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Correlation Between Hydrolysis of the β-Lactam Bond of the Cephalosporin Nucleus and Expulsion of the 3-Substituent

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The hydrolysis of two cephalosporins by three different β-lactamases has been studied. Each enzyme caused a decrease in ultraviolet absorption, a loss of biological activity, and the release of the leaving group from the 3-position. The changes occurred at the same rate and to the same extent with each enzyme, and it is inferred that the loss of the leaving group is a consequence of, and not a prerequisite for, hydrolysis of the β-lactam ring.

Gram-negative bacteria are capable of producing many different types of β-lactamase (EC 3.5.2.6), and in the *Enterobacteriaceae* alone there are at least eight distinct types of the enzyme to be found (2). These β-lactamases can hydrolyze both penicillins and cephalosporins to varying degrees (3, 5). The type of product found after hydrolysis of a cephalosporin depends on the particular cephalosporin used as substrate (1). Cephalosporin substrates investigated so far have all had substituents in the 3-position which can accept an electron and readily leave the nucleus when the β-lactam ring is opened (6). However, there is little quantitative data available concerning the relationship between the hydrolysis of the β-lactam ring of cephalosporins and the release of a potential leaving group from position 3. Neither is it known whether or not different β-lactamases bring about similar molecular changes in a particular substrate.

The present study relates the hydrolysis of the β-lactam bond as shown by changes in the ultraviolet (UV) spectrum and in the microbiological activity to the quantitative release of a leaving group from the 3-position. The substrates used, cephaloridine (CER) and 3-azidomethyl-7β-benzylthioacetamidoceph-3-em-4-carboxylic acid (BTCA), are both highly susceptible to β-lactamases from gram-negative organisms and both possess a leaving group, pyridine and azide, respectively, at position 3.

MATERIALS AND METHODS

Preparation of β-lactamases. Partially purified enzymes were prepared from *Enterobacter cloacae* P99, *Escherichia coli* TEM, and *Klebsiella aerogenes* 1082E. The properties of these enzymes were compared by Jack and Richmond (1970), and they were chosen for this work as being broadly representative of the types of enzymes that occur in the *Enterobacteriaceae*. The partially purified preparations were made essentially by Sephadex G-50 chromatography of the supernatant fluid produced by centrifugation of a cell suspension after sonic treatment (4). The freeze-dried enzymes were dissolved in 0.1 M phosphate buffer, pH 7, at concentrations found by experiment to give a complete reaction in a suitable period of time.

Substrates used. CER and BTCA (sodium salt) were used in this study. Their formulas are given in Fig. 1.

Sodium nafcillin was used as an enzyme inhibitor and was kindly provided by Wyeth Labs., Inc., Philadelphia, Pa.

Operating system. The substrates were dissolved in 0.1 M phosphate buffer, pH 6, and used at a final concentration of 1 mg/ml (CER) and 400 μg/ml (BTCA). The substrate solution was placed in a conical flask in a shaking incubator operating at 40 cycles/min at 37 C. Enzyme, also at 37 C, was added at time zero. Enzyme activity was sufficient to cause complete hydrolysis of the substrates.

Sampling. For assay of the leaving group and measurement of change in UV light absorption, the reaction mixture was continuously sampled. The reaction was stopped by the addition of an equimolar amount of sodium nafcillin, and the sample was split into two, one part then being assayed for either pyridine or azide and the other part for measurement of change in absorption.

For the determination of biological activity, samples were withdrawn at selected intervals from the reaction mixture and assayed as described below.

UV absorption assay. The β-lactam system in both substrates absorbs at 255 nm and decreases in absorption occur when the β-lactam ring is hydro-

lysed (4). After hydrolysis, there is a residual absorption for each substrate and it is necessary to prepare a calibration curve. The absorptions were measured in a Unicam SP 500 spectrophotometer.

Measurement of biological activity. The enzyme reaction in the samples was stopped by the addition of an equal volume of iodine solution (KI at 200 g/liter containing I_2 at 40.6 g/liter) followed by 1:20 dilution in 2 M sodium acetate buffer. A further 1:10 dilution was made in 0.05 M phosphate buffer, *p*H 7, at which concentration the iodine did not interfere with the assay. The residual antibacterial activity was measured by the large-plate agar diffusion method, against *Staphylococcus aureus* ATCC 7447. The medium used was Oxoid Tryptose agar, containing 0.0015% of 2,3,5-triphenyltetrazolium chloride, and the plates were read after 18 hr of incubation at 37 C.

Measurement of azide. Free azide was determined by the autoanalyzer system devised by Waller (*unpublished data*). In this system, azide ions are rapidly oxidized under mildly acidic conditions to gaseous nitrogen by an excess of nitrite ions generated continuously within the system from aqueous potassium nitrite and acetate buffer (2 M, *p*H 4.6). The reaction mixture, consisting of enzyme, substrate, and hydrolysis products, was treated with nafcillin (1.1 mM) to stop the reaction, cooled to 22 C, the assay temperature, and introduced as the sample for the assay system. After being mixed with the nitrite reagents at the same temperature, a solution of 4-aminosalicylic acid was added, and diazotization was allowed to continue for 4 min at 22 C. After being made alkaline with aqueous tetramethylammoniumhydroxide, coupling of the diazo-compound with excess aminosalicylate takes place, and a yellow-brown color—inversely proportional to azide concentration—is produced. The color was measured continuously at 440 nm, and the azide concentration was read from a calibration graph prepared with sodium azide.

Measurement of free pyridine. Free pyridine may be determined in the presence of CER by the conventional aniline phosphate-cyanogen bromide procedure. For the present work, an adaptation of the method for the autoanalyzer was employed. Cyanogen bromide was replaced by cyanogen chloride generated continuously within the system from aqueous solutions of potassium thiocyanate and chloramine-T. The sample, after the addition of inhibitor (2.4 mM) was mixed with aniline in phosphate buffer (*p*H 6.0), the cyanogen chloride reagent was then added, and, after a suitable delay period, the color was measured at 462 nm. The concentration of pyridine was measured from a calibration graph.

RESULTS

Figure 2 illustrates the rate of pyridine release, loss of biological activity, and loss of absorbance at 255 nm which occurs when CER is hydrolyzed by three different β-lactamases. Apart from the difference in the rates of hydrolysis of the different enzymes, all three variables changed at similar rates.

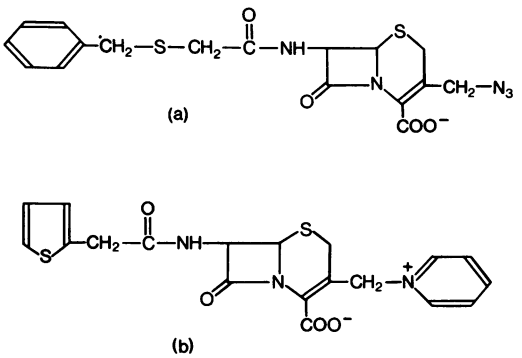

FIG. 1. *Structures of (a) BTCA and (b) CER.*

When a similar experiment was made with BTCA, it was again found that the three variables changed at similar rates with each enzyme, except that this time the azide was the grouping which was released from the cephalosporin (Fig. 3). There is a quantitative relationship, therefore, between hydrolysis of the β-lactam bond, release of a group from the 3-position, and reduction in antibacterial activity of the compound.

DISCUSSION

Earlier studies have shown that the hydrolysis of cephalosporin C and cephalothin by a β-lactamase produced by *Pseudomonas aeruginosa* resulted in the release of 1 mole-equivalent of acetic acid from these compounds (6). The present study has shown that the azide group in the 3-position of BTCA was released quantitatively upon hydrolysis of the β-lactam bond. Moreover, this release occurred at the same rate and to the same extent as the breakage of the β-lactam bond and the loss of biological activity with all three enzymes. This is in agreement with the observations made with the β-lactamase from *P. aeruginosa* with cephalosporin C and cephalothin (6).

In the case of CER, the same authors reported that hydrolysis resulted in the production of only 0.67 mole of pyridine for each molecule of cephaloridine hydrolyzed, the remaining 0.33 mole being unaccounted for. In the experiments reported here, however, exactly 1 mole of pyridine was released per mole of CER with all three β-lactamases, and its liberation was closely paralleled by changes in UV absorption and microbiological activity. The use of a direct assay for free pyridine may account for the detection of the stoichiometric amount of pyridine in these experiments.

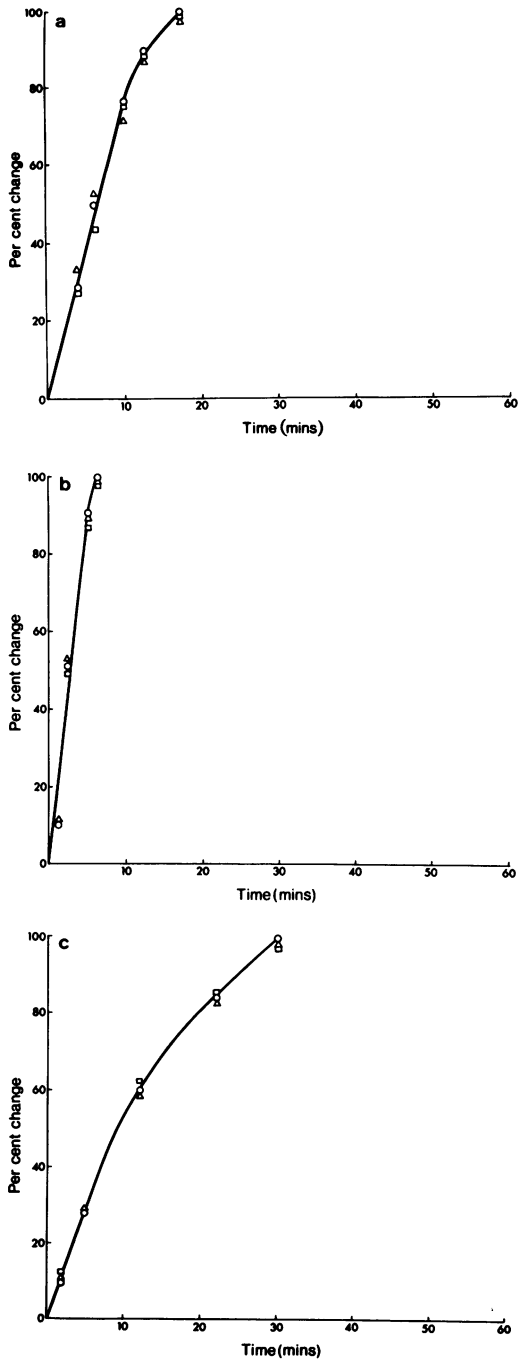

FIG. 2. *Effects of hydrolysis of CER by three β-lactamases: (a) Enterobacter cloacae P99; (b) Escherichia coli TEM; (c) Klebsiella aerogenes. Symbols: (△) decrease in absorption at 255 nm; (○) release of pyridine; (□) loss of biological activity.*

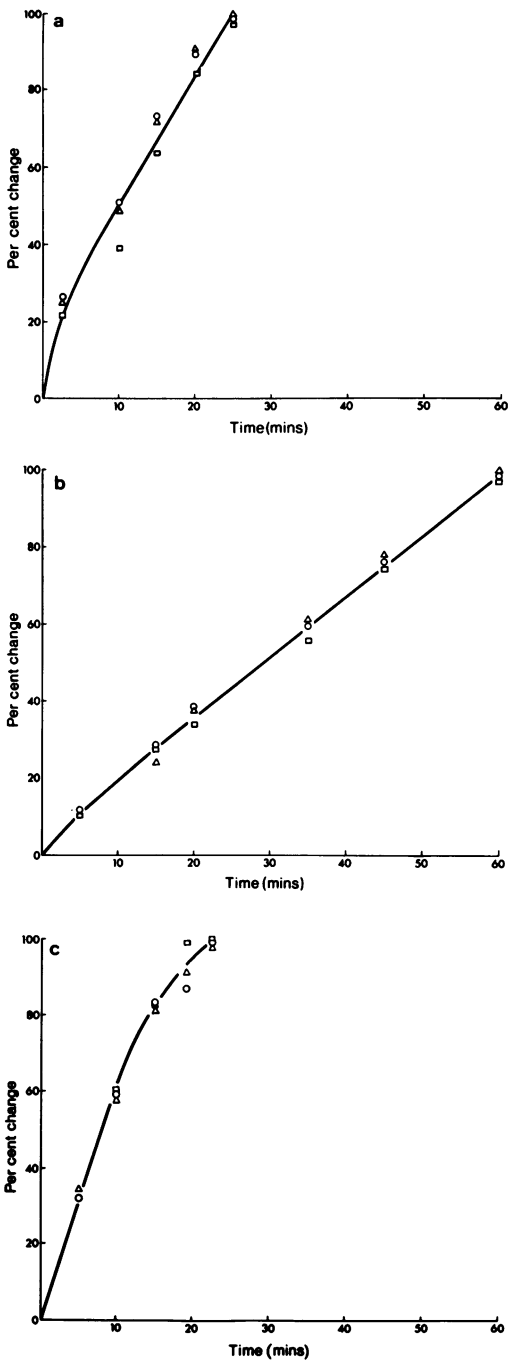

FIG. 3. *Effects of hydrolysis of BTCA by three β-lactamases: (a) Enterobacter cloacae P99; (b) Escherichia coli TEM; (c) Klebsiella aerogenes. Symbols: (△) decrease in absorption at 255 nm; (○) release of azide; (□) loss of biological activity.*

It is concluded, therefore, that whenever the β-lactam bond in a cephalosporin is hydrolyzed by a β-lactamase, there is a concomitant quantitative release of the group in the 3-position, unchanged, provided it can accept an electron. This strongly suggests that the mode of attack of the β-lactamases of gram-negative origin is the same, despite considerable differences between the enzymes, particularly with regard to their substrate profiles.

Other cephalosporins which do not have a good leaving group in the 3-position are also susceptible to β-lactamase hydrolysis. Compounds such as desacetylcephalothin (6) and cefazolin are readily hydrolyzed but do not give any readily identifiable product. A cephalosporin derivative in which the 3-substituent can accept an electron without leaving the nucleus (5) gives the corresponding cephalosporoic acid, in which the only change in structure which occurs is the opening of the β-lactam ring. It would seem, therefore, that susceptibility to these enzymes does not depend on the presence of a leaving group. Its loss, intact, is thus probably a consequence and not a cause of susceptibility to β-lactamase attack, and points to the occurrence of an electron shift from the β-lactam ring in the direction of the 3-position (4). Once the substituent is lost, the residual compound undergoes further changes (1).

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