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Monitoring β-Lactamase Activity In Vivo by ^{13}C Nuclear Magnetic Resonance Spectroscopy

SHAHRIAR MOBASHERY,[1]† STEPHEN A. LERNER,[2] AND MICHAEL JOHNSTON[1,3]*

Departments of Chemistry[1] and of Biochemistry and Molecular Biology,[3] Searle Chemistry Laboratory, University of Chicago, Chicago, Illinois 60637, and Department of Medicine, Wayne State University, Detroit, Michigan 48201[2]

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A ^{13}C-labeled cephalothin, 7β-(2-thienylacetamido)-3-[acetoxy-$^{13}C_1$]methyl-3-cephem-4-carboxylate (compound 1), has been prepared and used to monitor β-lactamase activities by ^{13}C nuclear magnetic resonance spectroscopy. Time-elapsed spectral analysis of the reaction of the labeled cephalothin with the TEM-2 β-lactamase purified from *Escherichia coli* revealed the progressive loss of the cephalothin acetyl resonance at 176.8 ppm and accumulation of an acetate signal at 184.3 ppm. Spectral results identical to those observed in the in vitro experiment were obtained when compound 1 was incubated with cell suspensions of *E. coli* JSR-O (pBR322), which contains the plasmid-encoded TEM-2 β-lactamase, and *Enterobacter cloacae* strains that contain a class I chromosomal β-lactamase. Pseudo-first-order rate constants for the lactamase-catalyzed formation of acetate from cephalothin in vivo were obtained by integration of the ^{13}C-acetyl resonances of compound 1 during timed incubations with cell preparations. These results constitute the first demonstration of the ability to monitor β-lactamase activity in viable cells by nuclear magnetic resonance spectroscopy.

In the course of examining the mechanisms of action of a novel set of cephalothin-derived antibacterial agents (14, 16, 17), we required a noninvasive method for monitoring β-lactamase-dependent fragmentation of cephems in intact, metabolically active microorganisms. It occurred to us that ^{13}C nuclear magnetic resonance (NMR) spectroscopy might provide an approach to this problem. Although in vivo NMR spectroscopy has now become recognized as a powerful analytic tool, the technique has not been used widely for the study of drug metabolism, nor has it been applied previously to the analysis of the intracellular fate of β-lactams. Two recent reports, however, document the utility of ^{19}F NMR spectroscopy in monitoring the metabolic disposition of flucytosine (8, 22).

The efficacy of in vivo NMR spectroscopy for metabolic studies is limited in part by the potentially small number of metabolites sufficiently concentrated to be observed. For studies with ^{13}C NMR spectroscopy, this problem is compounded by the intrinsically low sensitivity of the ^{13}C nucleus. Consequently, in vivo ^{13}C NMR experiments have been restricted generally to measurements of enriched compounds at concentrations above 0.5 mM (1, 18, 21). In application of NMR spectroscopy to the analysis of the in vivo metabolism of a β-lactam, the ^{13}C sensitivity problem may be aggravated further by the sometimes limited permeability of the outer membranes of gram-negative species to certain of these antibiotics (23, 25). Given these considerations, we sought first to ascertain whether ^{13}C NMR spectroscopy would be applicable to the simple detection of β-lactamase activity in vivo.

Reaction of the β-lactamases with Δ^3-cephalosporins leads both to cleavage of the β-lactam ring and to subsequent release of a heteroatom-linked functional group from the C_{10}-position of the cephem. For example, for enzymatic hydrolysis of cephalothin, acetate is formed quantitatively with scission of the lactam bond (9, 14, 19, 20). On the basis of this feature of the enzymatic mechanism, the [acetoxy-^{13}C]cephalothin (compound 1) (Fig. 1) seemed to be an ideal substrate for monitoring β-lactamase activity by NMR spectroscopy. Cleavage of compound 1 should be characterized by carbon spectral sets showing the loss of a [^{13}C]acetyl resonance together with the accumulation of a [^{13}C]acetate signal as the reaction progresses. We report here our studies of the processing of compound 1 both by purified β-lactamase in vitro and in viable and intact cells.

MATERIALS AND METHODS

Sodium cephalothin and potassium clavulanate were the gifts of Eli Lilly & Co., Indianapolis, Ind. Cefoxitin was generously supplied by Merck Sharp & Dohme, West Point, Pa. Sodium [1-^{13}C]acetate was purchased from Merck, Canada. *Escherichia coli* TEM-2 β-lactamase was obtained from Applied Microbiology and Research, Porton Down, United Kingdom. All other reagents were of the best grade commercially available.

Spectrophotometric analyses were carried out with a model 559 spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.). Proton NMR spectra were obtained at 500 MHz by using a DS-1000 instrument equipped with a model 1180 computer (Nicolet Instrument Corp., Madison, Wis.). Chemical shift values (δ) are given in ppm with tetramethylsilane in $CDCl_3$ or sodium β-trimethylsilylpropionate in D_2O as the internal reference. Infrared spectra were recorded with a model 283 spectrometer (The Perkin-Elmer Corp.). Fast-atom bombardment mass-spectral data were obtained with a VG 7070 spectrometer. Melting points were taken on a Hoover Uni-Melt apparatus and are uncorrected.

Syntheses. [^{13}C]cephalothin (compound 1) was prepared as outlined in Fig. 2. The cephem iodide (compound 2) is readily obtained from sodium cephalothin by methods described previously (5, 15). Reaction of compound 2 and sodium [1-^{13}C]acetate (compound 3) affords the ester (compound 4) as a mixture of Δ^2- and Δ^3-isomers. We routinely use the methods of Kaiser et al. (12) for converting Δ^2-cephems to their Δ^3-isomers. Thus, treatment of compound 4 with *m*-chloroperoxybenzoic acid affords the sulfoxide

* Corresponding author.
† Present address: Laboratory of Bioorganic Chemistry and Biochemistry, The Rockefeller University, New York, NY 10021.

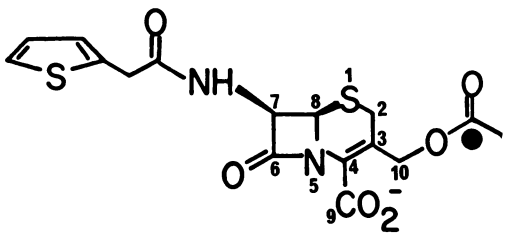

FIG. 1. Structure of [^{13}C]cephalothin (compound 1). Symbol: ●, position of ^{13}C label.

(compound 5) as a single Δ^3-isomer, and reduction of compound 5 with stannous chloride gives the nitrobenzyl ester of [^{13}C]cephalothin (compound 6). Catalytic hydrogenolysis converts compound 6 to the desired compound 1.

***p*-Nitrobenzyl-7β-(2-thienylacetamido)-3-[acetoxy-$^{13}C_1$]methyl-3-cephem-4-carboxylate (compound 6).** A solution of the iodide (compound 2) (700 mg, 1.17 mmol) and sodium [1-^{13}C]acetate (125 mg, 1.52 mmol) in 6 ml dimethyl formamide was stirred for 1 h at room temperature. The solution was then poured into a mixture of ethyl acetate and saturated $CaCl_2$. The organic layer was washed twice with saturated $CaCl_2$ and once each with water, 10% $NaHCO_3$, and water; it was then dried over $MgSO_4$. This solution was then evaporated to dryness in vacuo.

The residue was dissolved in 7 ml of $CHCl_3$ and cooled in an ice bath with vigorous stirring. A solution of *m*-chloroperoxybenzoic acid (230 mg) in 5 ml of $CHCl_3$ was then added dropwise over ca. 2 min. Subsequently, the solution was stirred at room temperature for 30 min and then washed twice with saturated $NaHCO_3$ and once with water. The organic layer was dried over $MgSO_4$ and evaporated to dryness.

A solution of the residue, stannous chloride dihydrate (640 mg, 2.84 mmol), and acetyl chloride (2.8 ml, 39.4 mmol) in 17 ml of dimethyl formamide was stirred at room temperature for 40 min. Ethyl acetate (ca. 100 ml) was added, and the solution was washed three times with saturated $CaCl_2$ and once with water. The organic phase was dried over $MgSO_4$ and evaporated to dryness to give 475 mg of the ester (compound 6). Yield, 76% (from the iodide, compound 2); melting point, 147 to 148°C; infrared peaks ($CHCl_3$) 1,791, 1,739, 1,735, 1,683, and 1,349 cm^{-1}; R_f 0.41 (3:1, benzene-ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.06 (d, 3H, acetyl methyl, J_{HC} = 6.9 Hz), 3.37, 3.54 (2d, 2H, C-2, J = 18.6 Hz), 3.85 (s, 2H, side-chain methylene), 4.78, 4.79 (dd, 1H, C-10, J_{HH} = 13.7 Hz, J_{HC} = 3.5 Hz), 4.96 (d, 1H, C-6, J = 4.8 Hz), 5.11, 5.12 (dd, 1H, C-10, J_{HH} = 13.6 Hz, J_{HC} = 2.8 Hz), 5.30, 5.35 (2d, 2H, benzylic, J = 13.0 Hz), 5.86 (m, 1H, C-7), 6.20 (d, 1H, NH side chain, J = 9.0 Hz), 6.98 (m, 2H, thienyl), 7.25 (m, 1H, thienyl), 7.55, 8.20 (2d, 4H, phenyl, J = 8.6 Hz); mass spectrum (fast atom bombardment$^-$), *m/z* 531 $(M-H)^-$; analysis, C, H, N.

Sodium 7β-(2-thienylacetamido)-3-[acetoxy-$^{13}C_1$]methyl-3-cephem-4-carboxylate (compound 1). A solution of compound 6 (400 mg, 0.75 mmol) in 60 ml of acetone was added to a preequilibrated suspension of sodium bicarbonate (128 mg, 1.5 mmol) and 10% Pd/C (400 mg) in 80% ethanol (40 ml) under a H_2 atmosphere at ambient temperature and pressure. The solution was stirred for 2 h, with one H_2 exchange after approximately 1 h. The suspension was filtered through Celite, and the filtrate was evaporated in vacuo to a volume of ca. 10 ml. Additional water (ca. 30 ml) was added, and the aqueous solution was washed twice with ethyl acetate. The aqueous layer was acidified to pH 1.7 to 1.9 with 1 N HCl at 0°C and washed three times with ethyl acetate, and the combined organic layers were washed with water, dried over $MgSO_4$, and evaporated to dryness to give 298 mg of compound 1 (in its free acid form) as a pale yellow solid. Yield, 83%; melting point, 148 to 150°C (decomposes); infrared peaks ($CHCl_3$) 1,775, 1,731, 1,710, 1,663 cm^{-1}; R_f 0.80 (85:15:5, $CHCl_3$-CH_3OH-CH_3CO_2H); ^{1}H NMR ($CDCl_3$) δ 2.07 (d, 3H, acetyl methyl, J = 6.8 Hz), 3.36, 3.55 (2d, 2H, C-2, J = 18.6 Hz), 3.85 (s, 2H, side-chain methylene), 4.90, 4.91 (dd, 1H, C-10, J_{HH} = 13.7 Hz, J_{HC} = 3.1 Hz), 4.97 (d, 1H, C-6, J = 4.9 Hz), 5.08, 5.09 (dd, 1H, C-10, J_{HH} = 13.7 Hz, J_{HC} = 3.0 Hz), 5.83 (m, 1H, C-7), 6.42 (d, 1H, NH side chain, J = 9.0 Hz), 6.98 (m, 2H, thienyl), 7.25 (m, 1H, thienyl); mass spectrum (FAB^-), *m/z* 396 $(M-H)^-$; analysis, C, H, N.

To prepare the sodium salt of the carboxylic acid, a solution of the free acid of compound 1 (290 mg, 0.73 mmol) in 2 ml of acetone was reacted with a solution of $NaHCO_3$ (67 mg, 0.80 mmol) in 4 ml of water. After the solution had been stirred at room temperature for 15 min, acetone was evaporated and the aqueous layer was washed three times

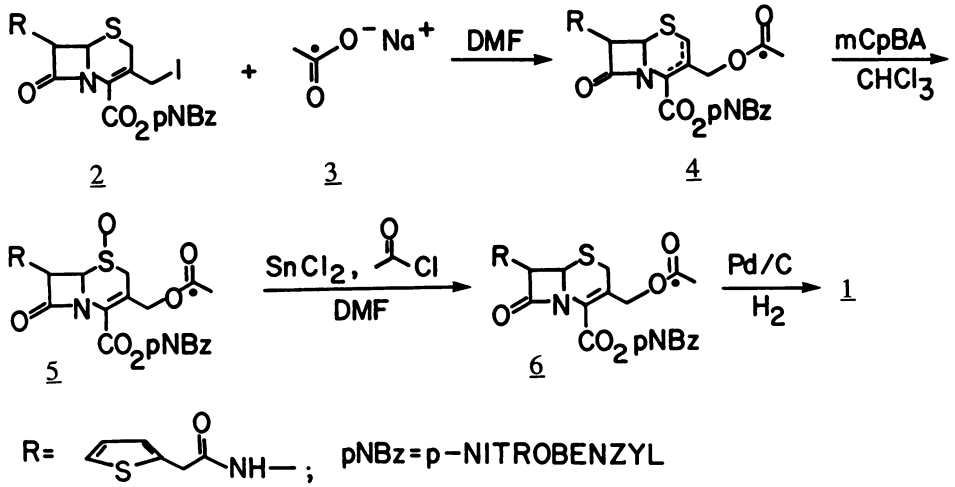

FIG. 2. Synthetic route for preparation of compound 1. Details of the synthesis are given in the text.

with ethyl acetate. The aqueous layer was then lyophilized to give a fluffy powder (263 mg, 86%).

Bacterial strains. *E. coli* JSR-O is a plasmidless laboratory strain of *E. coli* K-12; *E. coli* JSR-O(pBR322) contains the plasmid-borne gene encoding TEM-2 β-lactamase. *Enterobacter cloacae* 55 and its mutant derivative, 55M, which is constitutive for production of the chromosomally encoded class I β-lactamase, were generously provided by C. C. Sanders. All strains were subcultured on commercially prepared blood agar or chocolate agar (BBL Microbiology Systems, Cockeysville, Md.). Strains of *E. coli* were routinely cultivated on Davis-Mingioli minimal medium (7) supplemented with 50 μg of L-methionine per ml and 50 μg of L-proline per ml to satisfy the auxotrophic growth requirements of these organisms. The medium used for growth of *E. coli* JSR-O(pBR322) was further supplemented with 10 μg of ampicillin per ml. *Enterobacter* strains were cultivated on a defined peptide susceptibility medium (2). For induction of β-lactamase activity in *E. cloacae* 55, the growth medium was additionally supplemented with 20 μg of cefoxitin per ml.

Bacterial cells used for in vivo ^{13}C NMR experiments were prepared in the following way. Typically, a 20-ml culture was harvested after growth at 37°C to mid-logarithmic phase. The cells were then centrifuged (10,000 × *g*) for 10 min at 4°C. The resulting supernatant liquid was decanted, and the cells were suspended in 5 ml of 100 mM sodium phosphate buffer (pH 7.0) and centrifuged again. The pellet was suspended in 20 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 5% sucrose. A 600-μl portion of this cell suspension was then placed in a 5-mm NMR tube containing compound 1 (final concentration 50 mM), and spectra were acquired immediately.

For the NMR experiment shown in Fig. 4C, *E. coli* JSR-O(pBR322) was cultivated in the ordinary way, except that the washed cells were suspended in sodium phosphate buffer containing 20 μg of potassium clavulanate per ml and were incubated at 37°C for 30 min in this buffer before the NMR spectra were obtained with compound 1.

Determination of β-lactamase activity in cell homogenates. *E. coli* and *E. cloacae* strains were cultivated under conditions identical to those described for preparation of cells used in the NMR experiments. Typically, a 100-ml culture was grown to mid-logarithmic phase and harvested by centrifugation (10,000 × *g* for 10 min) at 4°C. The pellet was suspended in approximately 20 ml of 100 mM sodium phosphate buffer (pH 7.0) and centrifuged again. The cells were then suspended in 10 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 1.5 mM 2-mercaptoethanol and subjected to ultrasonic disruption on ice (10 30-s bursts separated by 45-s intervals). The disrupted cells were then centrifuged at 20,000 × *g* for 20 min at 4°C. The resulting supernatant liquid was assayed spectrophotometrically for β-lactamase activity with nitrocefin as the substrate.

A typical 1.0-ml assay mixture contained 67 μM nitrocefin and 50 μl of cell homogenate in 100 mM sodium phosphate buffer (pH 7.0) at 37°C. The reaction was monitored by measuring the loss of absorbance of the α,β-unsaturated carboxylate of the cephem (λ_{max} = 386 nm, $\epsilon = 1.75 \times 10^4$ M^{-1} cm^{-1}). Nitrocefin, rather than cephalothin, was specifically chosen for these experiments because of the greater sensitivity in assaying for β-lactamase afforded by the substrate with the higher extinction coefficient. The cephalothin chromophore absorbs with λ_{max} = 267 nm, $\epsilon = 0.65 \times 10^4$ M^{-1} cm^{-1}. Protein concentrations were determined by the method of Lowry et al. (13).

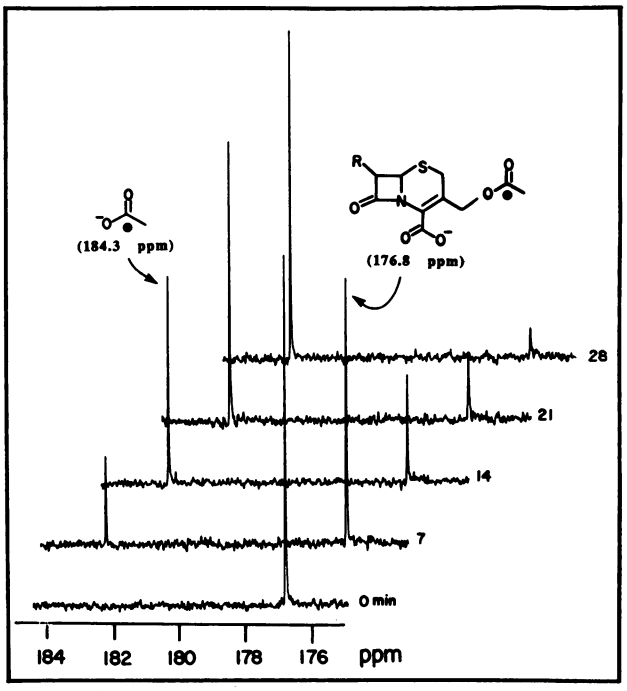

FIG. 3. ^{13}C NMR spectra of the reaction of compound 1 with *E. coli* TEM-2 β-lactamase. Spectra were recorded at 0 min (before addition of the enzyme) and then at 7, 14, 21, and 28 min after addition of the β-lactamase. In the stacked-plot presentation of the data shown here, each spectrum is plotted to appear slightly offset from the position of the spectrum previously recorded. This method avoids the problem of superimposition of identical spectral lines recorded at different time points. Reaction conditions are given in the text.

Reaction of TEM-2 β-lactamase with compound 1. A 600-μl solution of compound 1 (50 mM) was prepared in 100 mM sodium phosphate buffer (pH 7.0) and placed in a 5-mm NMR tube. After the acquisition of an initial spectrum, a 10-μl solution of *E. coli* TEM-2 β-lactamase (final concentration, 0.2 μM) prepared in 100 mM sodium phosphate buffer (pH 7.0) was added to the substrate solution. Spectra were acquired at timed intervals by using the parameters described below. The probe temperature was maintained at 36°C.

NMR experiments. ^{13}C NMR spectra were obtained at 100 MHz with a Nicolet XL-400 spectrometer. For both in vitro experiments with purified lactamase (see Fig. 3) and in vivo studies with whole cells (see Fig. 4 and 5), the spectra were acquired with a 5-s delay and a 50° flip angle. Each spectrum represents the accumulation of 10 transients and required 1.1 min for total acquisition. All reactions were carried out in protio-buffers, and the spectra were acquired without interlock and are broad-band decoupled. The probe temperature was maintained at 37°C during the course of each experiment. A signal-to-noise ratio of 60/1 to 75/1 was typically obtained for the initial spectrum of each experimental set.

Pseudo-first-order rate constants for the reaction of compound 1 in vivo were obtained by monitoring the rate of disappearance of the cephalothin acetyl resonance (δ 176.8). To ascertain the appropriate NMR parameters for reliable integration of the spectra, the spin-lattice relaxation times (T_1) for the acetyl resonance of compound 1 and of the

carboxyl resonance of [1-^{13}C]acetate were determined by an inversion-recovery experiment under the conditions used for nonquantitative spectral acquisition described above. The cephalothin ester carbonyl gave a T_1 of 12.3 s, and the carboxylate of acetate showed a T_1 of 7.6 s. Thus, when acquiring spectra for integration, we used a 65-s delay time with a 90° flip angle. Two transients were acquired, and the total acquisition time for each spectrum was 2.2 min. All other conditions were identical to those described above.

RESULTS

Reaction of TEM-2 β-lactamase with compound 1. We initially undertook a ^{13}C NMR analysis of the reaction of compound 1 with purified TEM-2 β-lactamase to obtain an in vitro calibration for the spectral events that might be observed in studies with compound 1 and β-lactamase-producing organisms in vivo. Figure 3, which is a stacked-plot presentation of the NMR spectra obtained at timed intervals, summarizes the in vitro results. Prior to addition of the enzyme to a solution of compound 1, the ^{13}C NMR spectrum (Fig. 3, spectrum at 0 min) contained a single line at 176.8 ppm, produced by ^{13}C- enrichment of the acetoxy ester carbon of compound 1. Upon addition of the TEM-2 β-lactamase, the signal at 176.8 ppm rapidly collapsed and, in parallel, was replaced by an acetate carboxyl resonance at 184.3 ppm (spectra at 7, 14, 21, and 28 min). During the reaction course of Fig. 3, compound 1 was nearly completely consumed; after 28 min of reaction, the principal spectral feature was the acetate resonance at 184.3 ppm.

In a control experiment, NMR spectra were acquired while [^{13}C]cephalothin was incubated for 30 min in the absence of β-lactamase. During this period, the acetyl signal of compound 1 did not collapse and no acetate resonance appeared (data not included).

^{13}C NMR spectra of the reactions of compound 1 in vivo. The ^{13}C NMR spectra acquired during incubation of compound 1 with whole-cell preparations of *E. coli* JSR-O strains are shown in Fig. 4. The spectra in Fig. 4A, obtained upon incubation of compound 1 with *E. coli* JSR-O(pBR322), reveal a gradual decrease in the acetyl signal at 176.8 ppm during the experimental period and a corresponding accumulation of an acetate resonance at 184.3 ppm. This is a spectral pattern like that seen in Fig. 3, indicative of β-lactamase-catalyzed fragmentation of cephalothin. *E. coli* JSR-O(pBR322) contains the plasmid encoding TEM-2 β-lactamase.

To demonstrate that the spectral pattern of Fig. 4A actually represents intracellular processing of compound 1, we conducted the following experiment. After the final 60-min spectrum of Fig. 4A had been acquired, the cells were removed by centrifugation, and the resulting supernatant solution was monitored by ^{13}C NMR spectroscopy for further metabolism of unreacted compound 1. During this period of additional spectral acquisition, the acetyl resonance of compound 1 did not collapse further and no acetate accumulated (data not shown). This result rules out the possibility that the data in Fig. 4A were obtained as a consequence of cell lysis and release of the β-lactamase to solution. Moreover, this solution contained no detectable β-lactamase activity when assayed spectrophotometrically with nitrocefin as the substrate.

The parental *E. coli* JSR-O strain used in these experiments lacks the β-lactamase-encoding plasmid, which is reflected by the spectral data of Fig. 4B. Note that during incubation of these cells with compound 1, the acetyl resonance at 176.8 ppm was only slightly diminished and a weak acetate signal at 184.3 ppm appeared, but this occurred late in the experimental course. At 60 min, the parental JSR-O strain (Fig. 4B) had produced only about 20% of the acetate formed by the plasmid-containing strain (Fig. 4A) during the same period.

Figure 4C shows the spectra acquired upon incubation of compound 1 with a preparation of *E. coli* JSR-O(pBR322) that had been previously incubated with the β-lactamase inactivator clavulanic acid (20 μg/ml) for 30 min. The spectral pattern of Fig. 4C duplicates that of the strain without β-lactamase (Fig. 4B). This finding supports the conclusion that the data of Fig. 4A can best be explained by a β-lactamase-catalyzed release of [^{13}C]acetate from [^{13}C]cephalothin.

We have also examined the reactions of compound 1 with whole-cell preparations of *E. cloacae*, whose β-lactamase is chromosomally encoded (Fig. 5). The β-lactamase in *E. cloacae* 55M is constitutive (10); thus, incubation of this strain with compound 1 gave a ^{13}C NMR spectral pattern (Fig. 5A) like that for reaction of the cephem in the presence of *E. coli* JSR-O(pBR322), whose TEM β-lactamase is also consititutive. By contrast, the lactamase of the parental *E. cloacae* 55 is inducible (10); in the absence of induction, these cells lack the ability to generate [^{13}C]acetate from [^{13}C]cepahlothin (Fig. 5B). However, when this strain was grown in the presence of the enzyme inducer cefoxitin (20 μg/ml), the resulting cell preparation formed acetate from compound 1 (Fig. 5C).

β-Lactamase activity in cell lysates. To verify that the spectra of Fig. 4 and 5 actually reflect β-lactamase processing of compound 1, we used a conventional nitrocefin assay with lysates of cells that had been treated identically to those used in the NMR experiments. β-Lactamase activity was detected by the spectrophotometric method in only three lysate preparations: *E. coli* JSR-O(pBR322), *E. cloacae* 55M, and *E. cloacae* 55 grown in the presence of cefoxitin. The specific activity values determined were 2.79, 19.45, and 4.58 μmol min^{-1} mg of protein^{-1}, respectively. The lysates found to contain lactamase activity by the nitrocefin assay are derived from the same cell populations in which the in vivo conversion of compound 1 to [^{13}C]acetate is attributable to the action of β-lactamase (Fig. 4A, 5A, and 5C). Essentially no β-lactamase activity was detected in lysates of *E. coli* JSR-O, *E. coli* JSR-O(pBR322) incubated with clavulanic acid, and *E. cloacae* 55; the specific activity values determined were 0.01, 0.01, and 0.03 μmol min^{-1} mg of protein^{-1}, respectively.

Kinetic constants for the processing of compound 1 in vivo. It is possible to obtain a quantitative evaluation of the rate of processing of compound 1 in vivo by acquisition of spectral data, like those shown in Fig. 4 and 5, under conditions that allow for reliable integration of the signal intensities. Figure 6, for which the reaction course was monitored as the log percent acetyl resonance (δ 176.8) remaining over time, presents rate data of this type. The data in Fig. 6 show that in each experimental case, the conversion of compound 1 to acetate appears to be a pseudo-first-order process. The slope of each line is the experimentally determined k_{obs}, and these values are summarized in Table 1. The concentration of compound 1 used in these experiments (50 mM) greatly exceeds the typical K_m value for reaction of a β-lactamase with a cephalosporin; for example, the K_m of cephalothin for the TEM-2 enzyme is 0.20 mM (14). Thus, the values for k_{obs} in Table 1 may approximate the V_{max} rates for reaction of compound 1 in vivo. However, we do not know the extent to

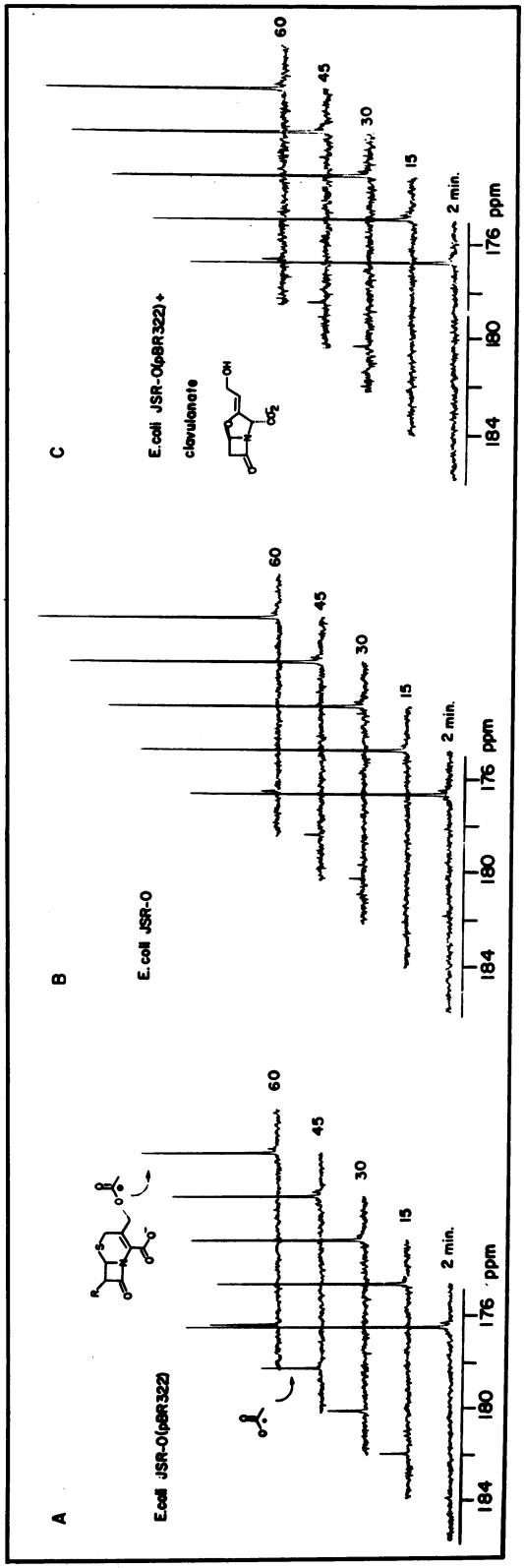

FIG. 4. ^{13}C NMR spectra of the reaction of compound 1 with whole-cell preparations of *E. coli* strains. (A) *E. coli* JSR-O(pBR322); (B) *E. coli* JSR-O; (C) *E. coli* JSR-O(pBR322) preincubated with 20 μg of sodium clavulanate per ml. Spectra were recorded at 2, 15, 30, 45, and 60 min after the addition of a cell suspension to a solution of compound 1. Other experimental details are given in the text.

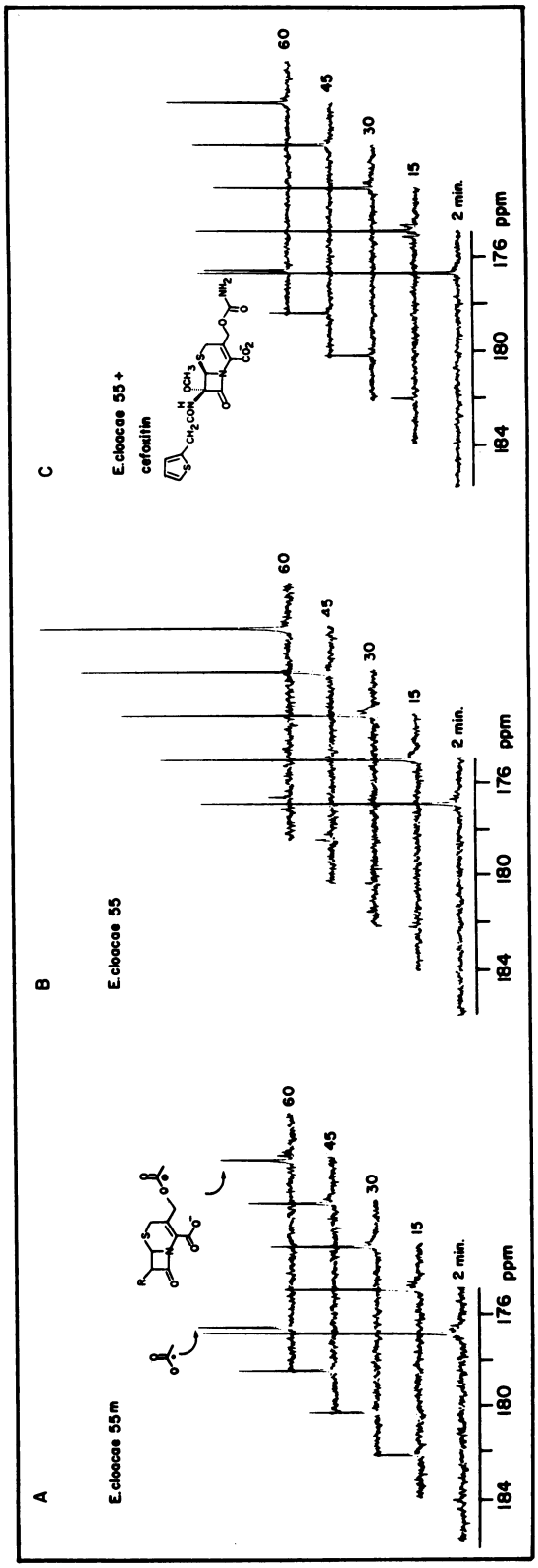

FIG. 5. ^{13}C NMR spectra of the reaction of compound 1 with whole-cell preparations of *E. cloacae* strains. (A) *E. cloacae* 55M; (B) *E. cloacae* 55; (C) *E. cloacae* 55 grown in the presence of 20 μg of cefoxitin per ml. Spectra were recorded at 2, 15, 30, 45, and 60 min after the addition of a cell suspension to a solution of compound 1. Other experimental details are given in the text.

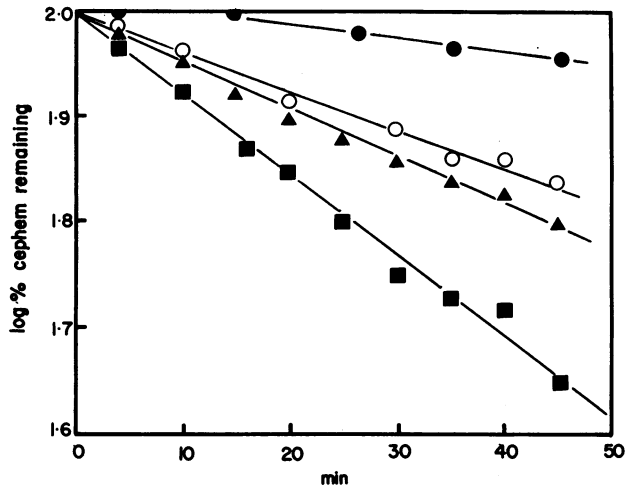

FIG. 6. Reaction rates for the processing of compound 1 by cell suspensions. Symbols: ●, *E. coli* JSR-O; ○, *E. coli* JSR-O(pBR322); ▲, *E. cloacae* 55 grown in the presence of 20 μg of cefoxitin per ml; ■, *E. cloacae* 55M. Rate data for the reaction of compound 1 with preparations of *E. coli* JSR-O(pBR322) pretreated with clavulanate and of *E. cloacae* 55 without cefoxitin induction were identical to those obtained for in experiment with *E. coli* JSR-O (see Table 1). Experimental details are given in the text.

TABLE 1. Rate data for the reaction of 50 mM compound 1 in whole bacterial cells

Cell prepn	k_{obs} (min^{-1}, 10^3)
E. coli JSR-O(pBR322)	8.54
E. coli JSR-O	2.05
E. coli JSR-O(pBR322) + clavulanate[a]	2.01
E. cloacae 55M	17.54
E. clocae 55	2.05
E. cloacae 55 + cefoxitin[b]	10.50

[a] The cells were incubated with sodium clavulanate (20 μg/ml) at 37°C for 30 min before the spectral data were acquired.

[b] The cells were cultivated in the presence of 20 μg of cefoxitin per ml. All other details are given in the text.

which the extracellular concentration of compound 1 is duplicated in the periplasmic space, nor are the copy numbers of β-lactamases known for the organisms examined. It may not be accurate, therefore, to assume that the enzyme has been saturated in vivo.

It is of interest that in cases in which β-lactamase activity was not detected by the nitrocefin assay [*E. coli* JSR-O, *E. coli* JSR-O(pBR322) incubated with clavulanate, and *E. cloacae* 55 (see above)], the values of k_{obs} measured for the disappearance of compound 1 (Table 1) were all identical. This finding with two genera implies that the in vivo reaction of cephalothin as monitored by NMR spectroscopy in lactamase-negative cells (Fig. 4B, 4C, and 5B) does not depend on a function of the organism, but is probably the result of a nonenzymatic, perhaps solvolytic, process.

DISCUSSION

We have described elsewhere (14, 17) the design, synthesis, and biological activity of a novel derivative of deacetylcephalothin, compound 7, which carries the antibacterial dipeptide β-Cl-L-Ala-β-Cl-L-Ala (compound 8) as a C-10 ester. Compound 7 was predicted to be bactericidal toward β-lactamase-producing organisms, since enzyme-catalyzed fragmentation of the cephem would be expected to release the antibacterial dipeptide (compound 8) within the cell (Fig. 7). Haloalanyl peptides, such as compound 8, lead to inactivation of alanine racemase in vivo (4, 6). The racemase is essential for peptidoglycan biosynthesis, and its inactivation gives rise to cell lysis (4).

In accord with predictions, compound 7 displays activity against β-lactamase-containing strains of bacteria (17). Furthermore, purified TEM-2 β-lactamase releases compound 8 from compound 7 in vitro (16). Despite these findings, however, there is as yet no direct evidence which links the antibacterial action of compound 7 to a lactamase-dependent fragmentation of the cephem in vivo. It occurred to us, however, that ^{13}C NMR spectroscopy might be useful in the analysis of the metabolism of compound 7. Specifically, we imagined the synthesis of a derivative of compound 7 bearing a single ^{13}C label, positioned, as in compound 1, in the ester carbon of the peptidyl fragment. Time-elapsed NMR spectrometry would then allow visualization of the events of Fig. 7 in whole cells. β-Lactamase processing of a labeled compound 7 should result in loss of a carboxyl ester signal and accumulation of a carboxylate resonance, both indicative of peptide release. It was necessary first, however, to assess the plausibility of this strategy with a synthetically accessible and inexpensive ^{13}C-labeled cephalothin that would serve as a model for compound 7. Thus, we undertook the

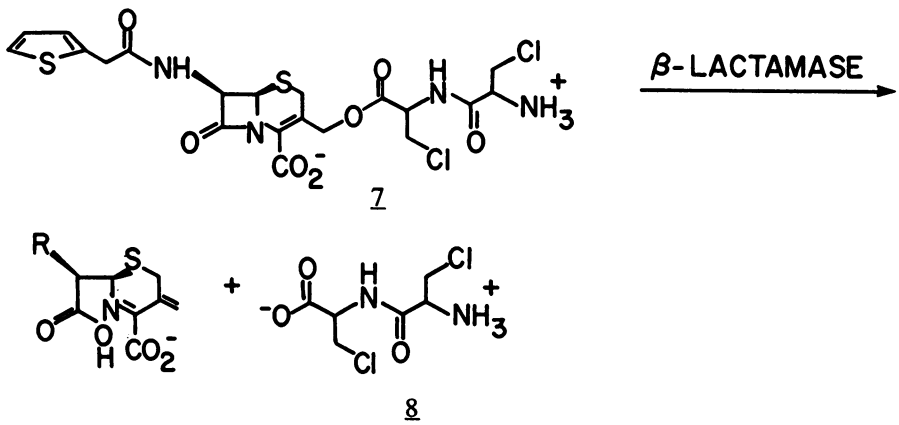

FIG. 7. Reaction of a chloroalanyl dipeptide ester of cephalothin (compound 7) with β-lactamase, showing release of the haloalanyl peptide (compound 8) upon cleavage of the β-lactam ring.

studies described in this paper with [^{13}C]cephalothin (compound 1).

The data in Fig. 4 and 5 demonstrate that it is indeed possible to "visualize" the activities of both plasmid-encoded and chromosomal β-lactamases in vivo by using ^{13}C NMR spectroscopy. In making this assessment, our interpretation of the in vivo data was informed by the processing of compound 1 observed in vitro with purified *E. coli* TEM-2 β-lactamase; enzymatic cleavage of compound 1 is characterized by loss of an acetyl resonance and accumulation of an acetate signal (Fig. 3). This spectral pattern was duplicated in experiments with whole cells in which β-lactamase is known to be expressed either constitutively (Fig. 4A and 5A) or as a result of induction (Fig. 5C). The spectra reveal that in cell preparations that lack β-lactamase activity (Fig. 4B and 5B) or in a lactamase-producing population exposed to clavulanic acid (Fig. 4C), there is no significant intracellular metabolism of compound 1.

However, Fig. 4B, 4C, and 5B do show small acetate signals, generally appearing late in the reaction course. It is possible that this phenomenon reflects the in vivo reaction of cephalothin with one of its usual intracellular targets, the penicillin-binding proteins. Acylation of a penicillin-binding protein by cephalothin would be expected, like reaction with a β-lactamase, to lead to release of acetate from the cephem nucleus. However, in *E. coli*, at least, there are relatively few copies of the penicillin-binding proteins within the cell, and only the D,D-carboxypeptidases are presumed to act catalytically against β-lactams (24). Thus, it seems reasonable that accumulation of [^{13}C]acetate in cells lacking β-lactamase would occur at concentrations sufficient for detection by NMR spectroscopy only after a long incubation with compound 1, as is generally observed in our spectra. It is also possible that the acetate resonances in Fig. 4B and 5B result from nonspecific esterolysis of compound 1 (3, 11) or from solvolysis. It is further conceivable in the special case shown in Fig. 4C that acetate formation could be attributed to residual β-lactamase activity that has survived treatment with clavulanic acid.

By contrast to the reaction of compound 1 with the purified enzyme (Fig. 3), it is obvious from the data in Fig. 4A, 5A, and 5C that the labeled cephalothin is not exhausted after prolonged incubation with whole cells. The peak height of the acetyl line of the final spectrum of Fig. 4A, for example, represents only about a 40% reduction in the signal intensity of the cognate resonance of the initial spectrum. These are not surprising observations, given both the high concentration (50 mM) of the cephem used in the experiments and the potentially restricted permeability of the gram-negative outer membrane to some β-lactams; estimates suggest that for certain β-lactams only about 1% of the extracellular concentration of the drug is achieved in the periplasmic space (23, 25). Indeed, the intense acetyl signals in all of the spectra in Fig. 4 and 5 most probably arise primarily from unreacted extracellular compound 1.

Quantitative estimates of the intracellular rates of reaction of compound 1 can also be made by the NMR spectrocopy approach. The data presented (Fig. 6) suggest that it might be possible, at least in principle, to determine the Michaelis constant for the reaction of β-lactamase in whole cells. This assumes, however, that the β-lactam substrate rapidly and completely partitions from the extracellular environment into the intracellular space. Such an analysis would be precluded, of course, if the (subsaturating) concentration range of the substrate, required for determining K_m accurately, were insufficient to produce a detectable ^{13}C signal. In fact, we required concentrations of compound 1 well in excess of the in vitro K_m values known for most β-lactamases to detect the rates of processing of our cephem. Thus, the determination of reaction kinetics in vivo by ^{13}C NMR spectroscopy may well remain limited by the low sensitivity of the carbon nucleus. Nonetheless, we have shown that ^{13}C NMR spectroscopy is applicable for an at least qualitative investigation of lactamase activity in intact, viable cells. This finding will clearly allow us to perform metabolic studies in vivo with the peptide cephem ester, compound 7.

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