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# The pH-Dependence and Group Modification of β-Lactamase I

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The pH-dependence of the kinetic parameters for the hydrolysis of the β-lactam ring by β-lactamase I (penicillinase, EC 3.5.2.6) was studied. Benzylpenicillin and ampicillin {6-[D(−)-α-aminophenylacetamido]penicillanic acid} were used. Both $k_{cat.}$ and $k_{cat.}/K_m$ for both substrates gave bell-shaped plots of parameter versus pH. The pH-dependence of $k_{cat.}/K_m$ for the two substrates gave the same value (8.6) for the higher apparent p$K$, and so this value may characterize a group on the free enzyme; the lower apparent p$K$ values were about 5 (4.85 for benzylpenicillin, 5.4 for ampicillin). For benzylpenicillin both $k_{cat.}$ and $k_{cat.}/K_m$ depended on pH in exactly the same way. The value of $K_m$ for benzylpenicillin was thus independent of pH, suggesting that ionization of the enzyme's catalytically important groups does not affect binding of this substrate. The pH-dependence of $k_{cat.}$ for ampicillin differed, however, presumably because of the polar group in the side chain. The hypothesis that the p$K$5 group is a carboxyl group was tested. Three reagents that normally react preferentially with carboxyl groups inactivated the enzyme: the reagents were Woodward's reagent K, a water-soluble carbodi-imide, and triethyloxonium fluoroborate. These findings tend to support the idea that a carboxylate group plays a part in the action of β-lactamase I.

β-Lactamases are enzymes that catalyse the hydrolysis of the β-lactam ring of penicillins and cephalosporins. They are surprisingly numerous and diverse, and in some instances they play a decisive part in the resistance of pathogenic micro-organisms to the β-lactam antibiotics. As enzymes, β-lactamases are among the most effective catalysts ($k_{cat.}$ about $10^4 s^{-1}$ for ampicillin, given below) and they are specific in that their action is apparently restricted to the hydrolysis of certain β-lactam rings, namely those in the fused ring systems of penicillins and cephalosporins. Histidine, tyrosine and carboxyl groups have all been suggested as playing a part in the reaction (Depue *et al.*, 1964; Patil & Day, 1973; Scott, 1973). Yet little is definitely known about the nature of the groups responsible for catalysis, or how they co-operate, or about the detailed mechanism (e.g. whether covalent intermediates play a part). The following investigation of the pH-dependence of the β-lactamase I (EC 3.5.2.6) from *Bacillus cereus* (Davies *et al.*, 1974) represents an initial step towards the more detailed characterization of one of these enzymes. The pH-dependence of an enzyme's kinetic parameters is both a feature to be accommodated by any full mechanism, and a tentative guide to the nature of the groups in the enzyme that play a part in catalysis. This guide has been followed in that several reagents, generally selective for carboxyl groups, have been tested for their ability to inactivate the enzyme.

## Materials and Methods

The β-lactamase I, prepared by the procedure of Davies *et al.* (1974) from *B. cereus* strain 569/H, was kindly provided by Dr. M. Brightwell. *N*-Ethyl-5-phenylisoxazolium-3′-sulphonate, Woodward's reagent K, was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; triethyloxonium fluoroborate was from Ralph N. Emanuel Ltd., Wembley, Middx., U.K.; 1-cyclohexyl-3-(2-morphilinoethyl)carbodi-imide metho-*p*-toluenesulphonate was from Fluka A.G., Buchs, Switzerland. The spectrophotometric assay was carried out as described by Waley (1974), at 30°C. Buffers were 0.05M-sodium formate (pH3.7, 4.0), 0.05M-sodium acetate (pH4.5–5.5), 0.05M-sodium phosphate (pH6–8), 0.05M-diethanolamine hydrochloride (pH8.5, 8.75) and 0.05M-$Na_2CO_3$ (pH9, 10). The assay solution was 0.3M in NaCl, which prevented large differences in the ionic strength with different buffers; separate experiments had shown that the rate was little influenced by changes in the ionic strength. In most of the experiments, EDTA (1mM) was present, so that any β-lactamase II would be inactivated, but the inclusion of EDTA did not seem to affect the rate. Adherence to Beer's Law was checked at each pH, and the change in extinction due to hydrolysis of the β-lactam ring was also determined at each pH. The spectrophotometric assay is better than the pH-stat assay below pH4.7, owing to protonation of the thiazolidine in the

penicilloate. The pH-stat assay was used for many of the experiments with ampicillin; relatively high concentrations of substrate were required at the lower pH values, and the spectrophotometric assay is less satisfactory when the concentration of substrate is greater than about 1 mM.

Initial rates were measured, and the correction of Lee & Wilson (1971) was used. Least-square fits of plots of $\bar{s}/v$ against $\bar{s}$ (where $\bar{s}$ is the mean concentration of substrate, and $v$ is the estimated initial rate) gave values for the kinetic parameters. The convenient direct linear plot (Eisenthal & Cornish-Bowden, 1974) was also much used for obtaining the kinetic parameters from the measured initial rates. The concentration of benzylpenicillin varied from 0.05 to 1 mM; four or five concentrations of substrate were used. The concentration of ampicillin varied from 0.05 to 3 mM, the range chosen depending on the pH; mostly, seven concentrations were used. For both substrates, the runs were carried out in duplicate or triplicate. The enzyme is less stable at low pH values, but the stability was adequate for the measurement of initial rates, which only took a few minutes. Solutions of the substrates were kept chilled until just before use; non-enzymic hydrolysis was appreciable above pH 9. No systematic deviation from Michaelis–Menten kinetics was observed.

The kinetic parameters estimated were $k_{cat.}$ and $K_m$, defined from the equation: $v = k_{cat.}[E][S]/(K_m+[S])$, where $v$ is the rate when the concentration of substrate is [S] and that of enzyme is [E]. The molecular weight of the enzyme was taken to be 28000 (Davies *et al.*, 1974).

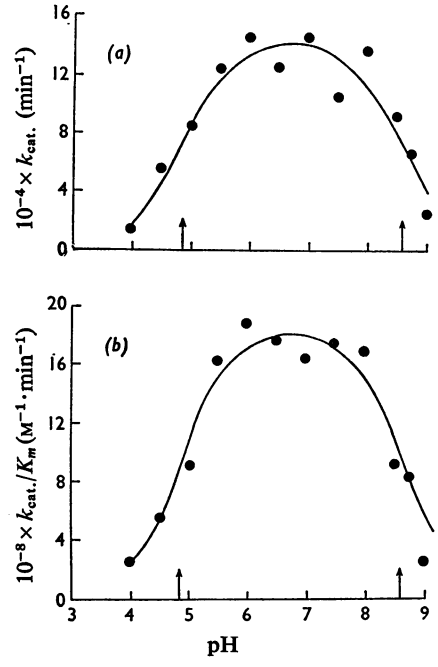


Fig. 1. *Plots of (a) $k_{cat.}$ and (b) $k_{cat.}/K_m$ versus pH for the hydrolysis of benzylpenicillin*

The hydrolysis of benzylpenicillin by β-lactamase I at 30°C was followed by the spectrophotometric assay (Waley, 1974). The curves are theoretical; the arrows show the p$K$ values used to calculate them.

## Results and Discussion

### *pH-dependence of kinetic parameters*

The plot of $k_{cat.}/K_m$ against pH for the hydrolysis of benzylpenicillin is shown in Fig. 1(*b*). Apparent p$K$ values of 4.85 and 8.6 are obtained from this plot. The plot of $k_{cat.}$ against pH (Fig. 1*a*) is very similar, and the apparent p$K$ values are again 4.85 and 8.6. Consequently, the parameter $K_m$ (the Michaelis constant) is independent of pH. Another substrate, ampicillin {6-[D(−)-α-aminophenylacetamido]penicillanic acid}, shares some of these features but differs in others. This substrate contains an amino group in the side chain as well as the carboxyl group of the thiazolidine ring, and has p$K$ values of 2.7 and 7.25 at 25°C (Hou & Poole, 1969). The plot of $k_{cat.}/K_m$ against pH (Fig. 2*b*) again gives apparent p$K$ values of 5.4 and 8.6, the latter being the same value as was obtained from Fig. 1(*b*) for benzylpenicillin. The plot for $k_{cat.}$, however, gives lower values, of 4.05 and 7.4 (Fig. 2*a*). Consequently, $K_m$ decreases with increase of pH, as was also observed for the hydrolysis of this substrate by the β-lactamase from a strain of *Staphylococcus aureus* (Hou & Poole, 1973). The significance of the Michaelis constant depends on the mechanism of the reaction. The fact that the $K_m$ for benzylpenicillin varies little with the pH whereas $k_{cat.}$ varies markedly is consistent with $K_m$ being a true dissociation constant for benzylpenicillin. However, the hydrolysis catalysed by β-lactamases may proceed by a mechanism in which there are two unimolecular steps. If the second step is rate-determining (whether this is hydrolysis of an acyl-enzyme or a change in conformation does not signify here) then $K_m$ may be independent of pH and yet its value may be different from that of the dissociation constant. This behaviour is exemplified in the hydrolysis of *p*-nitrophenyl acetate by Protease 3 from *Streptomyces griseus* (Bauer & Pettersson, 1974).

Schemes for explaining the pH-dependence of enzymic reactions usually start from the postulate that only three forms of the enzyme need to be considered; the three forms differ in charge by unity, and their interconversion is characterized by apparent p$K$ values that relate to the free enzyme. Correspondingly, there are three charged forms of the enzyme–substrate complex. Steady-state treatment of the simple Michaelis–Menten scheme, on the

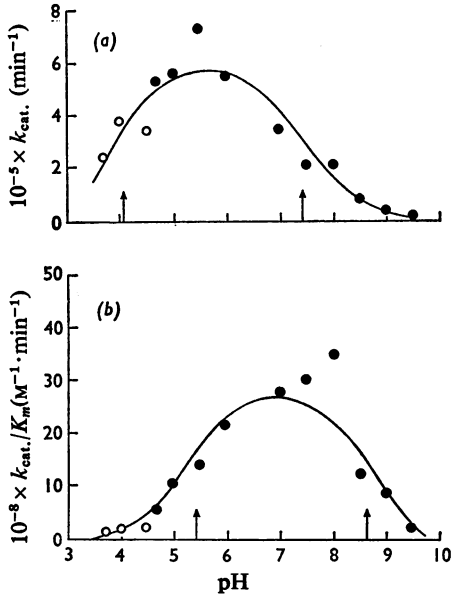


Fig. 2. *Plots of (a) $k_{cat.}$ and (b) $k_{cat.}/K_m$ versus pH for the hydrolysis of ampicillin*

The hydrolysis of ampicillin {6-[D(−)-α-aminophenylacetamido]penicillanic acid} by β-lactamase I was carried out as described in Fig. 1 (○), or in the pH-stat (●), with corrections below pH 7 for the incomplete uptake of alkali.

assumptions that proton-transfer reactions are fast, and that although the substrate may combine with any of the three charged forms of the enzyme it is only the intermediate form that gives products, gives equations for the overall rate that are easy to use and interpret (Waley, 1953). These equations give expressions for the pH-dependence of the kinetic parameters $k_{cat.}/K_m$ and $k_{cat.}$; the former gives apparent p$K$ values for the free enzyme, and the latter for the enzyme–substrate complex (Alberty, 1956; Peller & Alberty, 1959). The work on chymotrypsin gives a good example of the use of this approach (Bender *et al.*, 1964). It is, unfortunately, difficult to know whether the simplifying assumptions are valid; consequences of considering the rates of proton transfers are discussed by Ottolenghi (1971), and of parallel pathways by Laidler & Bunting (1973).

Both substrates, benzylpenicillin and ampicillin, gave an apparent p$K$ value of 8.6 in the $k_{cat.}/K_m$ plots (Figs. 1*b* and 2*b*). Thus this p$K$ may well characterize a group on the enzyme, for if the p$K$ value had been a complex expression containing ionization constants and rate constants it might be expected to differ for the two substrates. The situation is less clear-cut for the lower p$K$, which has the value 4.85 for benzylpenicillin and 5.4 for ampicillin.

The same p$K$ values feature in both plots for benzylpenicillin (Fig. 1). This would be so if the p$K$ values of the free enzyme and the enzyme–substrate complex were the same. The lower p$K$ values obtained from the plot of $k_{cat.}$ for ampicillin (Fig. 2*a*) may reflect an effect on the enzyme–substrate complex due to the amino group, which (at least at lower pH values) will be positively charged.

The chemical nature of the groups will now be considered. Although large perturbations (i.e. unexpected p$K$ values) are on record, for example the active-site lysine residue with p$K$6 in acetoacetate decarboxylase (Schmidt & Westheimer, 1971), it is uncertain how common they are. A large perturbation is also, eventually, informative. In the absence of a large perturbation, the obvious candidates for this group are either a carboxyl group or an imidazole group. The latter seems unlikely. Ferenc *et al.* (1971) reported that diethyl pyrocarbonate reacted with all the histidine residues without any loss of enzyme activity. Moreover, none of the histidine residues in the three homologous β-lactamases (from *Staph. aureus*, *Bacillus licheniformis*, *B. cereus*) is at the same position in all three sequences, i.e. there are no 'conserved' histidine residues (Ambler & Meadway, 1969; R. P. Ambler & D. R. Thatcher, personal communication). Hence the p$K$4.8 group may well be a carboxyl group, perhaps acting as a base (or a nucleophile if there is a covalent intermediate), as in carboxypeptidase A (Quiocho & Lipscomb, 1971).

The group of p$K$8.6 could be a lysine or tyrosine residue; nitration of tyrosine-77 in the homologous β-lactamase from *B. licheniformis* causes some decrease in activity (Meadway, 1969; Ambler & Meadway, 1969), and similarly nitration of a tyrosine in a β-lactamase from *Escherichia coli* caused 36% loss in activity (Scott, 1973). The changes seem rather modest for a group directly concerned with catalysis. Thus the nature of the group with p$K$8.6 remains an open question.

*Group modification*

We have seen that the kinetic results point to the involvement of a carboxyl group in catalysis. Group-selective reagents may be used to test this idea. These experiments are now described. They are of a preliminary nature: reliance was placed on the selectivity of the reagents (which is safer in acid solution than in neutral solution) and the extent of modification was not studied.

*Inactivation by N-ethyl-5-phenylisoxazolium-3′-sulphonate.* *N*-Alkyl-5-phenylisoxazolium salts react readily, and selectively, with carboxyl groups of proteins (Bodlaender *et al.*, 1969; Feinstein *et al.*, 1969); one of these salts (Woodward's reagent K) reacts with a carboxyl group at the active centre of carboxypeptidase A (Petra, 1971; Petra & Neurath, 1971). The reactive intermediate is a keto keten-

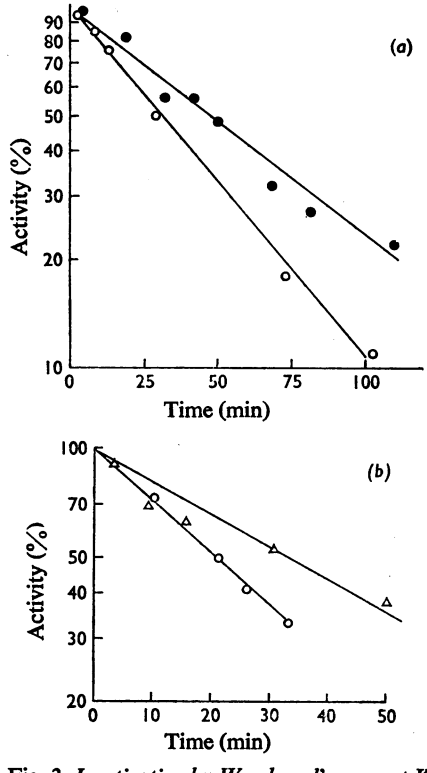


Fig. 3. *Inactivation by Woodward's reagent K*

Semilogarithmic plot of activity of β-lactamase I versus time of reaction. In (*a*) *N*-ethyl-5-phenylisoxazolium-3'-sulphonate [80μl of 12% (w/v) solution in 1mM-HCl] was added to 1mg of β-lactamase I in 2ml of 5mM-sodium phosphate–0.3mM-NaCl in a pH-stat at 10°C; the pH was maintained at 4.8 with 40mM-NaOH. Samples (10μl) were periodically withdrawn from the reaction mixture specified (○) or one containing 1mM-cephalosporin C (●). In (*b*) 20μl of 12% (w/v) reagent was used at pH6.4, 2°C; no additions (○); 1mM-cephalosporin C (△).

imine (Dunn *et al.*, 1974). The products are enol esters, which may rearrange to imides but are generally stable enough to be isolated. The inactivation of β-lactamase I by 19mM reagent at pH4.8, and at 10°C, was a first-order process (Fig. 3*a*), and the same applied to the reaction with 5mM reagent at pH6.4, 2°C (Fig. 3*b*). The rate of inactivation was less in the presence of cephalosporin C (Fig. 3). Cephalosporin C is only slowly hydrolysed by β-lactamase I; when tested as a competitive inhibitor the $K_I$ was of the same order as $K_m$ for benzylpenicillin (Abraham & Newton, 1956; Crompton *et al.*, 1962). At the end of the reaction shown in Fig. 3(*a*), appreciable amounts of cephalosporin C remained, as judged by paper electrophoresis at pH4.5. These results are consistent with inactivation being a consequence of the reaction of a carboxyl group at the above site, but no doubt many other carboxyl groups also react and the final reaction mixtures were quite turbid. Inactivation without precipitation of protein could be achieved at pH3.6, 2°C, but reaction was very slow.

*Inactivation by a carbodi-imide.* The inactivation of the β-lactamase by the water-soluble carbodi-imide that was used has a half-time of about 7min at pH4.5, 10°C. The rapid reaction of proteins with carbodi-imides is normally confined to carboxyl groups, at least when thiol groups are absent, as they are here: phenolic hydroxyl groups sometimes also react, but more slowly (Carraway & Koshland, 1972). The rapid inactivation here suggests reaction with carboxyl groups; inactivation was no less rapid when cephalexin or cephalosporin C was initially present, but they were quite largely decomposed during the reaction, probably by reaction with the carbodi-imide.

*Inactivation by triethyloxonium fluoroborate.* Triethyloxonium tetrafluoroborate (150μl) was added slowly to β-lactamase I (0.4mg) in 2ml of 0.3M-NaCl while the pH was maintained at pH3.5–4.5: there was about 70% inactivation. In a similar experiment in which cephalosporin C (1mM) was present there was about 40% inactivation. The high reactivity of this reagent towards water makes it difficult to obtain large extents of inactivation, but reaction is likely to be with carboxyl groups (Parsons *et al.*, 1969; Wilcox, 1972), and it is an advantage that the substituent (ethyl) is not as bulky as it is with the other reagents described.

## *Conclusions*

None of the lines of evidence put forward for the participation of a carboxyl group in the action of β-lactamase I would, by itself, carry much conviction. Yet taken together they seem reasonably persuasive, and form a basis for the design of reagents that will mimic substrates and react with carboxyl groups. Indeed, carboxyl groups are probably more often involved in enzyme action than any other class of side-chain group.

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