Biochem. J. (1981) **199**, 779–787
Printed in Great Britain

The inhibition of β-lactamases from Gram-negative bacteria by clavulanic acid

Christopher READING and Tony FARMER
Beecham Pharmaceuticals Research Division, Brockham Park, Betchworth, Surrey RH3 7AJ, U.K.

(*Received 17 July 1981/Accepted 24 July 1981*)

The β-lactamase from *Klebsiella pneumoniae* E70 behaved in a similar fashion to the TEM-2 plasmid mediated enzyme on reaction with clavulanic acid. Both enzymes produced two types of enzyme–clavulanate complex, a transiently stable species ($t_{\frac{1}{2}} = 4$ min at pH 7.3 and 37°C) and irreversibly inhibited enzyme. In the initial rapid reaction (2.5 min) the enzymes partitioned between the transient and irreversible complexes in the ratios 3 : 1 for TEM-2 β-lactamase and 1 : 1 for *Klebsiella* β-lactamase. Biphasic inactivation was observed for both enzymes and the slower second phase was rate limited by the decay of the transiently stable complex. This decay released free enzyme for further reaction with fresh clavulanic acid, the products again partitioning between transiently stable and irreversibly inhibited enzyme. This cycle continued until all the enzyme had been irreversibly inhibited. A 115 molar excess of inhibitor was required to achieve complete inactivation of TEM-2 β-lactamase. Hydrolysis of clavulanic acid with product release appeared to occur with the inhibition reaction, which explained this degree of clavulanic acid turnover. The stoichiometry of the interaction with *Klebsiella* β-lactamase was not examined. The penicillinase from *Proteus mirabilis* C889 was rapidly inhibited by low concentrations of clavulanic acid. The major product was a moderately stable complex ($t_{\frac{1}{2}} = 40$ min at pH 7.3 and 37°C); the proportion of the enzyme that was irreversibly inactivated was small. The cephalosporinase from *Enterobacter cloacae* P99 had low affinity for the inhibitor and only reacted with high concentrations of clavulanic acid ($k = 4.0\,\text{M}^{-1}\cdot\text{s}^{-1}$) to produce a relatively stable complex ($t_{\frac{1}{2}} = 180$ min at pH 7.3 and 37°C). No irreversible inactivation of this enzyme was detected. The rates of decay of the clavulanate–enzyme complexes produced in reactions with *Proteus* and *Enterobacter* enzymes were markedly increased at acid pH.

Clavulanic acid is a novel naturally occurring β-lactam with potent β-lactamase inhibitory properties (Reading & Cole, 1977). Studies on its interaction with the β-lactamase from the Gram-positive organism *Staphylococcus aureus* have already been reported (Cartwright & Coulson, 1979; Reading & Hepburn, 1979). The interaction of TEM-2 β-lactamase with clavulanic acid has been described in detail by Fisher *et al.* (1978). Comparative data on the inhibition of TEM-1 and -2 β-lactamases and the Pitton's type 2 (SHV-1) β-lactamase have also been described previously (Labia & Peduzzi, 1978). In the present paper we report in brief our findings on the interaction of clavulanic acid with TEM-2 β-lactamase, which agree with those of Fisher *et al.* (1978). These results are compared with those we have obtained for some further enzymes from Gram-negative bacteria, namely *Klebsiella pneumoniae* E70, *Proteus mirabilis* C889 and *Enterobacter cloacae* P99.

Materials and methods

Enzyme preparations

TEM-2 β-lactamase was supplied by the Microbiological Research Establishment, Porton, Wilts., U.K. This was an $(NH_4)_2SO_4$ precipitate of cell extracts of *Escherichia coli* W3110. Further purification was achieved by adsorption on DEAE-cellulose (Whatman DE-52) in a batchwise fashion, followed by water washing and then elution with 0.2 M-NaCl in 0.01 M-sodium phosphate buffer, pH 7.3. The eluate was saturated to 75% with $(NH_4)_2SO_4$ and the precipitate solid was then dissolved in water. After extensive dialysis at 5°C against water, the retained material was freeze dried

0306-3275/81/120779-09$01.50/1

to yield a crude preparation with a specific activity of 7.16 mg of benzylpenicillin hydrolysed/ml per min per mg of enzyme at pH 7.3 and 37°C. On the basis of the known molecular activity of TEM-2 β-lactamase (Fisher *et al.*, 1978) this represented a β-lactamase concentration of 0.34 μM.

Other enzyme preparations were simply cell-free extracts obtained by ultrasonication of bacterial cells as described previously (Reading & Cole, 1977).

Measurement of enzyme activity

Enzyme activity in control reactions or in inhibited reactions was measured by spectrophotometric assay in a Pye–Unicam SP. 800 spectrophotometer, either by adding reaction samples to benzylpenicillin (500 μg/ml) and measuring the decrease in A_{240} (Waley, 1974) or by adding reaction samples to nitrocefin (250 μg/ml) and monitoring the increase in A_{550}. All reactions were in 0.05 M-sodium phosphate buffer, pH 7.3, and at 37°C. Assay of *Enterobacter cloacae* P99 β-lactamase was carried out using cephaloridine as the substrate (40 μg/ml) by measuring the change in A_{255}.

Rates of hydrolysis of various β-lactams were compared relative to the rate of hydrolysis of benzylpenicillin (substrate profiles). These rates were determined spectrophotometrically as described previously (King *et al.*, 1980).

Measurements of rate of inhibition

Reactions of clavulanic acid and enzyme were sampled at time intervals and the samples were diluted into excess substrate to measure residual enzyme activity. Initial rates of substrate hydrolysis were measured as described above and were compared with an uninhibited control reaction.

Detection of transiently stable enzyme–inhibitor complexes

β-Lactamase was incubated for a suitable time period with an excess of inhibitor at pH 7.3 and 37°C. The presence of a relatively unstable complex was determined by diluting a reaction sample into excess substrate, which prevented further reaction of enzyme and inhibitor. This was followed by spectrophotometric assay of enzyme activity over a suitable time period. Return of enzyme activity was seen as an ever increasing rate of substrate hydrolysis. Alternatively, excess clavulanic acid was removed from the reaction by gel filtration of 0.5 ml of reaction sample at 5°C with Bio-gel P2 columns (12.5 mm × 250 mm; Bio-Rad) eluted with 0.5 M-sodium phosphate buffer, pH 7.3 (flow rate 1 ml/min). Fractions were collected (0.5 ml) and stored at 5°C. Those containing inhibited enzyme were combined on the basis of the position of enzyme activity present in fractions from an identical control column to which uninhibited enzyme had been applied. Inhibited enzyme was then incubated at 37°C and regenerated enzyme activity was measured with time by sampling into substrate.

Determination of clavulanic acid

Loss of clavulanic acid from reactions with β-lactamase was measured by gel filtration of reaction samples as described above. Fractions were assayed for clavulanic acid content by using an automated enzyme-inhibition assay (Reading & Hepburn, 1979) and staphylococcal β-lactamase.

The effect of pH on the stability of enzyme–inhibitor complexes

Inhibited enzyme was prepared by incubating with excess clavulanic acid. The reaction was then diluted into excess substrate at different pH values using the buffers described by Waley (1975). The return of enzyme activity was then measured spectrophotometrically as an increasing rate of substrate hydrolysis with time. Alternatively, inhibited enzyme was gel filtered at 5°C as described previously and the preparation of enzyme free of clavulanic acid so achieved was diluted into buffer at various pH values and then incubated at 37°C. Samples were removed and assayed for enzyme activity with time by measuring initial velocities of substrate hydrolysis.

Measurement of irreversible inactivation

The determination of the amount of enzyme that was inactivated in reactions with clavulanic acid was taken as the percentage activity that did not return after allowing unstable complexes to decay over a suitable time period. The decay time allowed for TEM-2 and *Klebsiella* enzymes was 30 min, for *Proteus* enzyme 60 min at pH 5.8 and for *Enterobacter cloacae* β-lactamase an overnight incubation, all at 37°C and pH 7.3, unless otherwise stated. Decay of the unstable complexes was monitored by dilution into substrate or by gel filtration of reaction samples as described previously. Irreversible inactivation was similarly followed and represented the enzyme activity that did not return even after prolonged incubation.

Isoelectric focusing

Analytical examination of the various β-lactamase preparations was carried out by isoelectric focusing on LKB Ampholine P.A.G. plates, pH 3.5–9.5, with LKB 2117 Multiphore equipment as described previously (King *et al.*, 1980). Enzyme bands were revealed by using nitrocefin.

Results

Inhibition of TEM-2 β-lactamase

The results we obtained for the interaction of clavulanic acid with TEM-2 β-lactamase agree with

those of Fisher *et al.* (1978). Rapid inhibition of the β-lactamase (2.5 min) was obtained with the formation of a transiently stable and irreversible enzyme–clavulanate complex. The transiently stable species was detectable by diluting reaction samples into excess benzylpenicillin substrate and monitoring spectrophotometrically as shown in Fig. 1. Alternatively reaction samples were gel-filtered to remove excess inhibitor and the return of enzyme activity was followed with time in the absence of substrate. Both methods revealed that the decay was first order with respect to time, with a $t_{\frac{1}{2}}$ of 4 min at pH 7.3 and 37°C ($k = 2.9 \times 10^{-3}$ s^{-1}). At a reaction time of 2.5 min, when all the enzyme had been inhibited, the transient and irreversible complexes were present in the ratio 3 : 1 respectively. The biphasic time course of irreversible inhibition is shown in Fig. 2. The molar excess of clavulanate to enzyme required to achieve complete irreversible inhibition was 115, which agrees with the results of Fisher *et al.* (1978).

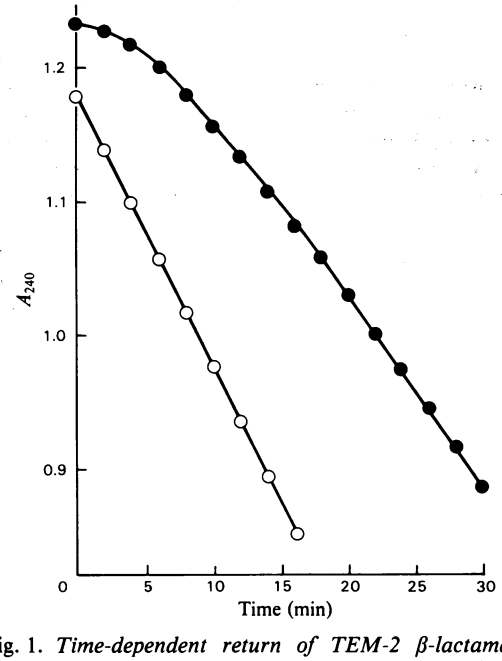


Fig. 1. *Time-dependent return of TEM-2 β-lactamase activity after inhibition by clavulanic acid and dilution into excess substrate*

TEM-2 β-lactamase (0.027 μM) was allowed to react at 37°C and pH 7.3 for 2.5 min with clavulanic acid (5 μM). Samples (80 μl) were then removed and diluted into 2.5 ml of benzylpenicillin (0.5 mg/ml) in 0.05 M-sodium phosphate, pH 7.3. Change in A_{240} was followed with time as a measure of the activity of the inhibited enzyme (●) in comparison with a control reaction of enzyme alone similarly diluted into benzylpenicillin (○).

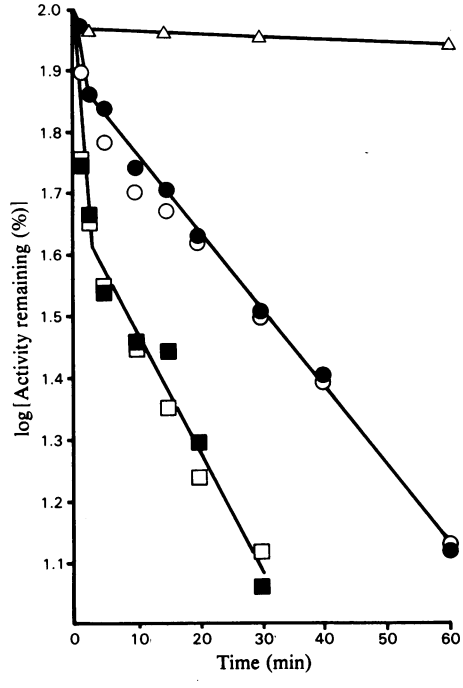


Fig. 2. *Time course for the irreversible inhibition of TEM-2, Klebsiella E70 and Proteus mirabilis C889 β-lactamases by clavulanic acid*

All reactions between β-lactamase and clavulanic acid were in 0.05 M-sodium phosphate buffer, pH 7.3, at 37°C. The amount of irreversible inhibition was measured relative to an uninhibited control as the stabilized enzyme activity after allowing any unstable complexes to decay. Both gel-filtration and substrate-dilution techniques were used to obtain the results as described in the Materials and methods section. TEM-2 β-lactamase (0.43 μM) was allowed to react with 40 μM- (●) and 100 μM-clavulanic acid (○). Samples were gel-filtered and assayed with benzylpenicillin as substrate at pH 7.3 to measure residual activity. *Klebsiella* E70 β-lactamase (activity, 1.3 mg of benzylpenicillin hydrolysed/ml per min at pH 7.3 and 37°C) was allowed to react with 3 μM- (□) and 6 μM-clavulanic acid (■). Reaction samples (25 μl) were diluted into 2.5 ml of nitrocefin (250 μg/ml) and residual enzyme activity was measured at pH 7.3 at the linear phase of substrate hydrolysis after decay of the transiently stable complex. *Proteus mirabilis* C889 β-lactamase (activity, 0.62 mg of benzylpenicillin hydrolysed/ml per min at pH 7.3 and 37°C) was allowed to react with 21 μM-clavulanic acid (△). Samples (25 μl) were removed at time intervals and diluted into 2.5 ml of benzylpenicillin (0.5 mg/ml) buffered at pH 5.8 (to increase the rate of decay of unstable complex). Stabilized enzyme activity was measured at the linear phase of substrate hydrolysis after decay of the unstable complex, in comparison with an uninhibited control also at pH 5.8.

Loss of clavulanic acid in the reactions was measured (Fig. 3) and illustrated the initial rapid, but short-lived, destruction of the inhibitor. By using [^{14}C]clavulanic acid (Stirling & Elson, 1979) it could be demonstrated that low-molecular-weight products were rapidly formed in reactions with TEM-2 β-lactamase (C. Reading & T. Farmer, unpublished work). These data supported the proposal (Fisher *et al.*, 1978) that clavulanic acid turnover was occurring at the same time as the reactions that produced the transiently stable and irreversible complexes. The co-occurrence of inhibitor turnover and enzyme inactivation has been previously reported for other enzymes and suicide inactivators (Abeles, 1978).

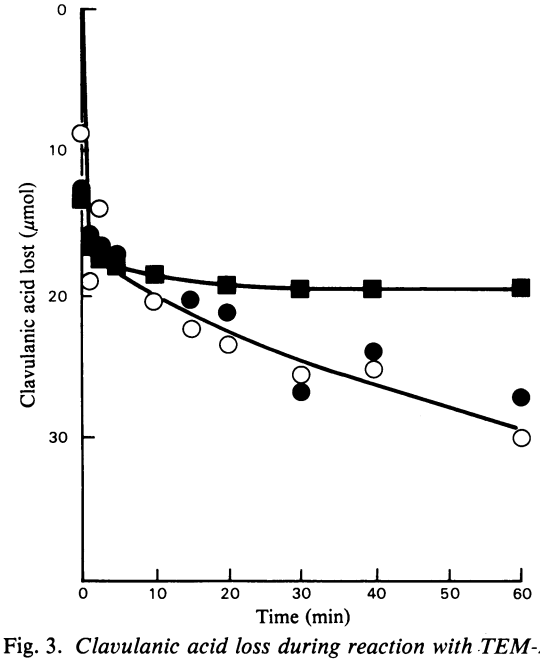


Fig. 3. *Clavulanic acid loss during reaction with TEM-2 β-lactamase*

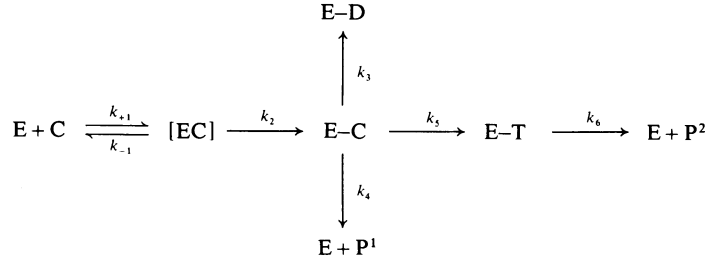
Clavulanic acid (20μM, ■; 40μM, ●, 100μM; ○) was incubated at pH7.3 and 37°C with TEM-2 β-lactamase (0.43μM). Samples were removed at time intervals and assayed for clavulanic acid content after gel filtration using the automated enzyme inhibition assay as described in the Materials and methods section.

We and Fisher *et al.* (1978) have proposed a minimal kinetic scheme to describe the interaction of clavulanic acid with TEM-2 β-lactamase and this is shown in Scheme 1.

Inhibition of Klebsiella β-lactamase

The enzyme from cell extracts of *Klebsiella pneumoniae* E70, when examined by analytical isoelectric focusing, had a main band at pI 7.0. Its substrate profile is shown in Table 1. The properties of this β-lactamase did not appear to coincide with those of any of the plasmid-mediated β-lactamases that are known to occur in strains of *Klebsiella* (Matthew, 1979). For this reason the enzyme was presumed to be a chromosomally mediated β-lactamase.

The results in Fig. 4 were obtained when reactions of clavulanic acid and *Klebsiella* β-lactamase were followed by diluting reaction samples into excess substrate (nitrocefin) and measuring enzyme activity by initial velocities. Enzyme activity was lost rapidly within 2.5 min. At high concentrations of clavulanic acid virtually complete inhibition was maintained for long periods when measured on the basis of initial velocities of substrate hydrolysis. At lower inhibitor concentrations (0.2μM) incomplete inhibition was obtained initially and furthermore a rapid return of activity then commenced. Fig. 4 shows that not all the enzyme activity returned and a level of stabilized inhibition was attained. These results were similar to those we and Fisher *et al.* (1978) have observed previously with TEM-2 β-lactamase and clavulanic acid and indeed the transiently stable complex formed between *Klebsiella* β-lactamase and clavu-



Scheme 1. *A possible interaction sequence for clavulanic acid and TEM-2 β-lactamase*

Abbreviations used: E, free enzyme; C, clavulanic acid; [EC], Michaelis complex; E–C, intermediary complex; E–D, irreversibly inhibited enzyme; E–T, transiently stable complex; P, product.

Table 1. *Substrate profiles of the β-lactamase enzymes*

Initial rates of substrate hydrolysis were measured spectrophotometrically at 37°C and pH 7.3. All substrates were tested at 500μg/ml. Change in A_{240} was used for ampicillin and carbenicillin, A_{250} for amoxycillin, A_{263} for oxacillin and A_{290} for cephaloridine and cephalothin. Rates were compared relative to benzylpenicillin as 100. After an initial burst the rates either slowed markedly (*) or slowed and then stopped completely (**).

	Rate					
β-Lactamase source	Amoxycillin	Ampicillin	Carbenicillin	Oxacillin	Cephaloridine	Cephalothin
Escherichia coli JT4 (TEM-1)	89	122	12	12	63	9
Escherichia coli W3110 (TEM-2)	83	121	12	12	55	8
Proteus mirabilis C889	142	198	102	8	5	3
Klebsiella E70	127	191	17	9*	55	7
Enterobacter cloacae P99	6	8	2	12**	5530	771
Staphylococcus aureus Russell	168	233	38	15**	<1	<1

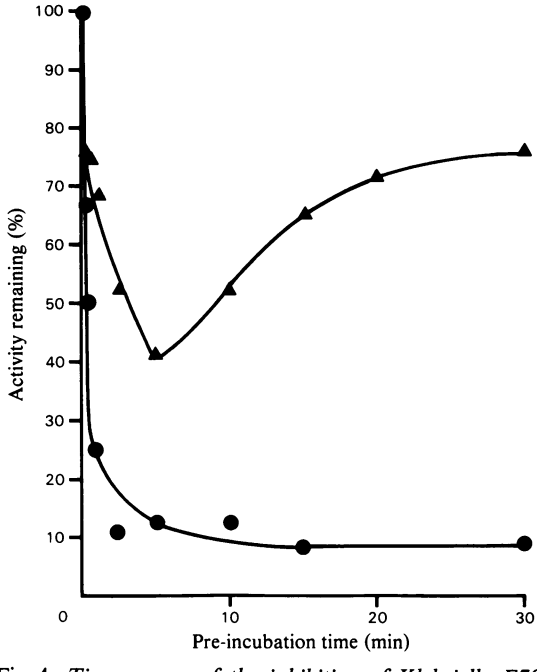


Fig. 4. *Time course of the inhibition of Klebsiella E70 β-lactamase by clavulanic acid*

Klebsiella β-lactamase (activity, 0.67mg of benzylpenicillin hydrolysed/ml per min) was allowed to react with 0.2μM- (▲) and 1.5μM-clavulanic acid (●) at pH 7.3 and 37°C in 0.05M-sodium phosphate buffer. Samples (50μl) were removed at time intervals and added to 2.5ml of nitrocefin (250μg/ml). Enzyme activity was measured as the initial velocity of substrate hydrolysis by following the change in A_{550} relative to an uninhibited control reaction.

lanic acid was found to have the same half-life as the TEM-2 β-lactamase complex ($t_{\frac{1}{2}} = 4.0$min at 37°C and pH 7.3). After complete inhibition had been attained (2.5min) with excess clavulanate, it was found that the inhibited enzyme existed as the transiently stable complex and the irreversible complex and these were present in the ratio 1:1. When the formation of irreversible complex was followed with time the results shown in Fig. 2 were obtained. Hence the consequences of a more favourable partition towards irreversibly inhibited enzyme are seen as a faster rate of second-phase inactivation ($k = 7.2 \times 10^{-4}$ s^{-1}) in comparison with TEM-2 β-lactamase ($k = 5 \times 10^{-4}$ s^{-1}).

In the absence of molecular-activity data for the *Klebsiella* β-lactamase it was not possible to consider the stoichiometry of its interaction with clavulanic acid. The *Klebsiella* enzyme appeared to share kinetic features with TEM-2 β-lactamase in that it produced a transiently stable complex with the same half life as well as displaying biphasic inactivation kinetics. The Scheme describing the detectable events in the interaction of clavulanic acid with TEM-2 β-lactamase (Scheme 1) may also be used therefore for the *Klebsiella* β-lactamase. Although the inclusion of the hydrolysis and product release event (k_4) must remain tentative, the formation of transiently stable complex E–T and inactivated enzyme E–I can be included in the scheme for the *Klebsiella* β-lactamase. The only differences between the two enzymes are the relative rates of formation of the two complexes. In the *Klebsiella* interaction these rates were similar, leading to equal proportions of the complexes after the initial rapid inhibition of the enzyme by clavulanic acid. This also explains the more rapid second phase of irreversible enzyme inactivation in comparison with TEM-2 β-lactamase, which initially partitions 3:1 in favour of the transiently stable complex to the inactive complex.

Inhibition of Proteus mirabilis C889 β-lactamase

The penicillinase from this strain of *Proteus* had a main activity band at pI 6.5 on isoelectric focusing.

The substrate profile was as shown in Table 1 and included the ability to hydrolyse the α-carboxypenicillin, carbenicillin. The inhibition of enzyme activity by various concentrations of clavulanic acid is shown in Fig. 5. Enzyme activity was monitored by removing samples at time intervals, diluting into excess benzylpenicillin and measuring initial velocities of substrate hydrolysis by using the decrease in A_{240}. As with the enzymes discussed above the rate of loss of enzyme activity was very rapid, yielding virtually complete inhibition within 2.5 min at the higher clavulanate concentration (0.53 μM) with an initial enzyme activity of 1.5 mg of benzylpenicillin hydrolysed/ml per min at 37°C and pH 7.3. At this higher inhibitor concentration the enzyme remained over 90% inhibited for the 5 h period of the experiment. With 0.26 μM inhibitor, however, after approximately a 60 min period during which the enzyme preparation remained inhibited, the activity started to return slowly. At the lower concentrations of 0.13 μM and 0.065 μM, 77% and 43% inhibition was achieved and activity started to return slowly almost immediately. The first-order rate of return of activity was considerably slower ($k = 2.75 \times 10^{-4}$ s^{-1}) than that seen for the transient complexes of TEM-2 β-lactamase and *Klebsiella* β-lactamases ($k = 3 \times 10^{-3}$ s^{-1}) at pH 7.3 and 37°C. Furthermore, the proportion of the enzyme activity that returned was higher. After a 2.5 min reaction period with excess clavulanate (6 μM) followed by dilution into substrate or gel filtration, less than 10% of the enzyme was irreversibly lost after allowing the activity to return and stabilize. When reactions of clavulanic acid and enzyme were monitored over longer periods and irreversible enzyme inactivation was specifically measured as described previously, there was a slow second phase of first-order enzyme inactivation as shown in Fig. 2. The rate was 1.73×10^{-5} s^{-1} at pH 7.3 and 37°C, this being considerably lower than the second-phase inactivation rates for TEM-2 and *Klebsiella* enzymes. The *Proteus* β-lactamase–clavulanate interaction yielded only 35% inactivation of the enzyme after 6.5 h at 37°C and pH 7.3.

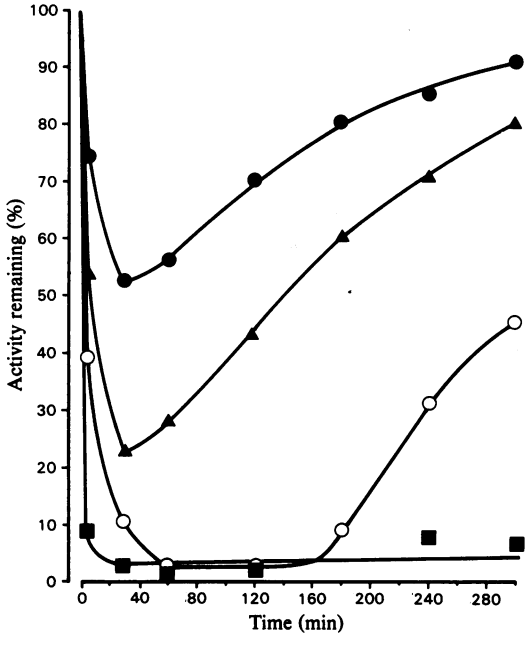


Fig. 5. *Time course of the inhibition of Proteus mirabilis C889 β-lactamase by clavulanic acid*

Proteus mirabilis C889 β-lactamase (activity, 1.5 mg of benzylpenicillin hydrolysed/ml per min at 37°C and pH 7.3) was allowed to react at 37°C and pH 7.3 with 0.065 μM- (●), 0.13 μM- (▲), 0.26 μM- (○) and 0.53 μM-clavulanic acid (■). Samples (25 μl) were removed at time intervals and added to 2.5 ml of benzylpenicillin (0.5 mg/ml) in 0.05 M-sodium phosphate buffer, pH 7.3, at 37°C. Enzyme activity was measured as initial velocities of substrate hydrolysis by following the change in A_{240}.

It was found that the rate of decay of the unstable *Proteus* β-lactamase–clavulanate complex was markedly affected by pH as shown in Fig. 6. As reported for clavulanate-inhibited staphylococcal β-lactamase (Reading & Hepburn, 1979) enzyme activity returns more rapidly at acid pH. The *Proteus* enzyme–clavulanate complex at pH 5.8 and 37°C gave a first-order rate of decay ($k = 1.75 \times 10^{-5}$ s^{-1}) that was approximately six times that obtained at pH 7.3. If, as with TEM-2 and *Klebsiella* β-lactamases, the second phase of irreversible inactivation is rate limited by the decay of the unstable complex as shown in Scheme 1, then the rate of inactivation of the *Proteus* enzyme could possibly have been elevated when reacting with clavulanate at acid pH. This was found to be so, the rate of formation of inactivated enzyme at pH 5.8 ($k = 5.9 \times 10^{-5}$ s^{-1}) being about 3.5 times that obtained at neutral pH.

The possibility of turnover of clavulanic acid by the *Proteus* β-lactamase during the interaction could not be answered satisfactorily without pure enzyme and molecular-activity data. Certainly some clavulanic acid was lost from the reaction via the slow decay of the unstable enzyme–inhibitor complex. This loss was confirmed in reactions (Fig. 5) where inhibition was maintained for long periods and activity then returned. Measurement of clavulanic acid concentrations in the reaction confirmed that the onset of return of activity coincided with disappearance of inhibitor. The rate of clavulanic acid loss via the very slow decay of the unstable complex would be extremely low and it is possible that a specific hydrolytic event also occurs in the earlier phase of the reaction as already discussed for TEM-2 β-lactamase.

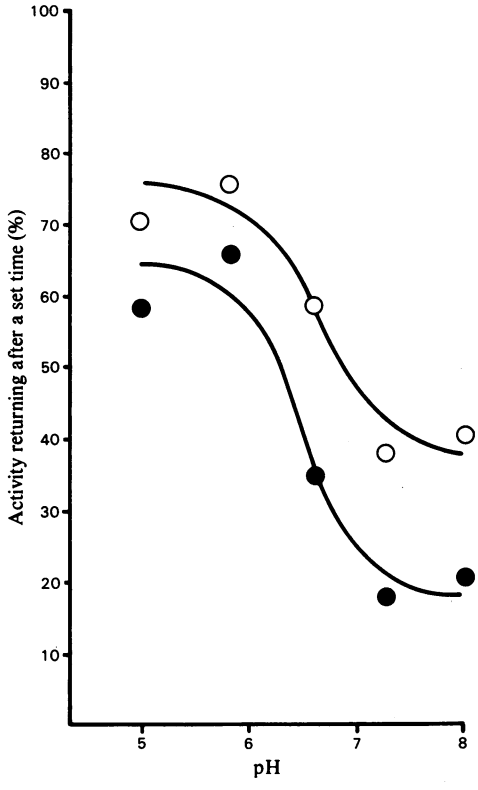


Fig. 6. *Effect of pH on the rate of reactivation of Proteus mirabilis C889 and Enterobacter cloacae P99 β-lactamases inhibited by clavulanic acid*

Completely inhibited *Proteus mirabilis* β-lactamase was prepared by incubation with excess clavulanic acid for 5 min in 0.05 M-sodium phosphate buffer, pH 7.3, at 37°C. *Enterobacter cloacae* enzyme was incubated for 30 min with excess clavulanic acid under the same conditions. The enzymes were then gel-filtered to remove excess inhibitor. Inhibited enzyme was diluted into buffers (Waley, 1975) at various pH values and incubated at 37°C. The β-lactamase activity was measured spectrophotometrically for the *Proteus mirabilis* β-lactamase (●) after 10 min incubation with benzylpenicillin as substrate and *Enterobacter cloacae* activity (○) was measured after 120 min incubation with cephaloridine as substrate. Activities were compared with uninhibited controls that had been treated identically to the inhibited enzyme samples.

Inhibition of Enterobacter cloacae P99 β-lactamase

The β-lactamase from this organism had a pI of 7.9 as determined by isoelectric focusing and gave the substrate profile shown in Table 1. This well known class-I cephalosporinase (Richmond & Sykes, 1973) has a high $V_{max.}$ value for cephaloridine relative to benzylpenicillin as substrate. The I_{50} value (the concentration of inhibitor required to inhibit substrate hydrolysis by 50%) obtained for this enzyme and clavulanic acid with pre-incubation was significantly poorer (Reading & Cole, 1979) than those obtained for the other enzymes and activity was even lower when the I_{50} was determined without the pre-incubation stage. This relative inability of clavulanic acid to inhibit the enzyme in the presence of high substrate concentrations suggested that clavulanic acid had a poor affinity for this enzyme relative to the substrate and in comparison with the other enzymes tested in this system. The *Enterobacter cloacae* enzyme was inhibited by clavulanic acid, but this time-dependent first-order loss of activity required very high concentrations of clavulanic acid to achieve reasonable rates in the absence of substrate. The second-order rate constant was $4.0\,M^{-1}\cdot s^{-1}$ compared with that for the inhibition of staphylococcal β-lactamase of $2.7\times10^{5}\,M^{-1}\cdot s^{-1}$ at 37°C and pH 7.3 (Reading & Hepburn, 1979). By using a double reciprocal plot (Kitz & Wilson, 1962) to determine the rate constant, the line obtained passed through the origin, indicating that little Michaelis-type complex was formed in this reaction at the inhibitor concentration used. This result supported the I_{50} data, which suggested that clavulanic acid had a poor affinity for this enzyme.

Totally inhibited enzyme was prepared by allowing reaction of clavulanic acid and *Enterobacter cloacae* P99 enzyme to go to completion and excess inhibitor removed using gel filtration. This inhibited enzyme slowly regained activity when incubated at 37°C and pH 7.3 ($t_{\frac{1}{2}} = 180$ min) and all of the original activity returned. No irreversible inhibition of the enzyme was detected even on prolonged incubation of this β-lactamase with excess inhibitor before gel filtration. The return of activity was first order with respect to time ($k = 6.3\times10^{-5}\ s^{-1}$ at pH 7.3 and 37°C). At acid pH this slow decay could be increased but even at pH 5.0 the half life of the complex was still about 1 h. The percentage return of enzyme activity as a function of pH over a 120 min period is shown in Fig. 6.

Discussion

Studies on the interaction of clavulanic acid with a number of β-lactamase enzymes has revealed the formation of a variety of covalent enzyme–inhibitor complexes as summarized in Table 2. These are distinguishable on the basis of their relative stabilities and, as seen for a number of enzymes, different types of complex can occur after the reaction of clavulanic acid with a single enzyme. The acid-labile complexes that have been detected in reactions of clavulanic acid with staphylococcal (Cartwright &

Table 2. *Mode of inhibition of various β-lactamases by clavulanic acid*

All rates were determined at pH 7.3 and 37°C as described in the Materials and methods section. * Indicates that rates of decay increased at acid pH. Abbreviation used: ND, not detected.

Source of β-lactamase	Types of complex detected ...	Transiently stable		Irreversible		Moderately stable	
		$t_{\frac{1}{2}}$ for formation (min)	$t_{\frac{1}{2}}$ for decay (min)	Time for 50% inactivation (min)	$10^5 \times$ Second-phase rate constant (s^{-1})	$t_{\frac{1}{2}}$ for formation (min)	$t_{\frac{1}{2}}$ for decay (min)
Escherichia coli W3110 TEM-2		<1	4	15	50	ND	—
Klebsiella E70		<1	4	3	72	ND	—
Proteus mirabilis C889		ND	—	510	1.73	<1	40*
Enterobacter cloacae P99		ND	—	ND	—	2.5 at 240 μg/ml ($k = 4.0 \text{ M}^{-1} \cdot \text{s}^{-1}$)	180*
Staphylococcus aureus Russell		ND	—	ND	—	2.5 at 0.2 μg/ml ($k = 2.75 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$)	160*

Coulson, 1979; Reading & Hepburn, 1979), *Proteus* and *Enterobacter cloacae* P99 β-lactamases appear to be of a similar type. These complexes, which are moderately stable at neutral pH but are acid-labile, may well represent the acyl-enzyme intermediate proposed by Cartwright & Coulson (1979), although other acid-labile covalent products remain a possibility (Reading & Hepburn, 1979).

The highly stable irreversible complexes, which are a feature of the reaction of clavulanic acid with TEM-2 and *Klebsiella* E70 β-lactamases and to a lesser extent with *Proteus* C889 enzymes, appear to represent a further type of complex. Charnas *et al.* (1978*a*) reported that with TEM-2 β-lactamase, the irreversibly inactivated enzyme could be further subdivided into three inactivated enzyme species, which were detected by isoelectric focusing. Charnas *et al.* (1978*b*) have proposed a number of reactive intermediates arising from the enzymic hydrolysis of clavulanic acid, which could lead to a variety of covalently labelled enzyme molecules. One such possibility was Michael addition to an enzymic carbanion, which appears to be a fairly common mechanism with inhibitors of the $k_{cat.}$ or suicide-inactivator type (Rando, 1978) and results in the formation of highly stable covalent products.

The transiently stable complexes that co-occur with the irreversible complexes in the TEM-2 β-lactamase and *Klebsiella* E70 β-lactamase reactions appears to represent a further type of product in the interaction of β-lactamases with clavulanic acid. With a short half-life (4 min) apparently unaffected by acid pH this type of complex can be distinguished from the moderately stable but acid-labile complexes and the irreversible species discussed above.

It is interesting that some derivatives and analogues of clavulanic acid react with TEM-2 β-lactamase differently from the parent compound (Bentley *et al.*, 1981). Such compounds are very potent inhibitors of the enzyme but form only the transiently stable complexes with no irreversible inactivation (C. Reading, unpublished work). The penicillanic acid sulphone (CP 45899) that is also reported to inhibit cell-free TEM β-lactamase (English *et al.*, 1978) yields only irreversibly inhibited enzyme and no transiently stable species (C. Reading & T. Farmer, unpublished work) and hence does not display biphasic inactivation kinetics. This compound, however, is a good substrate for TEM β-lactamase and high turnover accompanies the slow inactivation process, which results in a molar excess of inhibitor to enzyme of about 4000 being required to achieve irreversible loss of enzyme activity. Labia *et al.* (1980) have also reported high turnover of compound CP 45899 by plasmid-mediated β-lactamases.

The antibacterial synergy achieved when

clavulanic acid is combined with a β-lactamase-labile antibiotic (Hunter *et al.*, 1979) is superior to that observed previously when using penicillin and cephalosporins as inhibitors of β-lactamase. This improved activity *in vivo* relates to the potent broad-spectrum β-lactamase inhibitory activity of clavulanic acid but other factors may also make an important contribution. An ability to readily permeate the outer membrane of Gram-negative bacteria and so reach the β-lactamase within the periplasmic space may well distinguish clavulanic acid from its forerunners.

We thank Dr. A. G. Brown and Dr. M. Cole for both their encouragement and advice. We are also most grateful to Professor J. R. Knowles for interactive discussions during our study with TEM-2 β-lactamase in 1977 (see Fisher *et al.*, 1978).

References

Abeles, R. H. (1978) in *Enzyme-inactivated Irreversible Inhibitors* (Seiler, N., Jung, M. J. & Koch-Wester, J., eds.), pp. 1–12, Elsevier/North-Holland Biomedical Press, Amsterdam

Bentley, P. H., Berry, P. D., Brooks, G., Gilpin, M. L., Hunt, E. & Zomaya, I. I. (1981) in *Recent Advances in the Chemistry of β-Lactam Antibiotics* (Gregory, G. I., ed.), pp. 175–183, Royal Society of Chemistry, London

Cartwright, S. J. & Coulson, A. F. W. (1979) *Nature (London)* **278**, 360–361

Charnas, R. L., Fisher, J. & Knowles, J. R. (1978*a*) *Biochemistry* **17**, 2185–2189

Charnas, R. L., Fisher, J. & Knowles, J. R. (1978*b*) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, N., Jung, M. J. & Koch-Wester, J., eds.), pp. 315–322, Elsevier/North-Holland Biomedical Press, Amsterdam

English, A. R., Retsema, J. A., Girard, A. E., Lynch, J. E. & Barth, W. E. (1978) *Antimicrob. Agents Chemother.* **14**, 414–419

Fisher, J., Charnas, R. L. & Knowles, J. R. (1978) *Biochemistry* **17**, 2180–2184

Hunter, P. A., Coleman, K., Fisher, K., Taylor, E. & Taylor, E. (1979) *Drugs Exp. Clin. Res.* **5**, 1–6

King, J. D., Farmer, T., Reading, C. & Sutherland, R. (1980) *J. Clin. Pathol.* **33**, 297–301

Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249

Labia, R. & Peduzzi, J. (1978) *Biochim. Biophys. Acta* **526**, 572–579

Labia, R., Lelievre, V. & Peduzzi, J. (1980) *Biochim. Biophys. Acta* **611**, 351–357

Matthew, M. (1979) *J. Antimicrob. Chemother.* **5**, 349–358

Rando, R. R. (1978) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, N., Jung, M. J. & Koch-Weser, J., eds.), pp. 13–26, Elsevier/North-Holland Biomedical Press, Amsterdam

Reading, C. & Cole, M. (1977) *Antimicrob. Agents Chemother.* **11**, 852–857

Reading, C. & Hepburn, P. (1979) *Biochem. J.* **179**, 67–76

Richmond, M. H. & Sykes, R. B. (1973) *Adv. Microb. Physiol.* **9**, 31–88

Stirling, I. & Elson, S. W. (1979) *J. Antibiot.* **32**, 1125–1129

Waley, S. G. (1974) *Biochem. J.* **139**, 789–790

Waley, S. G. (1975) *Biochem. J.* **149**, 547–551