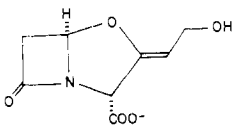
# Kinetic Studies on the Inactivation of *Escherichia coli* RTEM β-Lactamase by Clavulanic Acid†

Jed Fisher,‡ Robert L. Charnas,§ and Jeremy R. Knowles*

ABSTRACT: The kinetic details of the irreversible inactivation of the *Escherichia coli* RTEM β-lactamase by clavulanic acid have been elucidated. Clavulanate is destroyed by the enzyme and simultaneously inhibits it by producing two catalytically inactive forms. One of these is transiently stable and decomposes to free enzyme ($k = 3.8 \times 10^{-3}$ s$^{-1}$), while the other corresponds to an irreversibly inactivated form. The transient complex is formed from the Michaelis complex at a rate ($k \sim 3 \times 10^{-2}$ s$^{-1}$) which is some threefold faster than the rate of formation of the irreversibly inactivated complex. The transient complex is, therefore, the principle enzyme form present after short time periods. In the presence of excess clavulanate, however, all the enzyme accumulates into the irreversibly inactivated form. The number of clavulanate turnovers that occur prior to complete enzyme inactivation is 115.

The emergence within the past year of penicillin-resistant strains of *Neisseria gonorrhoeae* (Eisenstein et al., 1977; Roberts & Falkow, 1977) has provided the unnecessary reminder that the molecular nature of the enzyme primarily responsible for this resistance, the β-lactamase, is poorly understood. Since the clinical efficacy of most β-lactam antibiotics is largely determined by their susceptibility to this hydrolytic defense mechanism, the proliferation of β-lactamase-producing bacteria has led to the development of β-lactams that both resist hydrolysis by β-lactamases *and* are effective antibiotics. These properties (of resistance to β-lactamases, and antibacterial potency) have traditionally been obtained by altering the nature of the 6-acyl side chain of the penam. While these semisynthetic penicillins work well against gram-positive bacteria, they are relatively ineffective against gram-negative microorganisms (Nayler, 1973; Doyle & Nayler, 1964). Some of these compounds are good reversible inhibitors of β-lactamases in vitro, but have a restricted spectrum of bacteriocidal action and do not act usefully in vivo (Hamilton-Miller et al., 1964; Sutherland & Batchelor, 1964; Fraher & Jawetz, 1967; O'Callaghan et al., 1968; O'Callaghan & Morris, 1972; Cole et al., 1972; but see Greenwood & O'Grady, 1975).

This situation has changed dramatically with the discovery that clavulanic acid (I) (Howarth et al., 1976), a novel β-lactam isolated from *Streptomyces clavuligerus*, is a potent inactivator of β-lactamases from a variety of gram-negative and gram-positive bacteria in vitro and in vivo (Reading & Cole, 1977).

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Such an inactivator could extend the effective range of existing β-lactam antibiotics into resistant strains, and an understanding of the structure:function relationship for clavulanic acid is of the utmost importance. We report here results that lead to a minimal scheme for the inactivation pathway. This paper concentrates on the kinetic details and the following one outlines the preliminary chemical characterization of the inactivated enzyme species.

## Materials and Methods

*Sodium clavulanate* was the generous gift of Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, U.K. It was supplied as the crystalline tetrahydrate and stored desiccated at −20 °C. This material was examined before and during storage, by thin-layer chromatography and nuclear magnetic resonance, and showed no detectable impurities. Solutions of sodium clavulanate were prepared daily in 0.10 M potassium phosphate buffer, pH 7.0.

*β-Lactamase* was from an *Escherichia coli* RTEM strain described previously (Hall & Knowles, 1976). The enzyme (which is encoded by a plasmid) was purified by modification of the procedure of Melling & Scott (1972), and had a specific activity of 4150 μmol of benzylpenicillin hydrolyzed per $A_{281\ nm}$ per min (30 °C, pH 7.0). The enzyme was greater than 95% homogeneous as estimated by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate, and by nondenaturing polyacrylamide isoelectric focusing.

Sodium clavulanate solutions were made by weight using a 10-mg Roller-Smith precision microbalance. All kinetics were performed in 0.10 M potassium phosphate buffer, pH 7.0, at 30 °C using a Perkin-Elmer 575 spectrometer. Assays of enzyme activity involved following the change in ultraviolet absorbance as the β-lactam ring of either phenoxymethylpenicillin or benzylpenicillin was cleaved (Samuni, 1975; Citri et al., 1976).

*Irreversible Inactivation Kinetics.* A. Gel Filtration Assay. Portions (100 μL) of stock solutions of the enzyme (20 μM) and of clavulanate (7, 14, or 27 mM), equilibrated at 30 °C, were mixed in a vial. The incubation was maintained at 30 °C and then quenched by rapid cooling to 0 °C. The mixture was then quickly transferred to a column (1.0 × 8.5 cm) of Sephadex G-25, which was developed at 4 °C with a flow rate of 16 mL h$^{-1}$ of 25 mM potassium phosphate buffer, pH 7.0. All

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‡ National Institutes of Health Postdoctoral Fellow.

§ National Science Foundation Predoctoral Fellow.

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of the fractions containing enzymatic activity were pooled, and the total pool volume was determined by weight. The pooled fractions were warmed to 30 °C, and when the activity had stabilized (approximately 30 min) the total activity present was determined. Control runs in which buffer replaced the clavulanate solution gave at least a 95% recovery of the total enzyme activity regardless of the length of the incubation.

B. Dilution Assay. Portions (100 μL) of stock solutions of the enzyme (20 μM) and of clavulanate (7, 14, or 27 mM), equilibrated at 30 °C, were mixed in a vial. At the appropriate intervals, samples (20 μL) were removed and quenched by dilution into 500 μL of a solution of phenoxymethylpenicillin (3.6 mM). Portions of 10 μL (for early time points) or 20 μL (for late time points) were removed from this quenching solution, and added to an assay cuvette containing 3.0 mL of phenoxymethylpenicillin (3.6 mM) in 0.10 M potassium phosphate buffer, pH 7.0, at 30 °C. After 30 min the progress curve at 240 nm had become linear, and a final activity was calculated from this velocity. This limiting rate was compared with the control and the fraction of the catalytic activity remaining was thus obtained. In all of these experiments the final clavulanate concentration in the assay cuvette was approximately 0.5 μM. Additional controls established that an increase to 2 μM clavulanate did not alter the magnitude of the measured limiting velocity.

*Stoichiometry of Clavulanate Hydrolysis.* Buffered stock solutions of the enzyme (0.26 mM) and of clavulanate (4 mM), equilibrated at 30 °C, were combined in the appropriate quantities to give clavulanate (c) to enzyme (e) ratios of: ($c/e$) = 15, 30, 43, 60, 75, and 150. At the appropriate times, portions (5 μL) were withdrawn and diluted into an assay cuvette containing 3.0 mL of a benzylpenicillin solution (3.5 mM) and the initial velocity was determined and compared with the control. For the determination at $c/e$ = 15, the initial clavulanate concentration was 1.0 mM; for all of the others it was 2.0 mM.

## Results and Discussion

The kinetic and chemical mechanisms corresponding to the irreversible inactivation of the *E. coli* RTEM β-lactamase by sodium clavulanate are complex. The unusual features of this inactivation originate from the ability of clavulanate to exert *two* separate inhibitory activities simultaneously, only one of which involves the formation of an indefinitely stable, apparently covalent complex of the inhibitor with the enzyme. The second inhibitory activity is expressed as a transiently stable complex, that by virtue of its more rapid rate of formation relative to the irreversible complex dominates the kinetics of the inactivation process. Since this complex is transient and regenerates free enzyme, it only delays the eventual accumulation of the enzyme into the irreversibly inactivated form. The experiments described below allow the delineation of a minimal kinetic scheme for the interaction between enzyme and inhibitor.

*Kinetics of Irreversible Inhibition.* The capacity of sodium clavulanate to function as an irreversible β-lactamase inhibitor (Howarth et al., 1976; Reading & Cole, 1977) is clear. When the enzyme is incubated with clavulanate in 350-, 700-, and 1350-fold molar excess, the rate of loss of catalytic activity is identical, and less than 10% activity remains after 60 min. The activity remaining at different times was assayed in two ways. The first method involved gel filtration in the cold to separate the excess clavulanate from the enzyme. The enzyme fractions were pooled and assayed after the activity had stabilized (vide infra), and this activity was compared with the control. The identity of the rates of irreversible inactivation at different concentrations of clavulanate showed that the reaction was saturable with respect to clavulanate, thus eliminating the possibility of a nonspecific (bimolecular) inactivation reaction. The second method involved the successive dilution of a sample from the incubation into an assay mixture. The dilution of 3750-fold eliminates any further effect of clavulanate on the enzyme. A relatively small number of clavulanate molecules is required for complete enzyme inactivation. Treatment of the β-lactamase with as few as 350 molar equivalents of clavulanate for 90 min (pH 7.0, 30 °C) results in an enzyme having only 1 to 2% residual activity. The kinetic properties of this residuum (relative $V_{max}$ and $K_m$ for several substrates) were found to be identical with those of the native enzyme, indicating that small amounts of native enzyme, as distinct from substantial amounts of partially active enzyme, remained. The inactivated enzyme prepared in this way is irreversibly inactivated and shows only very small increases in activity (up to 3 or 4%) upon prolonged (72 h) dialysis at 4 °C or standing at 30 °C for several hours.

*Clavulanate Hydrolysis and Transient Complex Formation.* The fact that the enzyme can be completely inactivated by only 350 molar equivalents of inhibitor indicates that the turnover of clavulanate (presumably equivalent to the normal hydrolytic action of the enzyme) must be quite small. This question may be approached by monitoring the activity changes that occur when limiting amounts of clavulanate are added to the enzyme. Such an experiment provides not only the number of turnovers prior to inactivation, but also serves to demonstrate the complexity of the inactivation process. Using molar ratios of clavulanate to enzyme between 15 and 150, the fraction of enzymic activity remaining as a function of time was determined. These results are shown in Figure 1 (top). For all but the highest ratio, a rapid decrease in activity was observed initially, but this was then followed (after a variable lag period) by a *recovery* of activity to plateau levels corresponding to different extents of irreversible inactivation. The extent of irreversible inactivation increases with increasing quantities of clavulanate. A plot of the fraction of catalytic activity at the plateau shown in Figure 1 vs. the molar ratio of clavulanate to enzyme is linear and extrapolates to a ratio of clavulanate/enzyme of 115 at complete inactivation. This number represents the number of completed turnovers before the accumulation of *all* the enzyme in the irreversibly inactivated form. The family of curves shown in Figure 1 suggests two conclusions. First, the recovery of activity commences from the point at which there has been complete consumption of the available clavulanate; secondly, the initial loss of activity results from the partitioning of the enzyme into two forms, neither having catalytic activity, one being transiently stable (whence the enzyme recovery) and the other not. The first conclusion was confirmed directly by an experiment in which a second addition of enzyme was made to the clavulanate–enzyme incubation just at the point where the recovery of enzymatic activity began (see Figure 1 (bottom)). No inhibition of this second portion of enzyme was seen, and the kinetics of the recovery of enzyme activity were unaffected. The conclusion that the enzyme had dissipated the clavulanate is inescapable.

The second conclusion is less obvious and most of the experiments remaining to be described were designed to provide a better understanding of this partitioning process. It is apparent from Figure 1 that there is an approximately threefold preference in the partitioning of the enzyme to the transient complex relative to the irreversible complex (this is best illustrated by the curve corresponding to a clavulanate:enzyme ratio of 30), and that the decomposition of the transient complex is a first-order process with a rate constant of about 2 ×

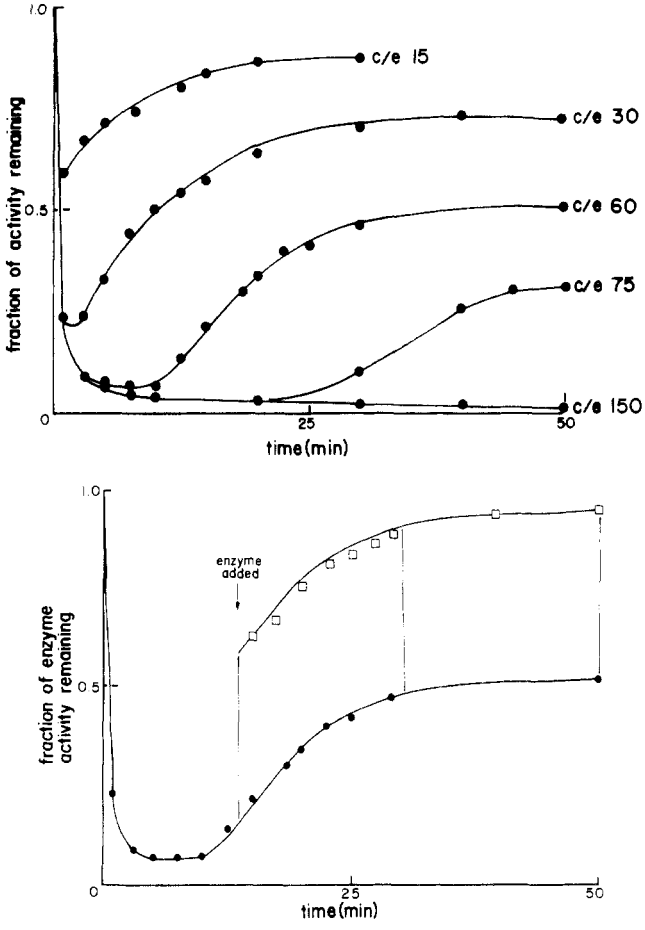



FIGURE 1: (Top) The time dependence of $\beta$-lactamase activity in the presence of clavulanic acid. Limiting quantities of clavulanate (c) were incubated with the enzyme (e) and, at the indicated times, portions were assayed and the fraction of remaining activity was determined from the initial velocity of substrate hydrolysis. For the determination at $c/e = 15$, [e] = 66.3 $\mu$M and [c] = 1.00 mM. In the remaining determinations, [c] = 2.00 mM and [e] was varied between 13.3 and 66.3 $\mu$M. All measurements were made at 30 °C in 100 mM potassium phosphate buffer, pH 7.0. (Bottom) $\beta$-lactamase-catalyzed decomposition of clavulanic acid. The lower curve (solid circles) is identical with the progress curve from Figure 1 (top) at $c/e = 60$. At 13.5 min after mixing the enzyme and clavulanic acid, an additional portion of enzyme was added (equivalent to 40% of the enzyme activity initially present). The upper curve is identical with the lower curve, offset by a fraction of 0.4. The open squares represent the experimentally measured enzyme activity.

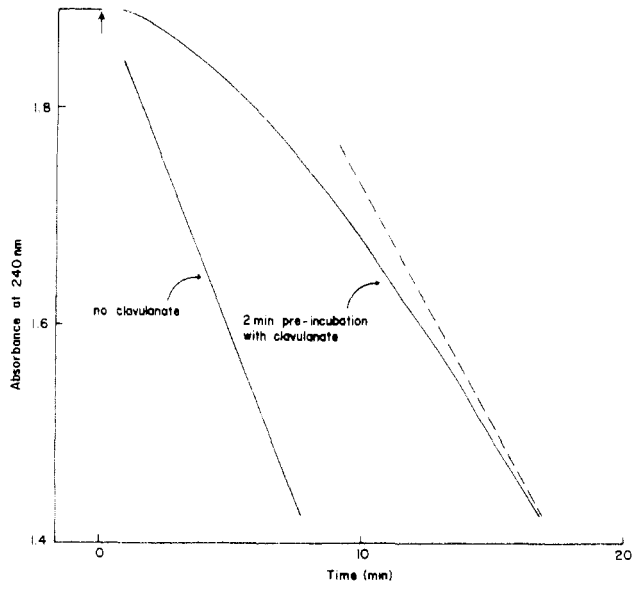



FIGURE 2: Time course of decomposition of the transiently inhibited complex between $\beta$-lactamase and clavulanic acid. Equal portions (20 $\mu$L) of a stock enzyme solution (19.5 $\mu$M) and stock clavulanate solution (2.00 mM) were combined and incubated at 30 °C for 120 s. The reaction was quenched with 1.00 mL of benzylpenicillin solution (2.4 mM). A portion (10 $\mu$L) of this solution was added to a cuvette containing 3.0 mL of benzylpenicillin (2.4 mM) for assay. The reaction rate accelerates to a limiting value (75% of the control velocity in this instance) as free enzyme is liberated from the transiently inhibited complex.

$10^{-3}$ $s^{-1}$. Since the rates of formation and decomposition of this complex are critical to the overall kinetic scheme, a more quantitative approach was undertaken.

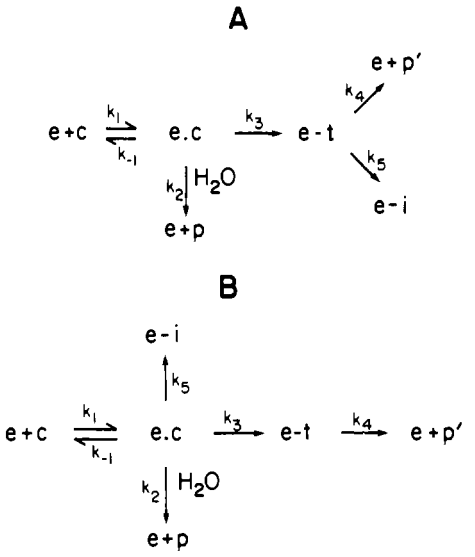
If clavulanate and enzyme are incubated together ($c/e$ ratio ~200) in a small volume (20 $\mu$L) for a short time (between 15 and 180 s) and then added to 3 mL of assay solution, it is possible from the initial rates of substrate hydrolysis to determine the approximate rate of formation of the transient complex. It was shown that this rate is independent of the concentration of clavulanate, and of the particular $\beta$-lactamase substrate used in the assay. The process appears to be first-order with a rate constant of $\sim 3 \times 10^{-2}$ $s^{-1}$ ($t_{1/2} \approx 20$ s). This rate is much too slow to reflect the diffusion of clavulanate to the active site of the enzyme and presumably corresponds to an event occurring after the initially formed Michaelis complex. That this process actually involves covalency changes involving the clavulanate is suggested by the spectral changes seen on formation and decomposition of the complex (Charnas et al., 1978).

*Decomposition of the Transient Complex.* The rate of decomposition of the transiently inhibited complex was determined analogously. The enzyme was incubated with a 100-fold molar excess of clavulanate for 120 s, at which point no catalytic activity remained (see Figure 1). By dilution into an assay mixture containing benzylpenicillin, the enzyme activity was monitored continuously (see Figure 2). Under these circumstances the rate of substrate hydrolysis measures the instantaneous concentration of active enzyme. It is evident that the concentration of free enzyme increases from the start of the assay and reaches a steady value after about 20 min. The final value obtained for the incubation illustrated in Figure 2 was 75% that of the control. The clavulanate concentration in the assay solution is negligible, and the activity difference (25%) corresponds to the *irreversible* inactivation that had occurred by the time that the enzyme was diluted (at 120 s) into the assay cuvette. It is also apparent that, at the start of the assay shown in Figure 2, all of the remaining enzyme was in the form of the transiently inhibited complex, since the *initial* velocity of the assay is essentially zero. After 120-s incubation, therefore, the enzyme exists partly as the transiently inhibited complex (75%) and partly in the form of irreversibly inactivated material (25%). There is no free enzyme left. From the rate of reappearance of enzyme activity, the rate of decomposition of the transient complex was shown to have a rate constant of $3.8 \pm 0.3 \times 10^{-3}$ $s^{-1}$, in good agreement with the approximate value derived from the results in Figure 1, obtained above. The rate of recovery of enzyme activity is independent of the assay substrate (benzylpenicillin, carbenicillin, or phenoxymethylpenicillin), and independent of the preincubation time with clavulanate (that is, of the extent of irreversible inactivation). It is unaffected by the presence of mM levels of either hydroxylamine or methanol. These data are most consistent with an enzyme-mediated reaction of clavulanate that results in the formation of a labile inhibited adduct that can spontaneously regenerate free enzyme.

*The Minimal Kinetic Mechanism.* With the basic kinetic

parameters established, how do we integrate the transiently inhibited complex into an overall kinetic scheme? Three possibilities exist. The transient complex may be an intermediate in "normal" hydrolysis, it may be the precursor to irreversible inactivation, or it may be formed independently of either hydrolysis or inactivation. The first possibility may be disposed of, since the rate constant for transient complex decomposition ($3.8 \times 10^{-3}$ s$^{-1}$) is much too slow to account for the number of turnovers which must occur immediately following the mixing of clavulanate with the enzyme (see Figure 1). To distinguish between the remaining two possibilities let us compare the two relevant schemes (Schemes IA and IB).

SCHEME I: Possible Kinetic Pathway for the Interaction of Clavulanic Acid and β-Lactamase.[a]




[a] The abbreviations used are: e, free enzyme; c, clavulanate; e·c, the Michaelis complex with clavulanate; p, the clavulanate product from normal turnover; p', the clavulanate product from decomposition of the transiently inhibited complex, e-t; e-i, irreversibly inactivated enzyme.

These differ only in the origin of the irreversible inactivation, which may occur either from the transient complex (Scheme IA) or from the Michaelis complex (Scheme IB). The basis on which these schemes can be distinguished lies in how they account for the kinetics of irreversible inactivation. In Figure 3, the rates of irreversible inactivation derived both from gel filtration experiments and from the dilution assay are plotted semilogarithmically. It may be seen that the inactivation is the sum of two first-order rate processes. The loss in catalytic activity occurs quickly at first ($k \gtrsim 10^{-2}$ s$^{-1}$) but within several minutes this gives way to a slower first-order rate ($k = 5.1 \times 10^{-4}$ s$^{-1}$) that characterizes the remainder of the inactivation. It is evident from this graph that irreversible inactivation of the enzyme is independent of the formation of the transient complex, since irreversible inactivation *slows down* as the enzyme accumulates into the transient complex. Scheme IA is thus not plausible. The rapid initial loss of activity (in Figure 3) occurs in competition with transient complex formation, and the slower rate corresponds to the steady state in which all of the enzyme that has not been irreversibly inactivated exists as the transient complex. Dissociation of the transiently inhibited complex to free enzyme is required in order for the enzyme to be presented with the opportunity to bind an intact clavulanate, the Michaelis complex so formed then partitioning in three ways, resulting in hydrolysis of clavulanate, irreversible inactivation of the enzyme, or regeneration of the transient

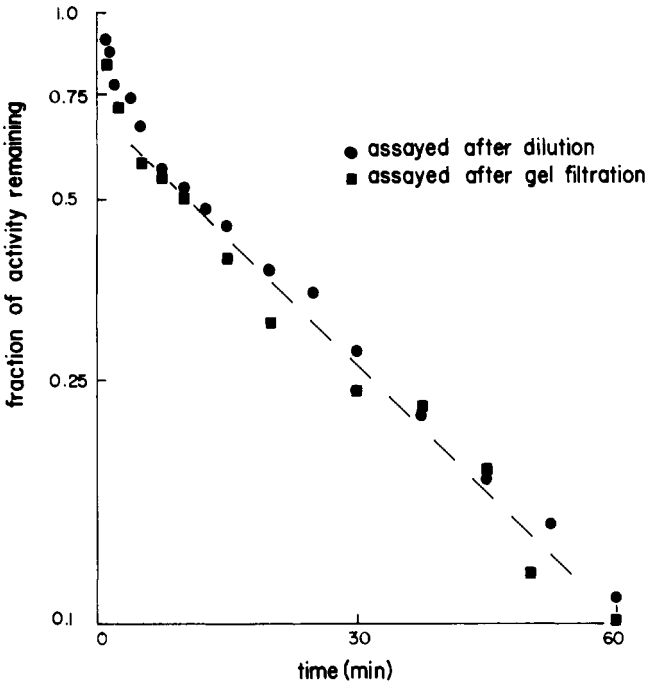



FIGURE 3: Semilogarithmic plot of the rate of irreversible inactivation of β-lactamase by clavulanic acid. The remaining catalytic activity after incubation with saturating levels of clavulanate ([enzyme], 10 μM; [clavulanate], 3.5, 7, or 13.5 mM) was determined after gel filtration (■) or by dilution assay (●). For details, see the text.

complex. The biphasic irreversible inactivation kinetics (Figure 3) are therefore the natural consequence of $k_4$ of Scheme IB becoming increasingly rate limiting as the steady state is established.

Although the above is the simplest interpretation of the results, two alternative explanations for the biphasic kinetics of Figure 3 must be mentioned. If the clavulanate were to contain a small quantity of a more potent inactivator, a faster rate of inactivation would be expected initially, up until the point at which the contaminant was consumed. If this were the case, however, increasing the total clavulanate concentration would result in greater quantities of enzyme activity being lost in the first phase, and this is not observed experimentally. The "two inactivator" hypothesis is thus eliminated. A second possibility is that the enzyme is slowly transformed by clavulanate into a conformational state that is less susceptible to inactivation. In studies on the enzyme from *B. cereus*, Citri et al. (1976) have shown that hysteretic behavior is observed by all substrates that have lower turnover rates than 6-aminopenicillanic acid. For the *E. coli* RTEM enzyme used here, no deviations from Michaelis-Menten behavior are seen for a number of substrates that are hydrolyzed more slowly than 6-aminopenicillanic acid (including phenoxymethylpenicillin, carbenicillin, cephalothin, and cephalosporin C), and if multiple conformational states of the enzyme exist for these substrates, their interconversion must be rapid compared with the several seconds required for mixing an assay solution. Only in such extreme cases as cloxacillin and methicillin are any deviations from simple kinetic behavior observed, and these anomalies are complete within 60 s. Moreover, hysteresis is apparently dependent on the size of the C-6 side chain (Citri et al., 1976), and this is missing completely in clavulanic acid. The possibility that the *absence* of a C-6 side chain results in hysteretic behavior was tested directly with desaminopenicillanic acid as a β-lactamase substrate. This compound cleanly obeys Michaelis-Menten kinetics, exhibiting a $V_{max}$ (relative to ben-

zylpenicillin) of 0.020 and $K_m$ of 350 $\mu$M. These findings do not rigorously exclude the possibility of multiple conformational states but the postulate is unnecessary as long as the simple explanation of Scheme IB suffices.

The most plausible kinetic mechanism is therefore Scheme IB, in which clavulanate hydrolysis, transient complex formation, and irreversible inactivation are competing events from the Michaelis complex. There are several implications. The limitation of 115 turnovers prior to complete inactivation demands that the rate constant for hydrolysis ($k_2$) be about 1 $s^{-1}$. [This value can be estimated from Figure 1, where it is seen that 30 equiv of clavulanate is consumed during the first 60 s after mixing (i.e., the rate appears to be about 0.5 $s^{-1}$). In this interval, however, approximately half the enzyme is inhibited or inactivated, so the true value for the hydrolysis rate will be about twice this, at 1 $s^{-1}$. This is a very much slower rate of hydrolysis than is seen for benzylpenicillin (which is 1480 $s^{-1}$).] It is also apparent that the product from transient complex decomposition (p') need not be identical with the product from hydrolysis (p), and that p will be produced in much larger quantities (perhaps tenfold) than p'.

Finally, while the rate of transient complex formation relative to inactivation (Scheme IB: $k_3/k_5$) determined from brief incubations is threefold (Figure 1), the difference between these rates as derived from the steady-state rate of irreversible inactivation is six- or sevenfold. This inconsistency may relate to the observation that the inactivated enzyme is heterogeneous (Charnas et al., 1978). There are in fact *three* irreversibly inactivated complexes that can be distinguished by their chemical reactivity and physical properties, and the experimental data that would isolate their rates of formation as separate kinetic events have not been acquired.

The interaction of clavulanate with $\beta$-lactamase from *E. coli* is not straightforward. While there are unanswered questions, and while it is apparent that there is a range of $\beta$-lactamase susceptibility to clavulanate (Reading & Cole, 1977), the pharmaceutical importance of this compound demands that these issues be clarified. It is conceivable that the complex kinetic pattern for inactivation may not be limited to clavulanate alone; and that clavulanate and olivanic acid (Brown et al., 1977) are all precursors to rather indiscriminate alkylating agents that attack and inactivate the $\beta$-lactamase (Charnas et al., 1978).

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