppolzer, W. *Tetrahedron Lett.* **1967**, 2325–8.

(16) Part 4: Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1981**, *103*. 4389–92.

(17) Bailey, J. L. "Techniques in Protein Chemistry"; Elsevier: Amsterdam, 1967; pp 340–1.

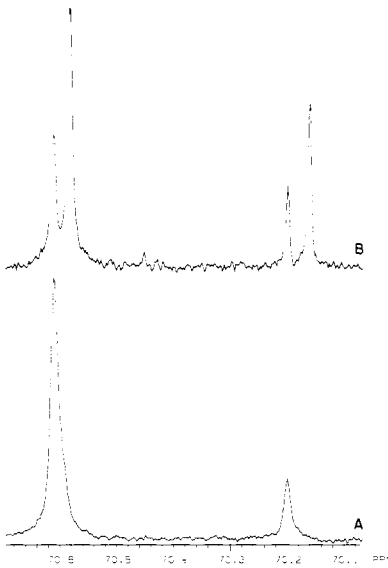

Figure 1. The natural-abundance ^{13}C NMR signals for the primary and tertiary ring carbon atoms of 2,2-dimethyloxirane (A) and of 2,2-dimethyl[^{18}O]oxirane (70 atom % ^{18}O) (B) in chloroform-*d* (99.8 atom % ^{2}H, Aldrich) were recorded on an NTC-200 spectrometer operating at 50.31 MHz. The primary carbon atom appears at 70.6 ppm and the tertiary carbon atom at 70.2 ppm downfield from Me_4Si (A). Upon substitution of ^{18}O for ^{16}O, the resonance signal of the primary carbon atom is shifted upfield 0.031 ppm and of the tertiary carbon atom 0.042 ppm with respect to the resonance signals of the unlabeled carbon atoms (B).

°C for 75 min. To this solution was added 2.5 mL of thawed mouse liver microsome preparation. After 5 min at 37 °C, 2,2-dimethyl[3-^{13}C,^{18}O]oxirane (90 atom % 3-^{13}C, 48 atom % ^{18}O) (3.6×10^{-4} mol) was added and mixed, and the hydrolysis reaction was transferred to the spectrometer. The pH of the reaction was 7.92 (uncorrected at room temperature). The instrumental parameters were the same as above; however, the acquisition time was 15.1 min per spectrum. A complementary solution was prepared that contained [^{18}O]water (0.5 mL), thawed mouse liver microsome preparation (0.5 mL), and 2,2-dimethyl[3-^{13}C]oxirane (90 atom % 3-^{13}C) (0.72×10^{-4} mol); this solution was incubated at 37 °C for the same length of time as the hydrolysis solution and diluted with deuterium oxide and water for analysis by NMR.

Homogeneous rat liver microsomal epoxide hydratase from male, Long–Evans rats was a gift from Dr. Anthony Y. H. Lu of Merck Sharp & Dohme, Rahway, NJ. It was purified by column chromatography[18] and had a specific activity of 499 nmol styrene glycol formed per min per mg of protein (1600 nmol of [^{3}H]styrene oxide in 0.16 M Tris, pH 8.7 at 37 °C) at a concentration of 4.24 mg protein per mL. The NTC-200 spectrometer operating at 50.31 MHz was fitted with a 12-mm probe equilibrated at 35 °C. In a 37 °C water bath, a 12-mm NMR tube containing microsome buffer (2.5 mL), water (1.5 mL), and deuterium oxide (1.0 mL) was equilibrated for 2 h. To this solution was added 2,2-dimethyl[3-^{13}C,^{18}O]oxirane (90 atom % ^{13}C, 48 atom % ^{18}O) (3.6×10^{-4} mol) and either 65 or 25 μL of enzyme. The solutions were mixed and transferred to the spectrometer. The pH of the reaction mixtures were 7.93 and 7.98, respectively (uncorrected at room temperature). The instrumental parameters and acquisition time were the same as in the mouse liver microsome hydrolysis reaction. A complementary solution was prepared that contained [^{18}O]water (0.5 mL), microsome buffer (0.5 mL), 2,2-dimethyl[3-^{13}C]oxirane (90 atom % ^{13}C) (0.72×10^{-4} mol), and 6.5 μL of enzyme; this solution was incubated at 37 °C for the same length of time as the first hydrolysis reaction and diluted with deuterium oxide and water for analysis by NMR.

A control reaction made up to contain microsome buffer (0.5 mL), water (0.5 mL), and 2,2-dimethyl[3-^{13}C]oxirane (90 atom % ^{13}C) (0.72×10^{-4} mol) was incubated at 37 °C and diluted with deuterium oxide and water for NMR analysis.

Data Analysis. The area under each peak was measured, and the relative concentration (as a percentage) of each species present was calculated. The oxirane hydrolysis data were plotted as for a first-order rate process, with the rate constants being obtained from the slope of a plot of ln (percent oxirane) against time.

(18) Lu, A. Y. H.; Ryan, D.; Jerina, D. M.; Daly, J. W.; Levin, W. *J. Biol. Chem.* **1975**, *250*, 8283–8. Lu, A. Y. H.; Levin, W. *Methods Enzymol.* **1978**, *52*, 193–200.

Table I. Pseudo-First-Order Rate Constants from ^{1}H and ^{13}C NMR Data for the Hydrolysis of 2,2-Dimethyloxirane at 40 °C and 2-2-Dimethyl[3-^{13}C,^{18}O]oxirane at 32 °C, Respectively

	NMR								
	^{1}H					^{13}C			
pH	3.1	4.1	4.5	4.9	6.1	4.6	4.8	4.9	5.3
$k \times 10^{5}$, s^{-1}	460	140	70	23	3.6	38	16	7	3

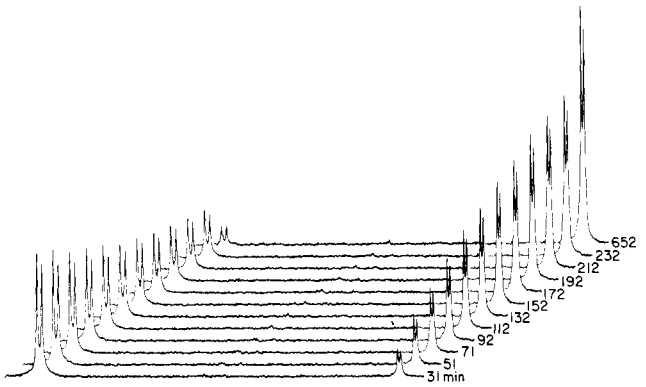

Figure 2. Acid-catalyzed hydrolysis of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane followed by ^{13}C NMR spectroscopy, using an NTC-200 instrument operating at 50.31 MHz. The solution contained 3.6×10^{-4} mol of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane (90 atom % 3-^{13}C, 48 atom % ^{18}O) in 40 mM sodium acetate buffer (pH 5.22) with 20% deuterium oxide (total volume 5.0 mL). Signals for the primary (^{13}C-enriched) carbon atom in the oxirane, [^{18}O]oxirane, diol, and [^{18}O]diol are observed at increasing magnetic field (left to right). The pseudo-first-order rate constant for this reaction at 19 °C was 8.1×10^{-5} s^{-1} (±2%) and the hydrolysis was followed for 4.5 half-lives. The stacked plot shows the spectrum recorded at 12 times during the reaction. The isotope shift on the primary carbon atom in the oxirane is 0.031 ppm and in the diol is 0.019 ppm upfield.

Results

The ^{18}O isotope-induced shifts in 2,2-dimethyl[^{18}O]oxirane are 0.031 ± 0.002 ppm upfield for the primary carbon atom and 0.042 ± 0.002 ppm upfield for the tertiary carbon atom (Figure 1). The ^{18}O isotope-induced shifts in the hydrolysis product, 2-methyl-[1,2-$^{18}O_2$]propane-1,2-diol, are 0.019 ± 0.002 ppm upfield for the primary carbon atom and 0.033 ● 0.002 ppm upfield for the tertiary carbon atom. Initial studies on the rates of acid-catalyzed hydrolysis of 2,2-dimethyloxirane were undertaken using ^{1}H NMR to define optimal conditions for subsequent studies using ^{13}C NMR. The pseudo-first-order rate constants calculated from both studies are given in Table I; the standard deviation in *k* from the linear least-squares analysis is ±10%. Figure 2 illustrates the time course of a typical acid-catalyzed reaction; the reaction was followed on an NTC-200 spectrometer operating at 50.31 MHz. Upon analysis by ^{13}C NMR spectroscopy, it was found that, in the diol produced in the acid-catalyzed hydrolysis of 2,2-dimethyl[^{18}O]oxirane in [^{18}O]water, the primary carbon atom was labeled with 65% ^{18}O and the tertiary carbon 60%. In the diol obtained from the base-catalyzed hydrolysis of 2,2-dimethyl-[^{18}O]oxirane in unenriched water, only the tertiary carbon atom was ^{18}O labeled.

Data for the mouse liver microsome-catalyzed hydrolysis of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane were obtained for three half-lives; the first-order rate constant was 7.21×10^{-5} s^{-1} (±2%) at 35 °C. The ^{13}C NMR spectra acquired during the reaction showed an ^{18}O isotope effect on the primary carbon atom of the substrate oxirane that was not observed on the primary carbon atom of the product diol. The ^{13}C NMR spectrum of the complementary reaction of 2,2-dimethyl[3-^{13}C]oxirane in [^{18}O]water in the presence of mouse liver microsomes is shown in Figure 3. Hydrolysis of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane by homogeneous rat liver microsomal epoxide hydratase is exemplified in Figure 4. In the complementary reaction, homogeneous rat liver microsomal

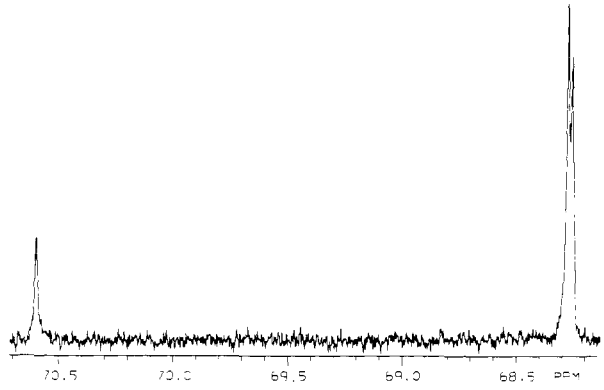

Figure 3. Hydrolysis of 2,2-dimethyl[3-^{13}C]oxirane in [^{18}O]water by a preparation of mouse liver microsomes from male Swiss mice. The ^{13}C NMR spectrum was recorded on an NTC-200 NMR spectrometer operating at 50.31 MHz. The solution contained 0.72×10^{-4} mol of 2,2-dimethyl[3-^{13}C]oxirane (90 atom % 3-^{13}C), 0.5 mL of [^{18}O]water, and 0.5 mL of mouse liver microsome preparation (3.45 mg of protein per mL) in 25 mM inorganic phosphate buffer (pH 7.92); the reaction mixture was diluted with 4.0 mL of 25% deuterium oxide (total volume 5.0 mL) for NMR analysis. Signals for the primary (^{13}C-enriched) carbon atom of the oxirane, diol, and [^{18}O]diol are observed (left to right). The ^{13}C NMR spectrum was recorded after incubation at 37 °C for 500 min. The isotope shift on the primary carbon atom in the diol is 0.019 ppm.

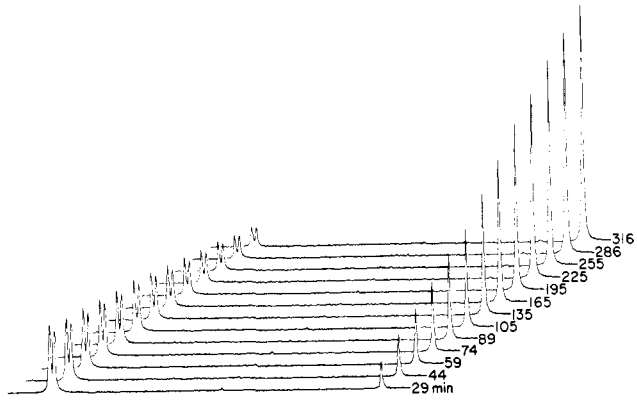

Figure 4. Homogeneous rat liver microsomal epoxide hydratase (EC 3.3.2.3) from male, Long–Evans rats catalyzes the hydrolysis of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane. The hydrolysis reaction was followed by ^{13}C NMR spectroscopy on an NTC-200 instrument operating at 50.31 MHz. The solution contained 3.6×10^{-4} mol of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane (90 atom % 3-^{13}C, 48 atom % ^{18}O) and 25 μL of enzyme (4.24 mg of protein per mL of specific activity 499 units per mg) in 25 mM inorganic phosphate buffer (pH 7.98) with 20% deuterium oxide (total volume 5.0 mL). Signals for the primary (^{13}C-enriched) carbon atom of the oxirane, [^{18}O]oxirane, and the diol are observed (left to right). The first-order rate constant for this reaction at 35 °C was 9.98×10^{-5} s^{-1} (±2%), and the hydrolysis reaction was followed for three half-lives. The stacked plot shows the spectrum recorded at 13 times during the reaction. The isotope shift on the primary carbon atom in the oxirane is 0.031 ppm.

epoxide hydratase catalyzed the incorporation of ^{18}O from the solvent onto the primary carbon atom of the diol; the extent of ^{18}O label present in the diol was the same as in the solvent. The base-catalyzed hydrolysis of 2,2-dimethyl[3-^{13}C]oxirane control reaction at 37 °C in 25 mM inorganic phosphate (pH 7.75) was exceedingly slow and, based on a minimal number of experimental data points (three), was found to proceed with a pseudo-first-order rate constant of 1×10^{-5} s^{-1}.

Discussion

The magnitudes of the ^{18}O-isotope-induced shifts on the ^{13}C resonance signals of the oxirane are the largest shifts that have been observed for ether derivatives. The upfield isotope-induced shifts are clearly seen in Figure 1 in the ^{13}C natural-abundance NMR spectra of the oxirane and the ^{18}O-labeled oxirane; the tertiary carbon resonance signal is particularly well resolved. The isotopic perturbation of 0.031 ppm upfield on the primary carbon atom in 2,2-dimethyloxirane is the same magnitude as has been observed (0.030 and 0.033 ppm) for the primary carbon atoms of three ^{13}C-enriched, ^{18}O-labeled spiro oxiranes.[5] However the tertiary carbon atom perturbation of 0.042 ppm is significantly larger and is the largest such shift that has been reported; nevertheless it is in keeping with the observation that tertiary carbon atoms generally display large ^{18}O-isotope-induced shifts.[19] The shifts observed in 2-methylpropane-1,2-diol—0.019 ppm for the primary carbon atom and 0.033 ppm for the tertiary carbon atom—are similar to the shifts reported for structurally related alcohols.[2,13,19,20]

The acid-catalyzed hydrolysis of 2,2-dimethyloxirane is quite easy to follow by ^{1}H NMR, and by ^{13}C NMR if a ^{13}C-enriched compound is used. The use of natural-abundance ^{13}C NMR is severely restricted by the relative insolubility of the oxirane in aqueous solutions, although acetone can be added to increase the solubility of the compound. The pseudo-first-order rate constants calculated from the ^{1}H NMR data at 40 °C (Table I) are in general agreement with the values obtained by Long and Pritchard[7a] at 25 °C. (Kinetic deuterium isotope effects[9c,21] on the rates of hydrolysis have not been included in the present rate constant calculations.) Figure 2 clearly demonstrates the extraordinary advantage possessed by the ^{18}O-isotope-induced shift technique in conjunction with ^{13}C enrichment, in comparison to discontinuous mass spectrometric techniques. In a continuous assay mode, it is very easy to follow directly the progress of the hydrolysis reaction; the signals of the isotopically labeled species are well resolved, and therefore it is possible to calculate the pseudo-first-order rate constant for the reaction and simultaneously to determine the position of bond cleavage as well as any accompanying oxygen exchange. The pseudo-first-order rate constants (not corrected for a kinetic deuterium isotope effect[9c,21]) were calculated on the basis of the loss of the oxirane, and these are listed in Table I for the hydrolysis reaction at 32 °C; the rate constants are of similar magnitude to those reported earlier.[7a] A plot of the data for the reaction illustrated in Figure 2 (21 points, slope -4.8×10^{-3} min^{-1}, y intercept 4.60, correlation coefficient 0.998) resulted in a pseudo-first-order rate constant of 8.1×10^{-5} s^{-1}. The isotopic labeling of the primary carbon atom did not change (±1%) upon hydrolysis, and therefore no oxygen exchange accompanied the cleavage of the tertiary carbon atom–oxygen bond of the oxirane. The control reactions demonstrated that in the acid-catalyzed hydrolysis, ^{18}O from the solvent appears on the tertiary alcohol, while in the base-catalyzed hydrolysis, it is the primary carbon atom–oxygen bond of the oxirane that is broken. Oxygen exchange at the tertiary carbon atom of the diol is not observed under these experimental conditions. The results obtained from these reactions support the mechanism of hydrolysis proposed by Long and Pritchard[7a] in which acid-catalyzed hydrolysis of 2,2-dimethyloxirane is accompanied by formation of the stable tertiary carbonium ion whereas base-catalyzed hydrolysis of 2,2-dimethyloxirane involves nucleophilic attack by hydroxide ion at the least sterically hindered position.

Microsomal epoxide hydratase (EC 3.3.2.3) is an important enzyme in the detoxification of potential hydrocarbon carcinogens in animal liver cells. Recent reviews[22] detail the properties of the enzyme. In general, the enzyme cleaves the epoxide (oxirane) substrate at the sterically favored position. However, the oxirane substrates that have been used in these studies have possessed large hydrophobic (lipophilic) groups and little research has been focused on simpler oxiranes as substrates,[23a] except for small, generally

(19) Part 3: Risley, J. M.; Van Etten, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 6699–702.
(20) Diakur, J.; Nakashima, T. T.; Vederas, J. C. *Can. J. Chem.* **1980**, *58*, 1311–5.
(21) Pritchard, J. G.; Long, F. A. *J. Am. Chem. Soc.* **1956**, *78*, 6008–13. Fischer, F.; Koch, H. *Chem. Ber.* **1966**, *99*, 2000–11.
(22) Lu, A. Y. H.; Miwa, G. T. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 513–31. Oesch, F. *Enzymat. Basis Detoxification* **1980**, *2*, 277–90.
(23) (a) Guengerich, F. P.; Mason, P. S. *Anal. Biochem.* **1980**, *104*, 445–51. (b) Oesch, F. *Biochem. J.* **1974**, *139*, 77–88. (c) Hanzlik, R. P.; Walsh, J. S. A. *Arch. Biochem. Biophys.* **1980**, *204*, 255–63.

halogenated, oxiranes that may serve as enzyme inhibitors.[23b,c] A significant problem in the use of small oxiranes as substrates is the analysis of the extent of enzyme-catalyzed hydrolysis. A recently described alcohol dehydrogenase coupled spectrophotometric assay[23a] offers a method whereby smaller oxiranes may be studied as substrates as conveniently as those oxiranes (and diols) containing large lipophilic groups. ^{13}C NMR spectroscopy, particularly in conjunction with ^{13}C-enriched compounds, can potentially facilitate the analyses with small oxiranes as substrates. The synthesis of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane afforded an opportunity to determine whether the smaller oxirane was a substrate, to measure the rate of hydrolysis of a small oxirane, and to deduce the point of bond cleavage for microsomal epoxide hydratase. A microsome preparation of the enzyme from mouse liver and a homogeneous preparation of the enzyme from rat liver were used in the analysis to demonstrate the potential differences between the two preparations of enzyme.

The incubation of mouse liver microsomes (a source rich in epoxide hydratase) (8.6 mg of protein) in the presence of 72 mM 2,2-dimethyl[3-^{13}C,^{18}O] oxirane in 25 mM inorganic phosphate buffer at pH 7.92 results in the hydrolysis of the sustrate to 2-methyl[1-^{13}C,2-^{18}O]propane-1,2-diol.[24] The first-order rate constant for this reaction at 35 °C was obtained from a plot of the first-order rate equation for the hydrolysis of the oxirane (33 points, slope -4.3×10^{-3} min^{-1}, y intercept 4.53, correlation coefficient 0.999) and was 7.21×10^{-5} s^{-1}. (No correction has been made for the presence of deuterium in the solvent, but by analogy with the rat liver enzyme,[9c,25] there should be little or no kinetic deuterium isotope effect.) The complementary reaction is illustrated in Figure 3. The ^{18}O label from the water is easily detected on the primary carbon atom of the product diol. Thus the enzyme in the microsome preparation catalyzes the hydrolysis of the sterically favored carbon–oxygen bond, analogous to substrates possessing large lipophilic groups. In the absence of enzyme, the base-catalyzed hydrolysis of the oxirane is very slow under these experimental conditions and does not measurably contribute to the rate of product formation.

Mouse liver microsomal epoxide hydratase has been purified to homogeneity.[26] The specific activity of the homogeneous preparation was measured at 37 °C with the substrate styrene oxide and is 131 nmol of styrene glycol formed per min per mg of protein; the enzyme is an oligomer made up of polypeptide subunits of unit molecular weight 49 500 that are immunologically identical with homogeneous rat liver microsomal epoxide hydratase. The microsome preparation of the mouse liver enzyme had a specific activity of 0.34 nmol of styrene glycol formed per min per mg of protein and if, by analogy, we assume that the microsomal enzyme used in the hydrolysis reaction of 2,2-dimethyl[3-^{13}C,^{18}O]oxirane would have the same specific activity, then the amount of homogeneous enzyme in the microsome preparation is 0.022 mg.

2,2-Dimethyl[3-^{13}C,^{18}O]oxirane is also a good substrate for homogeneous rat liver microsomal epoxide hydratase. Incubation of the enzyme (0.11 mg of protein) in 25 mM inorganic phosphate buffer (pH 7.98) with 72 mM 2,2-dimethyl[3-^{13}C,^{18}O]oxirane results in the hydrolysis of the substrate to 2-methyl[1-^{13}C,2-^{18}O]propane-1,2-diol as illustrated in Figure 4.[24] On the basis of the ^{18}O-isotope-induced shift of the primary carbon atom, it is evident that the enzyme also catalyzes the hydrolytic cleavage of the least sterically hindered carbon–oxygen bond because the isotope effect is present in the oxirane but absent in the diol (within the limit of detection of the isotopically labeled primary alcohol, which is approximately 10%). The first-order rate constant for the reaction at 35 °C shown in Figure 4 was obtained from a plot of the first-order hydrolysis of the substrate (22 points, slope -5.99×10^{-3} min^{-1}, y intercept 4.58, correlation coefficient 0.999) and was 9.98×10^{-5} s^{-1}. Under identical experimental conditions, the incubation of 0.28 mg of homogeneous rat liver epoxide hydratase with 2,2-dimethyl[3-^{13}C,^{18}O]oxirane at 35 °C resulted in a first-order rate constant of 2.45×10^{-4} s^{-1} (seven points, slope -1.47×10^{-2} min^{-1}, y intercept 4.63, correlation coefficient 0.999). Furthermore, the base-catalyzed hydrolysis of the oxirane in the absence of enzyme does not significantly contribute to the rate of product formation.

Rat liver microsomal epoxide hydratase has been purified to homogeneity from male Long–Evans rats,[18] male Wistar rats,[27] and male Sprague–Dawley rats.[28] The substrate styrene oxide has been used to measure the activity of these preparations where the unit is defined as the formation at 37 °C, pH 9 of 1.0 nmol of styrene glycol per min. The specific activity of the homogeneous enzymes is 465–685 units per mg of protein, and the molecular weight of the individual polypeptide subunit of the oligomers is 49 500.[18,27-29] The specific activity of the preparation of homogeneous rat liver microsomal epoxide hydratase used in our experiments was 499 units per mg of protein, which falls within the range given above. The pH optimum (of the activity) for the purified enzyme is dependent on the substrate used to measure the activity at 37 °C and varies over the pH range 6.9–8.9;[30] the pH used in our experiments is in the middle of this range.

The homogeneous enzymes from mouse liver and from rat liver have virtually identical known physical properties and are distinguishable only by the almost fourfold difference in specific activities of the homogeneous enzymes (in favor of the rat liver enzyme). However, under equivalent experimental conditions, the mouse liver microsomal preparation catalyzed the hydrolysis of the small oxirane significantly more rapidly (approximately four times) than the homogeneous rat liver enzyme. The microsome, and associated phenomena such as diffusion processes, most probably contributes significantly to the observed difference in the rate of hydrolysis of the small oxirane by providing a hydrophobic environment to solubilize the substrate for the enzyme, particularly under the experimental conditions of [S] << K_m used here. More extensive studies on the hydrolysis of the smaller oxirane substrates may be indicated.

Oxirane substrates that possess spectrally active components that undergo detectable changes upon hydrolysis can permit convenient, continuous methods of analysis of the enzyme, in contrast to discontinuous radiometric or mass spectral analysis.[25,31] The alcohol dehydrogenase coupled spectrophotometric assay[23a] of enzyme activity has provided the only alternate method of analysis for continuously monitoring the formation of product with substrates lacking absorbing or emitting groups. ^{1}H NMR spectroscopy can potentially be used to follow the enzyme-catalyzed hydrolysis of an oxirane substrate, although the ^{1}H resonance signal from water can interfere significantly with the analysis. A combination of ^{13}C-enriched substrates and ^{13}C NMR spectroscopy circumvents the problem inherent with ^{1}H NMR and, as we have demonstrated, may conveniently be used to measure

(24) The apparent Michaelis constant, K_m, for this substrate, and for most small substrates, is not known. Using ^{1}H NMR, we attempted to measure the K_m for isobutylene oxide. However, at substrate concentrations five to eight times the concentration (72 mM) used in these experiments, apparent first-order hydrolyses of the substrate both by the microsomal preparation and by the homogeneous enzyme were still observed. The limited solubility of the substrate in aqueous solutions precluded an accurate determination of K_m by going to yet higher substrate concentrations. However it is apparent from these data that a substrate concentration of 72 mM is still much less than K_m, and therefore an apparent first-order hydrolysis reaction should be observed. Assumptions regarding the magnitude of K_m and of the extent of saturation of such an enzyme are not without danger (Bellucci, G.; Berti, G.; Bianchini, R.; Cetera, P.; Mastrorilli, E. *J. Org. Chem.* **1982**, *47*, 3105–12). The presence of acetone should not inhibit the enzyme because water-miscible organic solvents are used to dissolve oxirane substrates in normal assay procedures; in fact, acetone helped the rapid dissolution of the oxirane and acetone is known not to inhibit purified rat liver epoxide hydratase.[23b]

(25) Westkaemper, R. B.; Hanzlik, R. P. *Arch. Biochem. Biophys.* **1981**, *208*, 195–204.

(26) Knowles, R. G.; Burchell, B. *Biochem. Soc. Trans.* **1977**, *5*, 731–2.

(27) Knowles, R. G.; Burchell, B. *Biochem. J.* **1977**, *163*, 381–3.

(28) Guenthner, T. M.; Bentley, P.; Oesch, F. *Methods Enzymol.* **1981**, *77*, 344–9.

(29) Lu, A. Y. H.; Thomas, P. E.; Ryan, D.; Jerina, D. M.; Levin, W. *J. Biol. Chem.* **1979**, *254*, 5878–81.

(30) Lu, A. Y. H.; Jerina, D. M.; Levin, W. *J. Biol. Chem.* **1977**, *252*, 3715–23.

(31) Dansette, P. M.; DuBois, G. C.; Jerina, D. M. *Anal. Biochem.* **1979**, *97*, 340–5.

the rate of the hydrolysis reaction. The alcohol dehydrogenase, coupled spectrophotometric assay may be used with little difficulty and with confidence at very small concentrations of substrate (<5 mM), whereas higher concentrations of substrate must be used in ^{13}C NMR in order to obtain reliable data. Thus, ^{13}C NMR may best be utilized in experimental situations where specific, clearly defined problems are conveniently and directly resolved such as the position of bond cleavage illustrated in the present study. At this time, ^{13}C NMR is probably not suitable for routine analyses of epoxide hydratase activity.

The ^{18}O isotope effect in ^{13}C NMR spectroscopy provides a continuous, direct method to evaluate simultaneously the rate of hydrolysis, the position of bond cleavage, and the extent of accompanying oxygen exchange in acid- and microsomal epoxide hydratase-catalyzed hydrolysis of 2,2-dimethyloxirane. This example further illustrates the applicability of this phenomenon in the analysis of a variety of research problems.

Acknowledgment. This investigation was supported by USPHS Research Grant GM 27003-10 from the National Institute of General Medical Sciences and by NIH Grant RR 01077 from the division of Research Resources. We thank Donna Radzik and Susan Hommel of Purdue University for a gift of mouse liver microsomes and Dr. Anthony Y. H. Lu of Merck Sharp & Dohme for the generous gift of homogeneous rat liver microsomal epoxide hydratase.

Registry No. ^{18}O, 14797-71-8; 2,2-dimethyloxirane, 558-30-5; epoxide hydratase, 9048-63-9; 2,2-dimethyl[3-^{13}C]oxirane, 84624-88-4; 2,2-dimethyl[3-^{13}C,^{18}O]oxirane, 84624-89-5; [^{13}C]methyldiphenylsulfonium perchlorate, 84624-91-9; acetone, 67-64-1; [^{18}O]acetone, 7217-26-7.

Communications to the Editor

Sequential Assignments for the ^1H and ^{31}P Atoms in the Backbone of Oligonucleotides by Two-Dimensional Nuclear Magnetic Resonance

Arthur Pardi,*‡ Roger Walker,† Henry Rapoport,† Gerhard Wider,§ and Kurt Wüthrich‡

Institut für Molekularbiologie und Biophysik
Eidgenössische Technische Hochschule
Hönggerberg, CH-8093 Zürich, Switzerland
Department of Chemistry and Lawrence Berkeley Laboratory
Univeristy of California, Berkeley
Berkeley, California 94720
and Spectrospin AG, CH-8117 Fällanden, Switzerland

Received November 10, 1982

A novel application of two-dimensional nuclear magnetic resonance (2-D NMR) for assignment of hydrogen and phosphorus nuclei in the sugar phosphate backbone of oligonucleotides is described and illustrated by the assignment of the tetranucleotide d-CpTpApG. The assignments are made by observation of homonuclear (^1H–^1H) and heteronuclear (^1H–^{31}P) scalar spin–spin couplings.

Proton NMR has been extensively used to study the conformation and dynamics of oligonucleotides in solution.[1] Although the coupling constants for the protons in the sugar rings provide information on the sugar and phosphate backbone conformation,[2] the difficulties involved in assigning these protons have limited the applications. 2-D NMR experiments overcome many of the problems of selective decoupling and extensive overlap of resonances observed in conventional one-dimensional studies. The approach of sequential assignments outlined here allows the complete assignment of the sugar phosphate backbone solely from the 2-D NMR experiments and knowledge of the covalent structure of the backbone.

The first step in the assignment procedure involves the identification of the proton spin systems of the individual sugar rings.

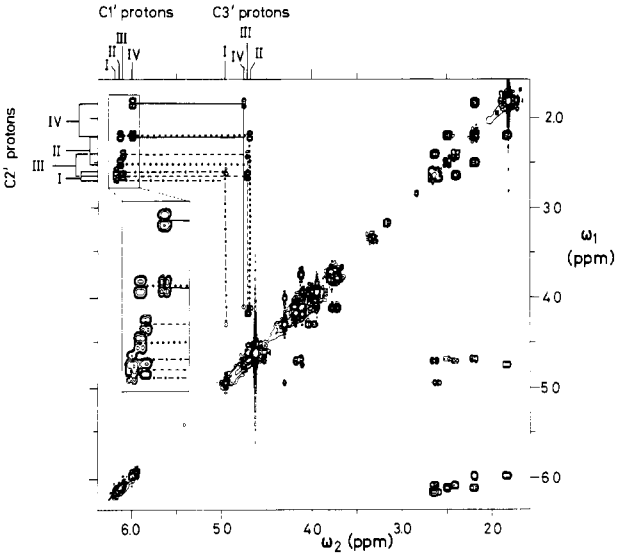

Figure 1. Contour plot of an absolute value 500-MHz ^1H COSY spectrum of 0.02 M d-CpTpApG in ^2H_2O, p^2H 8.0, T = 40 °C. The C1′C2′ proton cross peaks are also shown on an expanded scale in the inset. The chemical shifts of the C1′, C2′, and C3′ protons are indicated on the margins, where the four deoxyribose spin systems are arbitrarily labeled I (···), II (···), III (- - -), and IV (—).

This information was obtained with homonuclear correlated spectroscopy (COSY).[3,4] COSY spectra for d-CpTpApG[5] are shown in Figures 1 and 2. J connectivities between individual protons are manifested by cross peaks which appear symmetrically with respect to the diagonal. The deoxyribose spin system, which includes the lowest field C1′ proton at 6.16 ppm, was arbitrarily labeled "sugar I". It shows cross peaks to C2′ protons at 2.60 and 2.68 ppm (Figure 1). The C2′ protons then show coupling to the C3′ proton at 4.95 ppm (Figure 1), and this C3′ proton has

‡ETH Zürich.
†University of California, Berkeley.
§Spectrospin AG.

(1) (a) For a recent review see: Sarma, R. H. "Nucleic Acid Geometry and Dynamics"; Pergamon Press: New York, 1980. (b) Cheng, D. M.; Kan, L.-S.; Leutzinger, E. E.; Jayaraman, K.; Miller, P. S.; Ts'o, P. O. P. *Biochemistry* **1982**, *21*, 621–630. (c) Altona, C.; van Boom, J. H.; Haasnoot, C. A. G. *Eur. J. Biochem.* **1982**, *71*, 557–562. (d) Melleman, J. R.; Haasnoot, C. A. G.; van Boom, J.; Altona, C. *Biochim. Biophys. Acta* **1982**, *665*, 256–264. (e) Neumann, J.-M., Huynh-Dinh, T.; Kan, S. K.; Genissel, B.; Igolen, J.; Tran-Dinh, S. *Eur. J. Biochem.* **1982**, *121*, 317–323.

(2) Cheng, D. M.; Sarma, R. H. *J. Am. Chem. Soc.* **1977**, *99*, 7333–7348.

(3) (a) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2220–2246. (b) Nagayama, K.; Anil Kumar; Wüthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1980**, *40*, 321–334. (c) Bax, A.; Freeman, R.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 164–168.

(4) Detailed descriptions of the experimental parameters for the 2-D experiments are given in the supplementary material.

(5) (a) This tetramer was synthesized by the phospho triester method using *N*-benzyloxycarbonyl-blocked nucleosides in solution (see ref 6b and 6c). (b) Watkins, B. E.; Kiely, J. S.; Rapoport, H. *J. Am. Chem. Soc.* **1982**, *104*, 5702–5708. (c) Watkins, B. E., Rapoport, H. *J. Org. Chem.* **1982**, *47*, 4471.

0002-7863/83/1505-1652$01.50/0 © 1983 American Chemical Society