Cephalothin: Hydrolysis of the C-3′-Acetoxy Moiety of a 7-Aminocephalosporanic Acid; Observation of Both Acyl-Oxygen Bond Cleavage and Reversible Alkyl-Oxygen Bond Cleavage

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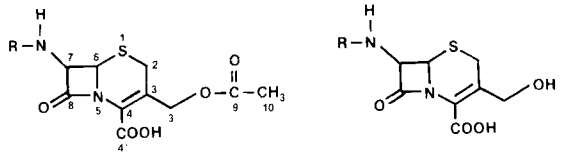
Abstract □ The aqueous solution chemistry of the C-3′-acetoxy moiety of cephalothin sodium (**1b**) was examined with the use of isotopically labeled $H_2^{18}O$ and [2-^{13}C]acetate anion. The ^{18}O incorporation studies indicate that the hydrolysis (at pH 4.7 ± 0.1) of **1b** to the deacetyl derivative of cephalothin (**2b**) proceeds via two pathways: alkyl-oxygen bond cleavage (55–63%) and acyl-oxygen bond cleavage accounting for the remainder. The incorporation of [2-^{13}C]acetate into **1b** suggests that the alkyl-oxygen cleavage pathway is a reversible reaction.

The chemical and/or enzymatic conversion of cephalosporin C[1] (**1a**) and of several clinically useful *N*-acylated 7-aminocephalosporanic acids, cephalothin[2] (**1b**), cephaloglycin[3,4] (**1c**), and cefotaxime[5,6] (**1d**), into the corresponding deacetyl analogues **2** and lactones **3** has been described. There are two pathways by which the nonenzymatic hydrolysis of these cephalosporins, or of any ester, may proceed. The more common path involves acyl-oxygen bond cleavage, but alkyl-oxygen bond cleavage may occur if the intermediate carbonium ion is particularly stable.[7]



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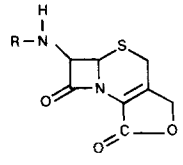

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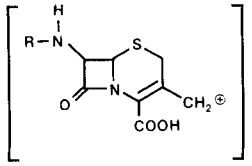

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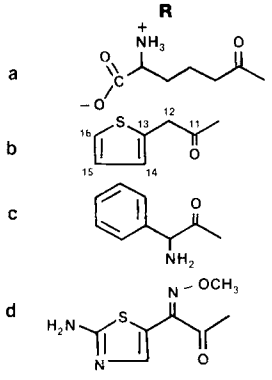

The mechanism for the aqueous formation of a deacetyl derivative of a 7-aminocephalosporanic acid from an acylated 7-aminocephalosporanic acid has received study only indirectly. Cocker et al.[8] and Taylor[9] suggested the existence of a resonance-stabilized allylic cation **4** (which would result from alkyl-oxygen bond cleavage) to explain sulfur and nitrogen nucleophilic displacement reactions at the allylic carbon in aqueous solution. More recently, Hatfield et al.[10] suggested that **4** is also an intermediate in similar nonaqueous nucleophilic displacements. Neither Cocker nor Hatfield, however, were able to isolate any of the β-lactam-containing products which could have been expected from reactions between **4** and oxygen nucleophiles such as alcohols or [CD_3]acetic acid. The only observed reaction was destruction of the β-lactam ring. For this reason, Hatfield et al. proposed that, when a deacetyl derivative of a 7-aminocephalosporanic acid **2** and its subsequent cyclization product, lactone **3**, form under aqueous conditions, they do so via acyl-oxygen bond cleavage and not alkyl-oxygen bond cleavage or allylic cation **4**.

We have reexamined the aqueous solution chemistry of the C-3′-acetoxy group of cephalothin sodium (**1b**) with isotopically labeled $H_2^{18}O$ and [2-^{13}C]acetate anion. The reaction conditions resembled those in aged parenteral cephalosporin solutions where deacetyl products (**2**) and their lactones (**3**) are known to be major degradation products.

Experimental Section

Cephalothin sodium (various bulk production lots, Eli Lilly & Co., Indianapolis IN) was used as supplied. Water-^{18}O (97 atom percent O-18) and [2-^{13}C]acetic acid (90 atom percent C-13) were obtained from MSD Isotopes (Merck Sharpe & Dohme Canada Ltd., Montreal). All other materials were reagent grade and were used as received. Electron impact (EI) and field desorption (FD) mass spectra were determined on a Varian-MAT 731 mass spectrometer, and the fast-atom bombardment (FAB) mass spectra were determined on a Varian VG-ZAB-3F mass spectrometer. The ^{13}C NMR spectra were recorded on a Brucker WM250 NMR spectrometer with an Aspect 2000 computer. Analysis of the cephalothin sodium degradation mixture was performed on an HPLC system composed of a Waters 6000A pump, a Rheodyne 7125 injector fitted with a 20-μL loop, a Lichrosorb RP-18 10-μm column, and a Tracor 970A detector set at 260 nm. The mobile phase contained methanol:buffer (30:70, v/v). The buffer was prepared by adding 24 ml of glacial acetic acid to 2.1 L of water and adjusting the pH to 3.5 with sodium acetate. The flow rate was 2.0 mL/min.

Isolation of Cephalothin Lactone 3b Prepared from Cephalothin Sodium (1b) in $H_2^{18}O$—Cephalothin sodium (**1b**) (125 mg) was dissolved in 0.5 mL of $H_2^{18}O$, and the solution was allowed to react in a screw-capped glass vial for 14 days at room temperature (~25°C). At that time, the precipitated crystalline cephalothin lactone (**3**) (~8 mg) was removed by filtration, washed with a few drops of $H_2^{18}O$, and air dried. The experiment was repeated three times, and the percent ^{18}O incorporation was determined each time by a different MS technique.

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Isolation of Cephalothin Lactone 3b Prepared from the Deacetyl Derivative of Cephalothin 2b in $H_2^{18}O$—The deacetyl derivative of cephalothin sodium[11] (**2b**) (100 mg) was dissolved in 1 mL of $H_2^{18}O$, and the solution was allowed to react in a screw-capped glass vial for 7 d at room temperature (~25°C). At that time, the precipitated crystalline cephalothin lactone **3b** (~5 mg) was removed by filtration, washed with a few drops of $H_2^{18}O$, and air dried. The percent ^{18}O incorporation was determined by FD and FAB MS.

Recovery of Crystalline Cephalothin Sodium (1b) Equilibrated in $H_2^{18}O$—Cephalothin sodium (**1b**) (250 mg) was dissolved in 1.0 mL of $H_2^{18}O$, and the solution was allowed to equilibrate for 4 d at room temperature (~25°C). At that time, 200 mg of sodium chloride (less than 100 mesh) was added with rapid stirring to the clear solution. The cephalothin sodium (**1b**) crystallized within a few minutes, and the crystals (120 mg) were removed by filtration and air dried. In a second experiment, 250 mg of cephalothin sodium (**1b**) was dissolved in 1.0 mL of $H_2^{18}O$, and the solution was allowed to equilibrate for 14 d (~25°C). On d 7 and 14, 0.5-mL aliquots were decanted from the crystalline cephalothin lactone **3b** now present in the mixture. The cephalothin sodium (**1b**) in the aliquots was precipitated by the above salting-out technique. The **1b** isolated on d 7 was crystalline, but that isolated on d 14 was an oil that failed to crystallize. The percent ^{18}O incorporation was determined by FD and/or FAB MS.

^{18}O Incorporation Analysis by Mass Spectrometry—EI measurements were made at 70 eV of ionizing energy by using a Varian-MAT 731 mass spectrometer with direct sample introduction to the ion source. FD spectra were obtained on the same instrument with carbon dendrite emitters and an emitter–extraction plate potential of 11 keV. FAB spectra were obtained from a VG ZAB-3F mass spectrometer using bombardment with 8 keV xenon atoms.

For FD, samples were dissolved in methanol, except for the sodium salt of cephalothin, which was dissolved in ammonium chloride solution; this yielded a molecular ion for the acid (m/z = 396). For FAB, samples were dissolved in glycerol or a mixture of five parts dithiothreitol and one part dithioerythritol ("magic bullet").

In determining peak heights to calculate isotopic ratios, the mass spectrometer collector slit was opened sufficiently to produce flat-topped peaks but not far enough to reduce the resolution, so as to give peak height interference from adjacent peaks. Peaks were recorded with either an oscillographic recorder (EI and FD) or an electrostatic recorder (FAB). Amplifier response time was 30 ms.

For unlabeled compounds, the ratios [(m + 1)/m] and [(m + 2)/m] for EI and FD spectra, and [(mH + 1)/m] and [(mH + 2)/m] for FAB spectra were in excess of the corresponding ratios calculated for the occurrence of natural isotopes.[12] Thus, in all three types of spectra, there took place multiple hydrogen addition which needed to be accounted for in the determination of ^{18}O in labeled compounds. Such multiple hydrogen addition to mH peaks to give (mH + H) and (mH + 2H) peaks in FAB spectra has recently been studied by Cerny and Gross,[13] who showed that for some compounds it leads to marked deviations from theoretically determined isotope patterns.

In order to overcome the effect of multiple hydrogen addition on ^{18}O determinations, experimentally determined peak height ratios for unlabeled compounds were used to correct the peak heights in the spectra of the corresponding ^{18}O-labeled compound. Implicit in this approach is the assumption that the amount of hydrogen addition is the same in both experiments. An internal check of this assumption is possible. Referring to Figs. 1A and 1B, if the ratio P_{338}/P_{337} for the unlabeled compound is used to correct the height of P_{338} in the labeled compound, a result of 0 should be obtained; i.e., all of P_{338} is due to isotopic P_{337} or calculable hydrogen addition. The result actually obtained is 5 (P_{339} = 100). The discrepancy is explained, however, by noting that, in the unlabeled compound, m/z 336 is 6% of m/z 337; taking 6% of P_{339} in the labeled compound and subtracting it from P_{338} essentially reduces P_{338} to 0. This shows the same degree of hydrogen addition in both experiments. Additionally, the good agreement among the values for ^{18}O incorporation into **3b** obtained by EI, FD, and FAB determinations speaks well to the accuracy of the ^{18}O determination.

Recovery of Crystalline Cephalothin Sodium (1b) from Aqueous Solutions Containing [2-^{13}C]Acetate Anion—[2-^{13}C]Acetic acid (0.5 g, 8.2 mmol), was added to 3 mL of distilled water, and the pH of the solution was adjusted to 5.2 with ~1.2 mL of 5 M KOH. Cephalothin sodium (**1b**) (1.18 g, 2.8 mmol) was dissolved (with the aid of sonication) in the above solution, and the mixture was allowed to equilibrate at room temperature (~25°C). On the following day and on d 4, 0.5-mL aliquots of the reaction solution were mixed with 100 mg of NaCl (less than 100 mesh) with rapid stirring. The cephalothin sodium (**1b**) crystallized within a few minutes, and the crystals were removed by filtration and air dried. On d 10 and 14, 1- and 2.5-mL aliquots, respectively, were decanted from the crystalline cephalothin lactone **3b** now present in the solution. The cephalothin sodium (**1b**) contained in the aliquots was crystallized by the above salting-out technique.

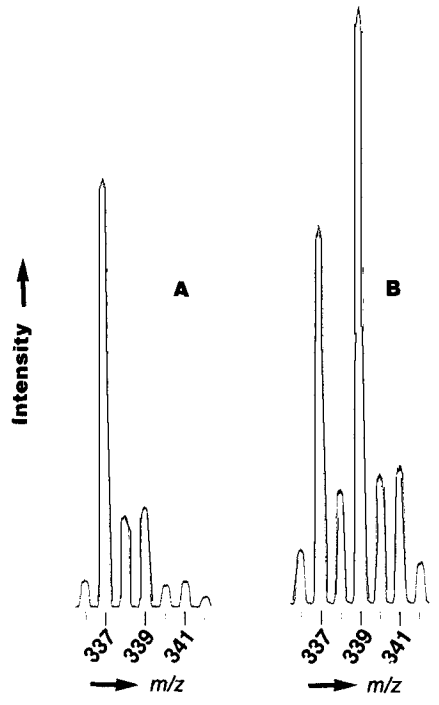


Figure 1—*Partial fast-atom bombardment mass spectra [near m + H ion] for* (A) *unlabeled cephalothin lactone (**3b**) and* (B) *isotopically labeled cephalothin lactone (**3b**) (isotopic mix: ~45% natural abundance and ~55% containing an ^{18}O atom).*

[2-^{13}C]Acetate Incorporation Analysis by ^{13}C Nuclear Magnetic Resonance Spectroscopy—The isotopic abundances were determined with ^{13}C NMR spectroscopy. Peak assignment was based upon published data for cephalothin sodium (**1b**)[14]. The samples were dissolved in H_2O with a small amount of D_2O added for a lock signal. Internal dioxane was used as a reference. The data were collected with a sweep width of 12,500 Hz, an acquisition time of 0.65 s, a data block of 16K, and a 45° pulse width. The FID was processed with a Lorentzian filter of 0.5 Hz. The number of acquisitions varied from 6,000 to 90,000 obtained with no delay between acquisitions. Although the spectra obtained under these experimental conditions were saturated and theoretically not ideal for obtaining quantitative ^{13}C results, the accuracy was considered to be adequate since the goal of the experiment was simply to demonstrate acetate exchange. Incorporation studies were also attempted with [1-^{13}C]acetic acid. Inconsistent data were obtained, however, possibly because of sensitivity of the carbonyl ^{13}C carbon relaxation time to experimental variables. In contrast, [2-^{13}C]acetate with protonated ^{13}C carbon gave consistent results.

In Fig. 2, the individual ^{13}C resonance peaks are not of the same magnitude because of differences in relaxation rate. If the experimental parameters of the NMR experiment are kept constant, however, then the ratio of the integrals for ^{13}C resonance peaks, such as peaks C-10/C-2, is a measurable constant (0.392), which may be expressed mathematically as:

$$\frac{\mathrm{INTG_{C\text{-}10}}}{\mathrm{INTG_{C\text{-}2}}} = \frac{\%\ ^{13}\mathrm{C\ at\ C\text{-}10} \times K_{\mathrm{C\text{-}10}}}{\%\ ^{13}\mathrm{C\ at\ C\text{-}2} \times K_{\mathrm{C\text{-}2}}} = 0.392 \qquad (1)$$

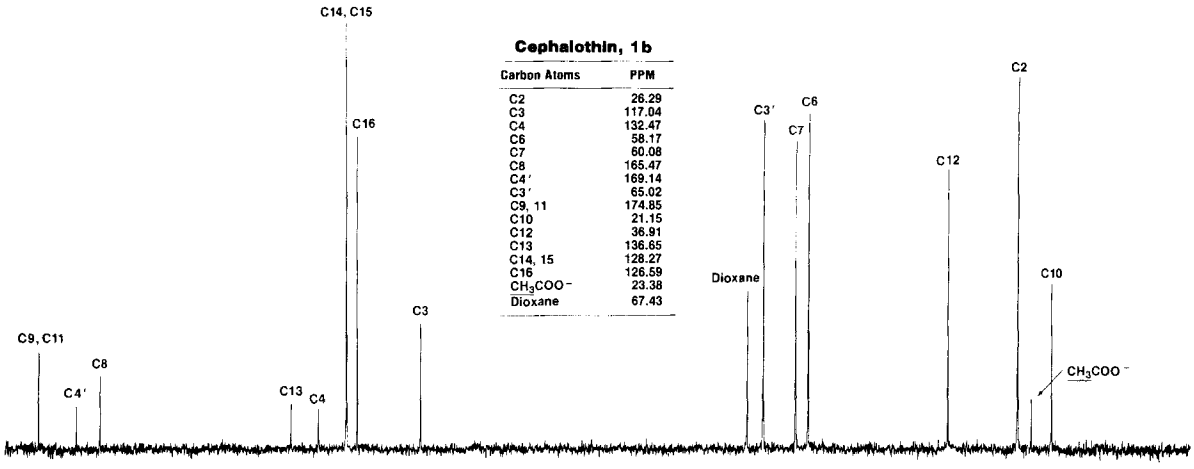


Figure 2—*^{13}C NMR spectrum (D_2O/H_2O) of cephalothin sodium natural abundance ^{13}C.*

where $INTG_{C\text{-}10}$ and $INTG_{C\text{-}2}$ are the integrals for peaks C-10 and C-2, respectively, and $K_{C\text{-}10}$ and $K_{C\text{-}2}$ are constants that reflect both the relaxation rate of C-10 and C-2 and the experimental conditions of the NMR experiment. Since the ^{13}C-labeled carbon content for all carbon atoms of unlabeled cephalothin is the same, 1.108%, then:

$$\frac{K_{C\text{-}10}}{K_{C\text{-}2}} = 0.392 \tag{2}$$

For the case of equilibrated cephalothin samples (1 to 10 d) where an atom percent excess in ^{13}C may be possible at C-10, an equation similar to eq. 1 may be written:

$$\frac{INTG_{C\text{-}10}\ (\text{exchangeable})}{INTG_{C\text{-}2}\ (\text{nonexchangeable})} = \frac{\text{percent } ^{13}C \text{ at C-10} \times K_{C\text{-}10}}{\text{percent } ^{13}C \text{ at C-2} \times K_{C\text{-}2}} \tag{3}$$

where the percent ^{13}C at C-2 is, again, natural abundance, 1.108%. Rearrangement of eq. 3 and substitution of eq. 2 allows for the empirical calculation of the atom percent excess of ^{13}C at C-10:

$$\%\ ^{13}C \text{ at C-10} = \frac{1.108\%}{0.392} \times \frac{INTG_{C\text{-}10}\ (\text{exchangeable})}{INTG_{C\text{-}2}\ (\text{nonexchangeable})} \tag{4}$$

Results and Discussion

Hydrolysis of the C-3′-Acetoxy Moiety of Cephalothin Sodium (1b)—The classic arguments for distinguishing between alkyl-oxygen and acyl-oxygen bond cleavage in an ester hydrolysis performed in ^{18}O-labeled water[7] apply to the hydrolysis of the C-3′-acetoxy moiety of cephalothin (Scheme I). If alkyl-oxygen cleavage occurs, then the intermediate allylic cation 4 will react with the labeled solvent, $H_2^{18}O$, and an increase in mass of 2 will occur in the alcohol fragment, the deacetyl derivative of cephalothin (**2b**). If acyl-oxygen cleavage occurs, then the ^{18}O label will be found in the acetate fragment and there will be no increase in mass of **2b**. In the present case, when subsequent intramolecular lactonization occurs, the isotopic distribution of the C-3′-oxygen atoms of **2b** will be retained in the lactone **3b**. Cephalothin lactone **3b** was chosen for examination of ^{18}O incorporation because it crystallizes directly from the mixture and may be isolated by filtration. Isolation of **2b** would have required chromatography, thereby raising the possibility of ^{18}O loss. The time course of cephalothin (**1b**), the deacetyl derivative of cephalothin (**2b**), and cephalothin lactone **3b** concentrations in the reaction mixture are listed in Table I.

Figure 1 shows the partial FAB mass spectra of cephalothin lactone **3b**, prepared in both H_2O and $H_2^{18}O$, from cephalothin sodium (**1b**). It is clear from these spectra that ^{18}O incorporation has occurred. The degree of incorporation was approximated from the relative peak heights after appropriate corrections. Similar spectra were obtained by FD and EI methods, and the degree of incorporation was once again approximated from the relative peak heights after appropriate corrections. The calculated percentage of ^{18}O incorporation, determined by the three different mass spectral methods on three different samples of **3b** prepared at different times, is shown in Table 2. Essentially the same result, ~55–63% incorporation of one ^{18}O atom, was obtained in all three experiments.

The ^{18}O incorporation argument in Scheme I is based upon the assumptions that only allylic cation 4 can react with the labeled $H_2^{18}O$ and that none of the other compounds in Scheme I (**1b**, **2b**, or **3b**) will undergo exchange at a significant rate. The alternate possibilities for ^{18}O exchange in Scheme I therefore need to be considered. (*a*) Oxygen exchange at the C-4′-carboxylic acid of either **1b** or **2b** is a possibility, but carboxylic acids in general exchange by

Table I—Time Courses for Cephalothin (1b) the Deacetyl Derivative of Cephalothin (2b) and Cephalothin Lactone (3b) during the Degradation of Cephalothin Sodium ~25% in H_2O at 25°C[a].

Time, days	Conc., mg/mL			pH
	1b	**2b**	**3b**	
0	221	1.6	0.2	5.5[b]
1	207	5.6	0.5	4.8
2	179	7.9	1.2	4.7
4	155	11.8	3.2	4.6
7	116	14.3	~3.2[c]	4.7
11	81.5	13.0	~3.2	4.7
14	64.6	11.1	~3.2	4.7

[a] Experimental values were measured by HPLC. [b] The pH of the mixture dropped from an initial value of 5.5 to 4.9 in 8 h when the system was internally buffered (RCOONa/CH_3COOH) upon the liberation of acetic acid from **1b**. [c] For time points 7, 11, and 14 d, the reported values represent only **3b** in solution and do not take into account precipitated crystalline **3b**.

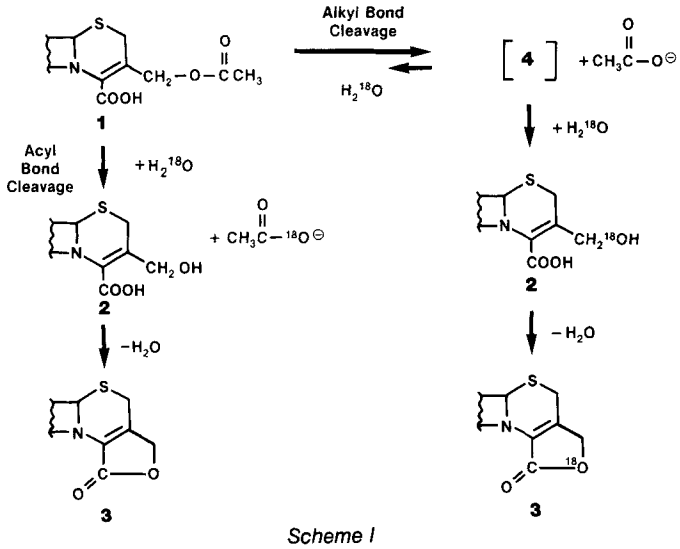
Table II—Incorporation of ^{18}O as Determined by MS[a]

Compound	Method of Preparation	Mass Spectral Method	Percentage of Incorporation of an ^{18}O Atom
1b	Recovered from $H_2{}^{18}O$ after 4 d	FD	≅6
		FAB	≅1
1b	Recovered from $H_2{}^{18}O$ after 7 d	FAB	≅0
1b	Recovered from $H_2{}^{18}O$ after 14 d	FAB	≅1
3b	Prepared from **2b** in $H_2{}^{18}O$	FD	≅5
		FAB	≅8
3b	Prepared from **1b** in $H_2{}^{18}O$	FD	≅55
		EI	≅63
		FAB	≅55

[a] Abbreviations and compound numbers: **1b**, cephalothin sodium; **2b**, deacetyl derivative of cephalothin; **3b**, cephalothin lactone; FD, field desorption; FAB, fast-atom bombardment, EI, electron impact.

nucleophilic attack of solvent–water on protonated carboxylic acid,[15] and the concentrations of such protonated species are negligible at the pH range, 4.7–5.5, encountered in the present reactions. (*b*) Oxygen exchange at the side-chain C-11-carbonyl carbon of **1b**, **2b**, or **3b** is also possible, although there is usually inappreciable oxygen exchange between solvent and substrate during the acidic hydrolysis of amides.[7] (*c*) Oxygen exchange might even be possible during the intramolecular lactonization of **2b** to **3b**, but a mechanism for ^{18}O exchange is unclear.

Although ^{18}O incorporation via these alternate mechanisms was considered to be minor for the reasons given above, two control ^{18}O exchange experiments were performed to approximate ^{18}O incorporation into molecules of Scheme I under conditions in which allylic cation **4** could not be a source of ^{18}O incorporation. First, to test the possibility of ^{18}O incorporation into cephalothin **1b** prior to its conversion to the deacetyl derivative of cephalothin **2b**, **1b** was dissolved in $H_2^{18}O$ and then isolated from the $H_2^{18}O$ solution after 4, 7, and 14 d of equilibration. The FAB mass spectral data (Table II) suggest little or no ^{18}O incorporation into any of the isolated cephalothin samples. (The FD mass spectral data, to be sure, suggest a trace of incorporation, ~6%, but quantitation of such a low level of uncorroborated incorporation should be viewed with caution.) Second, to test the possibility of ^{18}O incorporation into the deacetyl derivative of cephalothin **2b** prior to or during its lactonization to cephalothin lactone **3b**, unlabeled **2b** was dissolved in $H_2^{18}O$ and allowed to convert to **3b**. Both the FD and the FAB mass spectral data (Table II) on the isolated **3b** suggest ~5–8% incorporation of ^{18}O in this step of the reaction sequences in Scheme I. Although these control ^{18}O exchange experiments do not identify which of the three proposed alternate ^{18}O incorporation mechanisms are operative, they do demonstrate that ^{18}O incorporation by these mechanisms is minor relative to the 55–63% ^{18}O incorporation which occurs where cation **4** is a possible intermediate. The minor amount of ^{18}O incorporation by the alternate mechanisms has been ignored in the calculation of the relative importance of alkyl-oxygen versus acyl-oxygen bond cleavage in Scheme I because of the uncertainty in the quantitation of the low ^{18}O levels and because such a calculation would not change the conclusion that both pathways in Scheme I are significant.



Scheme I

Although both pathways in Scheme I are significant at the chosen reaction conditions (pH 4.7 ± 0.1), these pathways are competing reactions, the rates of which may not respond in the same way to changes in pH: the rate of alkyl-oxygen bond cleavage is relatively insensitive to pH (except for the inductive effect of an ionized versus an un-ionized C-4 carboxylic acid moiety, pK_a ~2.6)[9], whereas the rate of acyl-oxygen bond cleavage should be subject to general acid or base catalysis. The relative importance of each pathway in Scheme I may therefore change as experimental conditions are changed.

Aqueous Exchange of [2-^{13}C]Acetate Anion in Cephalothin (1b). The reversibility of the alkyl bond cleavage pathway in Scheme I was investigated with the use of isotopically labeled [2-^{13}C]acetate anion and ^{13}C NMR spectroscopy. Any incorporation of [2-^{13}C]acetate into cephalothin (**1b**) must occur via alkyl bond cleavage and cation **4**, because **1b** was not formed (in a control experiment) when the deacetyl derivative of cephalothin **2b** (the product of acyl bond cleavage) was allowed to react with acetate anion. The ^{13}C NMR

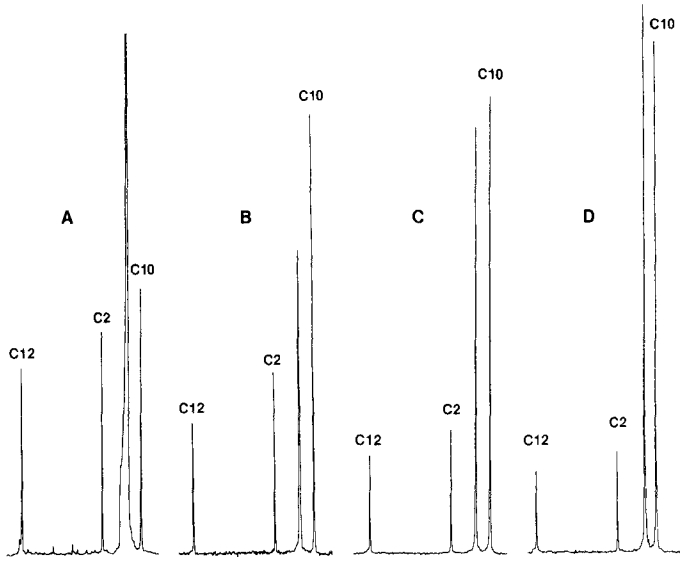


Figure 3—*Partial ^{13}C NMR spectra (D_2O/H_2O) (region carbon resonances C-12 to C-10) of cephalothin sodium exposed to aqueous [2-^{13}C]acetate anion. Key: reaction time A, 1 d; B, 4 d; C, 7 d; D, 10 d. The resonance peak which appears in each spectrum between resonance peaks for C-10 and C-2 is due to traces of [2-^{13}C]acetate anion, which contaminate all the recovered crystalline cephalothin sodium samples. Peak heights for the nonexchanged C-12 and C-2 vary in spectra A to D because no attempt was made to normalize recorded peak heights since it is the ratio of resonance peak heights C-2 to C-10 in each spectrum that reflects ^{13}C isotopic compositions.*

spectra of natural abundance cephalothin (1.108% ^{13}C) is shown in Fig. 2. Because the resonance peak for C-10 (21.15 ppm), the methyl carbon of the acetate moiety of cephalothin, is clearly resolved from the other cephalothin carbon atoms as well as from the methyl carbon of free acetate anion (23.38 ppm), the ratio of the peak height for C-10 to that of a nonexchangeable carbon atom such as C-2 (26.29 ppm) can easily be measured. Accordingly, the percentage of [2-^{13}C]acetate incorporation was approximated by comparing the ratio of peak heights C-10/C-2 for natural abundance cephalothin with the ratio of peak heights C-10/C-2 for cephalothin (0.53 M) exposed to [2-^{13}C]acetate anion (1.55 M) in aqueous solution at 25°C (Fig. 3). After 1, 4, 7, and 10 d of equilibration, 2.5, 5.7, 10.3, and 14.6%, respectively, excess ^{13}C at C-10 was observed, for an average incorporation rate of ~1.4% per day.

Conclusions

The conversion of cephalothin sodium **1b** into the deacetyl derivative of cephalothin **2b** proceeds by both pathways shown in Scheme I. Hydrolysis experiments (pH 4.7 ± 0.1, 25°C) in isotopically labeled $H_2^{18}O$ suggest that ~55–63% of the reaction occurs via alkyl-oxygen cleavage or allylic cation **4** and the remaining via acyl-oxygen cleavage. Exchange experiments between **1b** and isotopically labeled [2-^{13}C]acetate anion suggest that the alkyl-oxygen cleavage pathway in Scheme I is a reversible reaction. Similar chemistry may be expected with other *N*-acylated 7-aminocephalosporanic acids.

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