The Inhibition of Staphylococcal β -Lactamase by Clavulanic Acid

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Clavulanic acid inhibited both the extracellular and cell-extract β -lactamases of the four *Staphylococcus aureus* strains tested. The inhibition of *S. aureus* Russell cell-extract enzyme appeared to be active-site-directed and proceeded in a first-order fashion consistent with the formation of a covalent intermediate. Inhibited enzyme free of excess clavulanic acid was shown to regenerate enzyme activity slowly at pH 7.0, but the rate of reactivation increased at acid pH. When the enzyme was incubated with excess clavulanic acid complete inhibition was rapidly obtained, during further incubation clavulanic acid was shown to disappear slowly and complete loss of clavulanic acid from the reaction mixture coincided with the onset of the return of enzyme activity. A reactive enamine resulting from enzymic hydrolysis of the β -lactam ring of clavulanic acid has been proposed as a possible intermediate in the inhibitory mechanism.

Clavulanic acid is a novel β -lactam that was isolated from the culture broth of *Streptomyces clavuligerus* A.T.C.C. 27064 (Reading & Cole, 1977). The structure of this metabolite has been determined (Howarth *et al.*, 1976) and is shown in Fig. 1. Preliminary information on the β -lactamase-inhibitory properties and moderate antibacterial activity of clavulanic acid has been described previously (Hunter & Reading, 1976; Reading & Cole, 1977). The present paper describes in more detail the interaction of clavulanic acid with the β -lactamase from several strains of *Staphylococcus aureus*, but in particular the inducible enzyme from cell extracts of *S. aureus* Russell.

Materials and Methods

Organisms and enzyme preparation

S. aureus Russell (B.R.L. 1555), *S. aureus* B.R.L. 1083 (Batchelor *et al.*, 1961) and *S. aureus* MB9 (B.R.L. 2333) are all penicillin-resistant strains from the culture collection of our company. *S. aureus* PC1 is a constructed constitutive strain (Richmond, 1963). All cultures were grown, methicillin-induced

where necessary, and cell extracts were prepared as described previously for *S. aureus* Russell (Reading & Cole, 1977). The activity of cell-extract enzyme from *S. aureus* Russell is expressed as mg of benzylpenicillin hydrolysed/min per ml at 37°C and pH 7.3. To obtain an approximate indication of the enzyme/inhibitor ratios used in the various experiments, enzyme activities are also quoted in molarity. These, however, are estimates for crude-cell-extract enzyme based on the reported molecular activity of extracellular staphylococcal enzyme from *S. aureus* PC1 (Virden *et al.*, 1975). The molecular activity of enzyme from *S. aureus* PC1 was reported on assays at 25°C, and so a conversion factor was determined experimentally to adjust assay data at 37°C for *S. aureus* Russell cell-extract enzyme into molar concentrations. Extracellular enzyme preparations were cell-free 7 h culture broths.

I_{50} determination

The effect of inhibitor concentration on the level of inhibition after a set preincubation period of enzyme and inhibitor was determined by using a Pye-Unicam AC 30 discrete analyser. The hydroxylamine assay of residual substrate and hence enzyme activity, as described previously (Reading & Cole, 1977), was adapted for the automated system as shown in Scheme 1.

Percentage inhibition of enzyme activity was measured against a control reaction where inhibitor was replaced by buffer in the sample cup. The concentration of inhibitor giving 50% inhibition (I_{50}) was calculated from a plot of percentage inhibition against inhibitor concentration. The concentration

Fig. 1. Structure of clavulanic acid [(Z)-(2R,4R-3-(β -hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid]

Scheme 1. Automated assay of enzyme inhibition

The Scheme shows the flow sequence used for the assay of enzyme inhibition on a Pye-Unicam AC 30 discrete analyser.

of inhibitor is given as that present with the enzyme before dilution with substrate.

K_m determinations

These values were obtained by using the micro iodometric method of Novick (1962*b*). All reagents, however, were buffered at pH 7.3 in 0.01M-sodium phosphate, rather than at pH 5.8. Results were plotted by using the direct linear method of Eisenthal & Cornish-Bowden (1974).

Progressive inactivation of β -lactamase

Two methods were used to follow the time course of the reaction of clavulanic acid with *S. aureus* Russell β -lactamase. The automated assay described for the determinations of I_{50} values was adapted as follows. The preincubation period of enzyme and inhibitor was varied by altering the distance of the

initial addition point, where both these reactants were simultaneously added to the reaction tube, from the point at which substrate was added. For zero-time samples the substrate-addition point was removed from the process line. Substrate was added manually to the reaction tube so that on reaching the inhibitor/enzyme-addition point all three were simultaneously admixed. The position of the initial addition of enzyme and inhibitor was adjusted in these zero-time samples to give the same 10min enzyme-assay period as used in the preincubated samples. In this automated assay method inhibitor concentrations were in molar excess of an estimated enzyme concentration of $0.034 \mu\text{M}$.

The second method was that utilizing decrease in A_{240} of benzylpenicillin on hydrolysis to its penicilloic acid (Waley, 1974). Enzyme in sodium phosphate buffer was mixed with an equal volume of

inhibitor and incubated at 37°C. At various time intervals 0.1 ml samples were removed and added to 2.4 ml of benzylpenicillin, final concentration 0.5 mg/ml, in a 1 cm silica cuvette. The decrease in A_{240} was then followed on a Pye-Unicam SP.800 recording spectrophotometer over a 3–5 min reaction period in a water-jacketed cell holder at 37°C. Amounts of inhibition were calculated from a comparison of the initial linear rate of change in absorbance with respect to that of an uninhibited control. In this experiment inhibitor concentrations were lower than the estimated enzyme concentration of 1.95 μ M (equivalent to an activity of 8.6 mg of benzylpenicillin hydrolysed/min per ml).

Preparation of inhibited enzyme

Enzyme preparations hydrolysing 1 mg of benzylpenicillin/min per ml at 37°C (estimated 0.23 μ M-enzyme) were reacted with 224 μ M-clavulanic acid, giving an approximate enzyme/inhibitor concentration ratio of 1:1000. A further sample of enzyme without added inhibitor was treated identically, as a control. The enzyme solutions (2 ml) were then chromatographed at 5°C on two identical Bio-Rad Bio-Gel P₂ columns (12.5 mm $\times$ 300 mm) in water. Fractions (1 ml) were collected. Enzyme activity was monitored by adding 0.1 ml of the fractions to 2.4 ml of benzylpenicillin in sodium phosphate buffer at a final concentration of 1 mg/ml, which after 30 min at 37°C was assayed by using the normal hydroxylamine assay (Boxer & Everett, 1949). Fractions eluted after the peak of enzyme activity were assayed for clavulanic acid by using inhibition of staphylococcal β -lactamase with the automated enzyme-inhibition assay. Fractions from the control column that contained enzyme activity were combined and made up to 15 ml with sodium phosphate buffer. Fractions from the column to which inhibited enzyme had been applied were selected with reference to the elution position of the enzyme from the control column. Protein content of the two preparations was assessed by measuring A_{280} to ensure that even sampling of the two columns had been achieved.

Effect of pH on inactivation of β -lactamase by clavulanic acid

In all experiments the buffers described by Waley (1975) were used to give a range of pH values. Where necessary enzyme preparations were adjusted to the required pH before dilution into the buffer. Determination of the pH optimum for the hydrolysis of benzylpenicillin by *S. aureus* Russell cell extract was achieved by using the spectrophotometric assay (Waley, 1974).

The effect of pH on the inhibition of *S. aureus* Russell enzyme was determined by incubating enzyme (activity equivalent to 64 μ g of benzylpenicillin hydrolysed/min per ml) estimated at

0.015 μ M with 1 μ M-clavulanate in buffers of various pH values at 37°C. Samples were removed (2 ml) at various times and added to 0.5 ml of benzylpenicillin (2.5 mg/ml) at the same pH value and incubated at 37°C in a 1 cm silica cuvette. The decrease in A_{240} was then followed on a Pye-Unicam SP.800 recording spectrophotometer with a water-jacketed cell holder. The initial velocities were measured and percentage inhibition calculated against a control reaction of enzyme alone that had been incubated at the appropriate pH for an identical reaction period before addition of substrate. The stability of clavulanic acid at the various pH values used was measured by incubating 1 μ M-clavulanate in the buffers and then adding 0.5 ml samples to enzyme (1.5 ml) at the same pH. After allowing a 1 min reaction period between enzyme and clavulanic acid, 0.5 ml of benzylpenicillin (2.5 mg/ml) was added and the enzyme activity measured. No gross change in the inhibitory activity of 1 μ M-clavulanic acid solutions were measured over a 5 min incubation period of inhibitor alone in the various pH buffers.

Effect of pH on regeneration of inhibited enzyme

This was followed in two ways. Reactions were carried out as described above with 0.015 μ M-enzyme and 1 μ M-clavulanate and, after a 5 min reaction period, 2 ml samples were added to benzylpenicillin (0.5 ml) at the same pH value to give a final substrate concentration of 0.5 mg/ml. Restoration of enzyme activity was then followed spectrophotometrically at 240 nm as an increasing rate of absorbance change. Also, portions of inhibited enzyme and control enzyme from the gel-filtration columns previously described were diluted with equal volumes of buffers of various pH values. Restoration of enzyme activity at 37°C was followed by removing 0.1 ml samples and assaying against benzylpenicillin with the normal hydroxylamine assay.

Hydrolysis of clavulanic acid

Reaction mixtures of enzyme and clavulanic acid in buffer at pH 6.0 were monitored by removing 0.5 ml samples and loading these on Bio-Rad Bio-Gel P₂ columns (12.5 mm $\times$ 300 mm) at 5°C in water. Fractions (0.5 ml) were collected and assayed for enzyme activity by incubation with benzylpenicillin and the hydroxylamine assay. Samples (0.1–0.2 ml) of the fractions were also assayed for clavulanic acid content by using the automated enzyme-inhibition assay against staphylococcal β -lactamase with appropriate standards.

Results

Inhibition of staphylococcal enzymes

The I_{50} values obtained by using the automated inhibition assay were virtually identical for all four

S. aureus strains tested. The values obtained for extracellular enzyme are consistently lower (0.04–0.056 $\mu\text{g/ml}$) than for cell-extract enzyme (0.1–0.12 $\mu\text{g/ml}$). Differences between cell-extract and extracellular enzymes have been reported previously (Dyke, 1967). It has been suggested that cell-extract β -lactamase remains bound to cell debris (Richmond, 1963) and that the small differences in catalytic properties between extracellular and cell-extract preparations may be due to conformational effects resulting from such an association (Dyke, 1967).

Fig. 2. Plot showing linearity of the first-order rates with respect to the concentration of sodium clavulanate

First-order rates were measured by the automated methodology as described in the Materials and Methods section. Sodium clavulanate concentrations (μM , quoted as those present with the enzyme before substrate addition) were: $\circ$, 0.1; $\blacktriangle$, 0.2; $\square$, 0.4; $\bullet$, 0.75; $\triangle$, 1.5. The enzyme, at an activity of 150 μg of benzylpenicillin hydrolysed/min per ml (estimated 0.034 μM -enzyme), gave approximate inhibitor/enzyme ratios of 3 ($\circ$), 6 ($\blacktriangle$), 12 ($\square$), 22 ($\bullet$) and 44 ($\triangle$). The derived rate constant is $2.7 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$.

S. aureus Russell cell-extract β -lactamase

The enzyme preparation gave a K_m of 10 μM for benzylpenicillin, which was identical with that obtained at the same time for the cell-extract enzyme of *S. aureus* PC1. The pH optimum was broad between pH 5.0 and 6.4 with an optimum of about pH 5.7. This is similar to that found for the extracellular enzyme (Novick, 1962a; Richmond, 1963).

Progressive inhibition of β -lactamase

The enzyme activity remaining at various times in reactions of β -lactamase and clavulanic acid was measured. These enzyme assays, as described in the Materials and Methods section, relied on the measurement of residual substrate after a set reaction period. Substrate was shown to be hydrolysed by the enzyme in a linear fashion with time under the conditions used, as shown in the control reactions of Fig. 5. For this reason it was considered satisfactory to use residual substrate concentrations after a fixed reaction time as a measure of enzyme activity. Loss of enzyme activity in reactions of clavulanic acid and enzyme was found to proceed in a first-order fashion with respect to time. At zero time when enzyme is added to premixed clavulanic acid and excess benzylpenicillin substrate (1 mg/ml) no inhibition was obtained. The ability of high substrate concentrations to protect the enzyme would suggest, as do its structural features, that clavulanic acid is active-site-directed. The rate of enzyme inactivation is shown in Fig. 2 to be directly related to the concentration of clavulanic acid and the derived rate constant is $2.7 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$.

It would appear that the dissociation constant for clavulanic acid and this enzyme is relatively high. It was not possible to obtain a K_i value from plots of t_4 against $[I]^{-1}$. The straight line so obtained passed through the origin, indicating that little Michaelis-type complex had been formed (Kitz & Wilson, 1962). Further indication of the poor affinity of clavulanic acid for this enzyme is the protective effect obtained with a good substrate such as benzylpenicillin. This can be illustrated by comparing degrees of inhibition obtained when enzyme and inhibitor were preincubated with those obtained without preincubation when both substrate and inhibitor were present at zero time (Table 1). The effect of substrate type is also shown where penicillins with lower affinity for the enzyme than benzylpenicillin, such as ampicillin and amoxycillin, give less protection in the assay with no preincubation, resulting in greater inhibition. At the concentrations of substrate used (1 mg/ml) I_{50} values for clavulanic acid with preincubation are seen to be independent of substrate type.

The semisynthetic penicillins methicillin and cloxacillin, which have previously been reported as being inhibitors of this enzyme (Batchelor *et al.*, 1963), are compared with clavulanic acid in Table 1.

Table 1. *The inhibition of S. aureus Russell β -lactamase*

Results were obtained with and without a 5 min preincubation stage of enzyme and inhibitor by using the automated procedure described in the Materials and Methods section. Data for clavulanic acid were obtained by using various substrates (all at 1 mg/ml). Abbreviations used: G, benzylpenicillin; A, ampicillin; Amx, amoxycillin.

Inhibitor	Substrate	No preincubation		Preincubation	
		Inhibitor concentration ($\mu\text{g/ml}$)	Inhibition (%)	Inhibitor concentration ($\mu\text{g/ml}$)	Inhibition (%)
Sodium clavulanate	G	20	31	0.12	50
		43	50		
	A	6.2	50	0.12	50
		7.0	50		
Methicillin	G	200	0	200	0
Cloxacillin	G	200	0	200	23

Fig. 3. *Inactivation of staphylococcal β -lactamase by sodium clavulanate at low inhibitor/enzyme ratios*
Samples from the reaction mixture (pH 7.3, 37°C) were assayed by using the spectrophotometric method described in the Materials and Methods section. The enzyme activity in the reaction was 8.6 mg of benzylpenicillin hydrolysed/min per ml (estimated as 1.95 μM -enzyme). Clavulanate concentrations were (μM): ▲, 1.67; ●, 1.25; △, 0.83, ○, 0.45. These concentrations give estimated inhibitor/enzyme ratios of 0.86 (▲), 0.64 (●), 0.43 (△), and 0.215 (○).

Fig. 4. *Percentages of stabilized inhibition attained at various concentrations of inhibitor*
Stabilized inhibition percentages from the results shown in Fig. 3 are plotted against inhibitor concentration. These results show, by extrapolation of the apparent linear relationship, that 100% inhibition is achieved at 1.8 μM -clavulanate with an enzyme activity of 8.6 mg of benzylpenicillin hydrolysed/min per ml (1.95 μM -enzyme).

At concentrations of 200 $\mu\text{g/ml}$ these penicillins were virtually inactive under the assay conditions used. Only cloxacillin gave some inhibition after preincubation with the enzyme, as shown in Table 1.

The results obtained by using the spectrophotometric assay (Waley, 1974) to follow the time course of the inactivation are shown in Fig. 3. The inhibitor concentrations in this experiment were not in excess of the estimated molar concentration of enzyme present in the reaction giving enzyme/inhibitor

ratios of less than 1:1 (as shown in Fig. 3). Under these conditions the rate of inactivation slows to yield a final degree of stabilized inhibition. A plot of percentage stabilized inhibition against inhibitor concentration (Fig. 4) shows a linear relationship. From these results, which show that $1.8\text{ }\mu\text{M}$ -clavulanate is required to completely inhibit a calculated molar enzyme concentration of $1.95\text{ }\mu\text{M}$, it can be estimated that 0.92 mol of clavulanate was required to inactivate 1 mol of enzyme. Obviously such stoichiometric considerations for the cell-bound staphylococcal enzyme are not satisfactory as they are based on the molecular activity of *S. aureus* PC1 exocellular enzyme. These results, however, do indicate that large molar excesses of clavulanic acid are not required to inactivate staphylococcal β -lactamase, in contrast with the situation found for the R-TEM enzyme (Fisher *et al.*, 1978; C. Reading & T. Farmer, unpublished work).

In experiments where inhibited enzyme was incubated over longer time periods a restoration of enzyme activity was observed. By preparing inhibited enzyme free of excess clavulanic acid, as described in the Materials and Methods section, it was possible to show spontaneous regeneration of enzyme activity on incubation at pH 7.3 and 37°C . Even when stored at -20°C inhibited enzyme regenerated enzyme activity appreciably within 24 h at pH 7.3.

Fig. 5. Effect of pH on the restoration of enzyme activity. After a 5 min reaction of enzyme and inhibitor at various pH values (see the Materials and Methods section), samples were added to benzylpenicillin (0.5 mg/ml) buffered at the same pH and hydrolysis of substrate was followed by decrease in A_{240} at 37°C . Symbols: ●, enzyme + inhibitor; ○, control enzyme. The amount of enzyme activity present 10 min after addition to substrate was measured. These results as percentages of enzyme activity restored (compared with control uninhibited activity) as a function of pH are shown in Fig. 6. pH values were: (a) 4.5; (b) 5.0; (c) 6.0; (d) 7.50.

Effect of pH on inhibition and regeneration

The inhibition of the enzyme was measured at various pH values. In reactions where the inhibitor was estimated as being in 66-fold excess of the enzyme, percentage inhibition was measured against a control reaction (no inhibitor) at identical pH. Measurements of enzyme inhibition after 30, 60 and 120 s reaction times were made over the pH range 4.5–8.0. The pH optimum for enzyme inactivation under these conditions is between pH 5.5 and 6.0.

It can be seen from Fig. 5 that the hydrolysis of benzylpenicillin by inhibited enzyme is not linear with respect to time. The spectrophotometric assay shows an increase in the rate of decrease in absorbance with time, indicating a slow restoration of enzyme activity. In the experiment shown in Fig. 5 enzyme and inhibitor were allowed to react at the various pH values for 5 min. After addition of sample to substrate there is initially no change in absorbance at any of these pH values. At this stage therefore the enzyme is completely complexed with inhibitor.

Fig. 6. Effect of pH on the rate of reactivation of inhibited enzyme.

Results from Fig. 5 (●) and from experiments described in the Materials and Methods section with inhibited enzyme free of excess clavulanate (○) were used to obtain this plot.

Subsequently enzyme activity returns at various rates depending on the pH value. The rate of regeneration is most marked at acid pH. At pH 4.5 all the enzyme activity was restored with respect to the control within 12 min. The influence of pH on the rate of regeneration is summarized in Fig. 6, where percentage restoration of enzyme activity was measured 10 min after substrate addition. These data were obtained from the experiment shown in part in Fig. 5.

Similar results were obtained when inhibited enzyme free of excess clavulanic acid (by gel filtration) was incubated in buffers of various pH values as described in the Materials and Methods section.

Fig. 7. Loss of clavulanic acid and reactivation of inhibited enzyme

Reactions of enzyme (activity, 27 mg of benzylpenicillin hydrolysed/min per ml, equivalent to 6.1 μM) and clavulanic acid (49 and 9.7 μM) at pH 6.0 and 37°C were followed by gel filtration of reaction samples and assay of the separated reactants (see the Materials and Methods section). (a) Concentrations of clavulanic acid in the presence of enzyme are followed with time. Zero-time concentrations were 49 μM (●) and 9.7 μM (■). Clavulanate alone (no enzyme) was also followed with time at 49 μM (○) and 9.7 μM (□). (b) Enzyme activity was followed with time for the reactions initially containing 49 μM - (■) and 9.7 μM -clavulanate (△). The enzyme control (no inhibitor) is also shown. (▲)

Despite enzyme instability over longer incubation periods at the two pH extremes used (pH 4.3 and 8.0) the rates of regeneration in the absence of clavulanic acid compared favourably with those observed in the spectrophotometric assay. The percentage return of enzyme activity in 10 min by this methodology is also shown in Fig. 6.

Hydrolysis of clavulanic acid

The disappearance of clavulanic acid on reaction with cell-extract enzyme is shown in Fig. 7. By separating enzyme and clavulanic acid by gel filtration it was possible to monitor both of them. By using inhibition of the staphylococcal enzyme with the automated method it was possible to assay very low concentrations of clavulanic acid in the column fractions and at the same time follow the activity of the enzyme, free of inhibitor, by using its activity against benzylpenicillin. The results show an initial rapid loss of clavulanate. This rate was too fast to measure by the gel-filtration method, despite immediate cooling of the reaction samples to 4°C. In this initial phase 7.8 nmol of clavulanate was lost relative to an estimated 6 nmol of enzyme in a reaction containing 49 nmol of inhibitor/ml. Hence this initial reaction is consistent with the earlier estimations indicating an approximate 1:1 binding of enzyme and inhibitor. After the initial formation of enzyme-inhibitor complex, clavulanic acid disappears very slowly in a linear fashion with time. This rate of loss was about 0.4 $\mu\text{g}/\text{ml}$ per h (0.54 $\text{nm}\cdot\text{s}^{-1}$). With the same enzyme concentration and 9.7 nmol of clavulanate, most of the inhibitor disappeared rapidly. The remainder was slowly lost and after 4 h no detectable clavulanate was present. This complete loss coincided with the onset of return of enzyme activity. The above data are consistent with the rapid formation of a relatively stable intermediate between enzyme and clavulanic acid that then breaks down slowly to release free enzyme and a product, as shown in Scheme 2.

Discussion

The progressive inactivation of staphylococcal β -lactamase by clavulanic acid is first-order with respect to time. This is consistent with the formation

Scheme 2. A possible interaction sequence for clavulanic acid and staphylococcal β -lactamase

Abbreviations used: E, free enzyme; C, clavulanic acid; [EC] Michaelis complex; E-C, covalent intermediate; P, products.

of a relatively stable covalent intermediate. The demonstration of the regeneration of enzyme activity

and data suggesting a slow hydrolysis of clavulanic acid indicate that this compound may also be a poor

Fig. 8. Possible intermediates in the interaction of clavulanic acid and staphylococcal β -lactamase

The product (enamine NA 19840) of base-catalysed methanolysis of clavulanic acid is shown and this is used as a basis for the initial enzymic product. The potentially reactive β -oxo group resulting from oxazolidine ring cleavage is postulated as reacting with a free amino group on the enzyme to form a relatively stable Schiff base. This intermediate can break down, especially at acid pH, to yield reactivated enzyme and product(s).

substrate for staphylococcal β -lactamase. It has also been suggested that the semisynthetic penicillins are inhibitors of staphylococcal β -lactamase by virtue of being poor substrates (Sagai & Saito, 1973; Virden *et al.*, 1975) and that a relatively stable acyl bond via the β -lactam carbonyl group may be formed. However, whereas some similarities occur between the interaction of certain penicillins and clavulanic acid with the enzyme, the mechanisms involved may well be different. The criterion for good or poor substrates in the penicillins would appear to depend on the acylamino side chain. Clavulanic acid has no acylamino side chain, but hydrolysis of its β -lactam bond can result in further reactions not possible in the penicillins. Base-catalysed methanolysis of sodium clavulanate has led to the detection of the enamine NA 19840, as shown in Fig. 8 (T. T. Howarth, unpublished work). Spontaneous decarboxylation is well known in β -oxo acids and this is assumed to occur to give the enamine shown. This is therefore a chemical analogy to the enzymic hydrolysis of clavulanic acid, assuming that an enzyme hydroxy group and base are present at the active site. It is possible therefore that the formation of such a reactive enamine could place clavulanic acid in the k_{cat} or suicide-inactivator category (Rando, 1974). In Fig. 8 the unmasking of a reactive β -oxo group is postulated after β -lactam hydrolysis and oxazolidine cleavage. The reaction sequence then shows condensation with a free ϵ -amino group of (say) a lysine residue to form a Schiff base. The demonstration of complete regeneration of enzyme activity shows that the presumed covalent linkage between clavulanic acid and enzyme is not a highly stable one. The acid-catalysed regeneration of enzyme activity is consistent with the known acid-lability of Schiff bases. Such a reaction sequence, however, is hypothetical and the identification of the covalent bond involved is still awaited.

In order that some estimation of enzyme/inhibitor ratios could be given in the experimental results in the present paper, the published molecular activity of staphylococcal β -lactamase from *S. aureus* PC1 has been used to estimate enzyme concentrations present in preparations of *S. aureus* Russell β -lactamase. Although such estimations do not allow comment on the absolute stoichiometry of the interaction the results do indicate that no marked loss of inhibitor occurs rapidly before the formation of the relatively stable covalent complex. This is in contrast with the interaction of clavulanic acid with R-TEM β -lactamase (Fisher *et al.*, 1978; C. Reading & T. Farmer, unpublished work), where an initial rapid loss of inhibitor has been observed far in excess of a 1:1 binding of enzyme and clavulanic acid. Also, with R-TEM β -lactamase irreversible inactivation of the enzyme has been observed and again this is in contrast with the β -lactamase from

S. aureus, where 100% enzyme regeneration can be obtained under the conditions described in the present paper.

The potential chemotherapeutic use of clavulanic acid in protecting β -lactamase-labile antibiotics centres on its improved inhibitory properties compared with previously known penicillins and cephalosporins. The results in the present paper show the rapid formation of a tightly bound enzyme-inhibitor complex at low concentrations of inhibitor. The complex is relatively stable, giving only low rates of enzyme regeneration at normal physiological pH when excess inhibitor is removed. The rate of hydrolysis of clavulanic acid is extremely low and is only detectable at massive enzyme concentrations that would not be encountered in the whole-cell situation or *in vivo*. The performance of clavulanic acid in protecting β -lactamase-labile compounds against whole cells is readily demonstrable (Hunter & Reading, 1976; Reading & Cole, 1977), and low concentrations of inhibitor decrease the minimum inhibitory concentration of ampicillin against β -lactamase-producing staphylococci to virtually that of ampicillin alone against non- β -lactamase-producing strains.

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References

- Batchelor, F. R., Cameron-Wood, J., Chain, E. B. & Rolinson, G. N. (1961) *Proc. R. Soc. London Ser. B* **154**, 514-521
- Batchelor, F. R., Cameron-Wood, J., Chain, E. B. & Rolinson, G. N. (1963) *Proc. R. Soc. London Ser. B* **158**, 311-328
- Boxer, G. E. & Everett, P. M. (1949) *Anal. Chem.* **21**, 670-673
- Dyke, K. G. H. (1967) *Biochem. J.* **103**, 641-646
- Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715-720
- Fisher, J., Charnas, R. L. & Knowles, J. R. (1978) *Biochemistry* **17**, 2185-2189
- Howarth, T. T., Brown, A. G. & King, T. J. (1976) *J. Chem. Soc. Chem. Commun.* 266-267
- Hunter, P. A. & Reading, C. (1976) *Abstr. Int. Conf. Antimicrobial Agents and Chemotherapy 16th*, abstr. no. 211
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245-3249
- Novick, R. P. (1962a) *Biochem. J.* **83**, 229-235
- Novick, R. P. (1962b) *Biochem. J.* **83**, 236-240
- Rando, R. R. (1974) *Science* **185**, 320-324

Reading, C. & Cole, M. (1977) *Antimicrob. Agents Chemother.* **11**, 852-857
Richmond, M. H. (1963) *Biochem. J.* **88**, 452-459
Sagai, H. & Saito, T. (1973) *J. Antibiot.* **26**, 315-319

Virden, R., Bristow, A. F. & Pain, R. H. (1975) *Biochem. J.* **149**, 397-401
Waley, S. G. (1974) *Biochem. J.* **139**, 789-790
Waley, S. G. (1975) *Biochem. J.* **149**, 547-551