Inactivation of TEM-1 β -lactamase by 6-acetylmethylenepenicillanic acid

Mikio ARISAWA* and Rudolf THEN

Pharmaceutical Research Department, F. Hoffmann–La Roche and Co. Ltd., CH-4002 Basel, Switzerland

(Received 6 July 1982/Accepted 21 October 1982)

6-Acetylmethylenepenicillanic acid is a new kinetically irreversible inhibitor of various β -lactamases. Interaction between 6-acetylmethylenepenicillanate and purified TEM-1 β -lactamase during the inactivation process was investigated. 6-Acetylmethylenepenicillanate inhibited the enzyme in a second-order fashion with a rate constant of $0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$. The apparent inactivation constant decreased in the presence of increasing concentrations of the substrate benzylpenicillin. Native enzyme (pI 5.4) was converted into two inactive forms with pI 5.25 and 5.15, the latter form being transient and readily converted into the more stable form with pI 5.15. Even a 50-fold excess of inhibitor over enzyme did not produce any other inactivated species of the enzyme. All the results obtained suggest that 6-acetylmethylenepenicillanate is a potent irreversible and active-site-directed inhibitor of TEM-1 β -lactamase.

Bacterial pathogens resistant to various β -lactam antibiotics are an important problem in the clinical field. It is generally accepted that β -lactamases are frequently involved in and responsible for this resistance. Therefore, in addition to attempts to synthesize new intrinsically stable β -lactams, efforts to discover β -lactamase inhibitors have been made in order to overcome the resistance problem.

Although several β -lactamase inhibitors have been found or synthesized, their spectra of activity are not broad enough to inhibit all β -lactamases from clinically important bacterial species (Cole, 1981). Clavulanic acid, for example, exhibits a poor activity against cephalosporin-hydrolysing β -lactamases and exerts preferential activity against penicillinases.

From a wide series of 6-substituted acylmethylenepenicillanic acid derivatives, recently synthesized in our laboratories, 6-acetylmethylenepenicillanic acid (AMPA, compound Ro 15-1903; Fig. 1) was found to be the most potent inhibitor of various types of β -lactamases.

Its potency was seen to be higher than that of clavulanic acid or of penicillanic acid sulphone (Then *et al.*, 1982), and it inhibited both plasmid- and chromosomally mediated β -lactamases. These findings led us to investigate the mechanism of action of AMPA in order to discover the reason for

such broad and high activity, information that should further aid the rational synthesis of chemically useful drugs.

In the present paper we describe kinetic and biochemical studies on the activation of the TEM-1 β -lactamase by AMPA. Inactivation proceeded very rapidly in a second-order fashion, producing one species of inactivated enzyme with pI 5.15 via a transitional inactive form with pI 5.25.

Materials and methods

Materials

Escherichia coli TEM-1, harbouring the R6K plasmid (Matthew *et al.*, 1979), was kept in Tryptic Soy Broth (Difco Laboratories, Detroit, MI, U.S.A.) in the presence of 12.5% (v/v) of glycerol at -70°C .

Sodium benzylpenicillanate was a product of Novo Industri, Copenhagen, Denmark. AMPA was synthesized by Dr. S. Adam of F. Hoffmann–La Roche and Co., Basel, Switzerland (for details see Hoffmann–La Roche, 1982). AMPA was freshly dissolved on the day of each experiment in 0.1 M-sodium phosphate buffer, pH 7.0, and kept on ice.

Coomassie Brilliant Blue R250 and sodium dodecyl sulphate were purchased from Serva (Heidelberg, West Germany) and Sigma Chemical Co. (St. Louis, MO, U.S.A.) respectively.

DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., Sephadex G-75 was from Pharmacia (Uppsala, Sweden), and hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Abbreviation used: AMPA, sodium 6-acetylmethylenepenicillanate.

* To whom correspondence should be addressed. Present address: Nippon Roche Research Center, Kajiwara 200, Kamakura, Kanagawa 247, Japan.

Fig. 1. Structure of sodium 6-acetylmethylenepenicyllanate {compound Ro 15-1903; sodium (2*S*,5*R*)-6-acetonylidene-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylate}

Ready-prepared thin-layer polyacrylamide gel (LKB Ampholine PAGplate), pH range 4–6.5, was supplied by LKB (Bromma, Sweden).

Methods

The microcomputer-aided double-beam spectrophotometer UVIKON 810 (Kontron, Zürich, Switzerland) was used for all spectrophotometric work. All reactions were conducted at 37°C in 0.1M-sodium phosphate buffer, pH 7.0, in either a water bath or a water-jacketed cuvette-holder.

Enzyme assay. Hydrolytic activity of the β -lactamase was measured spectrophotometrically as described by Ross & O'Callaghan (1975). In all β -lactamase assays, sodium benzylpenicillin was used as the substrate and its hydrolysis was monitored by measuring the decrease in absorbance at 240 or 236 nm.

Purification of β -lactamase. *E. coli* TEM-1 was grown in Tryptic Soy Broth, harvested and stocked at -70°C. Three litres of crude extract were prepared from 400g of cells by the osmotic-shock method (Fisher *et al.*, 1980). This material was applied to a DEAE-cellulose column (970 ml bed volume) equilibrated with 10 mM-Tris/HCl buffer, pH 7.3. This column was developed with a linear gradient (3 litres + 3 litres) of NaCl (0–0.5 M) in 10 mM-Tris/HCl, pH 7.3. The fractions containing activity were pooled, concentrated to 40 ml by ultrafiltration (Amicon UM 10 membrane) and subjected to gel chromatography in four equal portions on a column of Sephadex G-75 (450 ml bed volume). The active fractions obtained from four runs were combined. The enzyme was further purified over a hydroxyapatite column (250 ml), from which the enzyme was eluted with a linear gradient of 5–250 mM-sodium phosphate buffer, pH 7.0. After dialysis against 10 mM-sodium phosphate buffer, pH 7.0, the final yield of β -lactamase was 18 mg. The homogeneity of the preparation was checked both by electrophoresis in the presence of 0.1% sodium dodecyl sulphate and by isoelectrofocusing in polyacrylamide gels. Purity was over

95%. Although the molecular weight was determined to be 27 500 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a molecular weight of 29 800, which had been reported by Ambler & Scott (1978), was adopted for the calculation of enzyme concentration. The purified enzyme solution was stored at -70°C until being used.

Determination of K_m and k_{cat} . K_m and k_{cat} , determined graphically by using double-reciprocal plots, were 26.4 μ M and 665 s⁻¹ respectively.

Measurement of stoichiometry of reaction of AMPA. TEM-1 β -lactamase (16 μ M; 10 μ l) was mixed with the same volume of AMPA solutions to give various concentration ratios of AMPA to enzyme. After incubation for 90 min at 37°C, 5 μ l of the reaction mixture was diluted into 995 μ l of ice-cold 0.1 M-sodium phosphate buffer, pH 7.0. Then 20 μ l of the diluted enzyme solution was transferred to a cuvette containing 1 ml of 2 mM-benzylpenicillin in order to measure the residual activity. The percentage inhibition was calculated as $(v_0 - v)/v_0$, where v_0 is the velocity of hydrolysis obtained with enzyme alone (control) and v is that obtained with the treated enzyme.

Inactivation of the enzyme in the absence of substrate. Prewarmed enzyme solutions (0.04–0.16 μ M; 100 μ l; 37°C) were rapidly mixed with the same volume and the same concentrations of prewarmed AMPA solutions. Upon mixing, a 5 μ l sample of the reaction mixture was taken out at appropriate time intervals and diluted into 1 ml of 2 mM-benzylpenicillin at room temperature. The amount of residual active enzyme was calculated from the hydrolysis rates measured after the cuvette had been kept at 37°C for 10–30 min. The maximum concentration of AMPA (0.16 μ M) used for the inactivation did not affect the enzyme assay under the condition employed. The concentration of native enzyme [E] at time t with enzyme activity of v was calculated by the equation:

$$[E] = [E]_0 \cdot v/v_0$$

where $[E]_0$ and v_0 stand for initial enzyme concentration and its initial velocity respectively.

Inactivation of the enzyme in the presence of substrate. Benzylpenicillin-hydrolysing velocities at various times during progressive inhibition were measured by essentially the method described by Knott-Hunziker *et al.* (1980). To 990 μ l of a mixture (37°C) containing an appropriate amount of benzylpenicillin (0.2–1 μ mol) and AMPA (0.1–0.3 nmol), 10 μ l of the enzyme solution (0.16 μ M) was added to start the reaction. The molar concentration ratios of inhibitor to enzyme were between 62.5 and 187.5. The decrease in absorbance at 236 nm was recorded over a period during which hydrolysis in control runs proceeded at constant rates (usually 0.8–3 min).

Scheme 1.

As indicated in the Results section, this scheme explains best the interaction between AMPA and benzylpenicillin and TEM-1 enzyme. E, TEM-1 β -lactamase; B, benzylpenicillin; EB, Michaelis complex; P, degradation product of benzylpenicillin; A, AMPA; EA, inactivated complex; k , inactivation constant.

From the progressive inhibition curve, individual velocities were calculated by using the equation:

$$v_i = (A_{i+1} - A_i) / \Delta t$$

where v_i is the velocity at time t_i , Δt is a constant period (0.2 or 0.4 min), and A_i and A_{i+1} are absorbances at time t_i and $t_i + \Delta t$ respectively. The apparent inactivation constant ($K_{app.}$) was determined graphically in accordance with eqn. (1) (Knott-Hunziker *et al.*, 1980; Kitz & Wilson, 1962) (which is derived from Scheme 1):

$$\ln(v_0/v) = \frac{k \cdot K_m \cdot [A]}{K_m + [B]} \cdot t \quad (1)$$

where v_0 is the initial velocity, v is the velocity at time t , k is the inactivation constant, K_m is the Michaelis constant, $[A]$ is the concentration of inhibitor (AMPA) and $[B]$ is the concentration of substrate (benzylpenicillin). $K_{app.}$ was determined as the slope of the straight line from the plot of $\ln(v_0/v)$ against t .

Therefore the following equation may be derived:

$$\frac{1}{K_{app.}} = \left(\frac{1}{k} + \frac{[B]}{k \cdot K_m} \right) \cdot \frac{1}{[A]} \quad (2)$$

Isoelectric focusing. Analytical isoelectric focusing was performed on a flat-bed apparatus (Pharmacia), being cooled by tap water, with Ampholine PAGplates, pH range 4–6.5.

Samples (1–7 μ g of protein in 5–20 μ l of 0.1 M-sodium phosphate buffer, pH 7.0) were applied after 30 min of prefocusing and were focused for 60 min. Gels were fixed, stained for protein, destained and dried by the procedure of Wrigley (1971). Densitometry of gels was performed with the Zeiss PMQII spectrophotometer assembly.

Protein determination. Protein concentration was determined by the method of Bradford (1976) with the commercially available Protein Assay kit (Bio-Rad Laboratories), with bovine serum albumin (Bio-Rad Laboratories) as the protein standard.

Fig. 2. Second-order inactivation of TEM-1 β -lactamase by AMPA

Equimolar solutions of enzyme and inhibitor were separately equilibrated at 37°C, and equal amounts of each were mixed to start the inactivation. Inactivation was followed by measuring residual activity with time. The amount of native enzyme remaining $[E]$ was calculated by using the initial enzyme concentration $[E]_0$ and the catalytic activities before and after inactivation, as described in the Materials and methods section. Concentrations were: ●, 0.04 μ M; ■, 0.08 μ M; ▲, 0.16 μ M.

Results

Inactivation in the absence of substrate

Since AMPA in excess over TEM-1 β -lactamase inactivated the enzyme too fast for the reaction to be followed, inactivation experiments were performed with equimolar amounts of enzyme and inhibitor. As shown in Fig. 2, all the results obtained from the experiments with three different initial concentrations fitted one straight line. This indicates that the inactivation obeyed second-order kinetics with a rate constant of $0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$. The inactivation constant of $0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ was large as compared with the second-order rate constant of $3.0 \times 10^{-7} \mu\text{M}^{-1} \cdot \text{s}^{-1}$ obtained for the degradation of AMPA by neutral hydroxylamine. The loss of activity of enzyme inactivated to various extents was not reversed after 10–30 min incubation at 37°C in the presence of 2 mM-benzylpenicillin. This indicates that inactivated enzyme would not be in an equilibrium that easily allows the enzyme–AMPA complex to dissociate.

As a consequence of the second-order mode of inhibition, two deductions could be made: (1) one molecule of AMPA inactivates one molecule of the enzyme; (2) dissociation of the inactivated complex to regenerate the active enzyme form is negligibly slow. Both items were reinforced by the result shown in Fig. 3. As derived from the straight line

Fig. 3. Determination of the number of AMPA molecules hydrolysed before inactivating one molecule of the enzyme

TEM-1 β -lactamase ($16\mu\text{M}$) was incubated with various concentrations of AMPA and incubated for 90 min at 37°C . Residual enzyme activity was measured with the use of benzylpenicillin as substrate after dilution of enzyme, and the percentage inhibition was calculated as described in the Materials and methods section.

in this Figure, the number of AMPA molecules that reacts with TEM-1 β -lactamase before inactivation is 1.04:1. This stoichiometry of close to unity was not affected by the incubation time (30–90 min), indicating the stability of the inactivated complex.

Inactivation in the presence of benzylpenicillin

By assuming bimolecular interaction between TEM-1 β -lactamase and AMPA, the inactivation in the presence of benzylpenicillin may be simply formulated as in Scheme 1. This scheme implies that a high concentration of benzylpenicillin would decrease K_{app} , because of the competition between the substrate and the inhibitor for the enzyme. This would make it easier to follow the inactivation provoked by an excess of inhibitor. Under such conditions the concentration of AMPA may be regarded as constant, and therefore eqns. (1) and (2) in the Materials and methods section could be derived.

Fig. 4 shows the correlation between K_{app} and $[A]$ at five substrate concentrations between 0.2 and 1 mM. The lines passing through the origin suggest the absence of a Michaelis complex in the reaction or a very short half-life of such a complex (Kitz & Wilson, 1962), which is consistent with the second-order kinetics observed. Fig. 5 gives the effect of benzylpenicillin concentrations on K_{app} . K_{app} decreased as the concentration of benzylpenicillin, a good substrate, increased.

Fig. 4. Dependence of the apparent inactivation constant K_{app} on the concentration of AMPA

Inactivation in the presence of benzylpenicillin was initiated by the addition of $10\mu\text{l}$ of $0.6\mu\text{M}$ -enzyme solution to a cuvette containing 0.2 (▲), 0.3 (○), 0.5 (□), 0.8 (■) or 1 (●) μmol of benzylpenicillin and from 0.1 to 0.3 nmol of AMPA. The molar concentration ratios of AMPA to enzyme were between 62.5 and 187.5. The decrease in absorbance at 236 nm was recorded over a period of time (0.8–3 min) during which hydrolysis in controls proceeded at a constant rate. Individual velocities were calculated in accordance the equation:

$$v_t = (A_{t+1} - A_t) / \Delta t$$

where v_t is the velocity at time t , Δt is a constant interval (0.2 or 0.4 min) and A_t and A_{t+1} are absorbances at time t and $t + \Delta t$ respectively. The apparent inactivation constant (K_{app}) was determined as the slope of the line obtained by plotting $\ln(v_0/v)$ against t (see the Materials and methods section).

The validity of the two graphs shown in Figs. 4 and 5 was confirmed by comparing kinetic parameters obtained from them with those obtained from direct measurement. As is to be inferred from eqn. (2), the slope of the straight lines from Fig. 4 will be $(K_m + [B])/kK_m$. The straight line in Fig. 5 will have intercepts on the $[B]$ and $1/K_{\text{app}}$ axes of $-K_m$ and $1/k[A]$ respectively, allowing K_m and k to be determined. The values derived in this way were: $k = 0.66 \pm 0.04 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ from five lines in Fig. 4 by using K_m measured directly, and $0 < K_m < 50 \mu\text{M}$ and

Fig. 5. Dependence of K_{app} on the substrate concentration

Experiments of the kind shown in Fig. 4 were performed at different concentrations of substrate. The concentrations of enzyme and inhibitor were 1.6 nM and 0.25 μ M respectively.

$k = 0.60 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ from Fig. 5. These values were closely similar to those determined directly ($K_m = 26.4 \mu\text{M}$ and $k = 0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$).

Molecular species of inactivated enzyme

AMPA-inactivated TEM-1 β -lactamase was subjected to isoelectric focusing in order to evaluate the number of species of inactivated enzyme. A short treatment (1 min at 10°C) of the enzyme with the inhibitor was found to produce two different bands, with pI 5.25 and 5.15 (Fig. 6). However, the amount of the latter band increased with incubation time, and, finally (within 30 min at 37°C), only this band was observed. This indicates that the molecular species with pI 5.25 was a transient form and that AMPA ultimately formed only one kind of inactivated complex, differing from the native enzyme in that its pI was 0.25 unit lower. Even when the enzyme was incubated with a 50-fold excess of AMPA, only one band could be seen, which also provides supporting evidence for a 1:1 binding of AMPA to the enzyme molecule.

Neither of the two inactivated forms was enzymically active, since all the activity remaining after inactivation with various amounts of inhibitor could be explained by the amount of protein focusing with the pI of the native enzyme (Table 1). When molar ratios of inhibitor to enzyme of 1:3 or 2:3 were allowed to react, 65% and 30% of residual activity respectively were detected. The relative amount of protein corresponding to the native enzyme band within each sample showed good agreement with the catalytic activity remaining. This is expressed as

Fig. 6. Densitogram of native and inactivated enzyme separated by isoelectric focusing

Inactivation was started by mixing the same volume of solutions containing enzyme (5 μ M in 0.1M-sodium phosphate buffer, pH 7.0) or inhibitor (1.67–250 μ M in the same buffer). The sample with 1 min inactivation was inactivated at 10°C for 1 min just before isoelectric focusing. After reaction, samples were kept in ice/water for no longer than 3 min. Then 20 μ l of each sample was applied on an LKB Ampholine PAGplate, pH range 4–6.5. Densitometer traces were obtained on a Zeiss PMQII spectrophotometer assembly. (a) Native enzyme; (b) briefly inactivated enzyme (1 min at 10°C) at [AMPA]/[E] molar ratio 1:1; (c) 37°C–30 min-inactivated enzyme at [AMPA]/[E] molar ratio 1:3; (d) 37°C–30 min-inactivated enzyme at [AMPA]/[E] molar ratio 50:1.

Table 1. Correlations between the amount of inhibitor used for inactivation, remaining native enzyme and residual catalytic activity

Enzyme solution (5 μ M; 100 μ l) was mixed with the same volume of 0.1 M-sodium phosphate buffer, pH 7.0, or inhibitor solution (1.67–250 μ M) and incubated for 30 min at 37°C. Samples were then subjected to both activity measurement and isoelectric focusing. Remaining activity was measured with the use of 2 mM-benzylpenicillin as substrate. Analytical isoelectric focusing was performed on a ready-prepared LKB Ampholine PAGplate, pH range 4–6.5. Quantification of the native-enzyme band was performed by densitometry, after protein staining with Coomassie Brilliant Blue R250.

Molar ratio of AMPA to enzyme	Amount of protein associated with native-enzyme band within each sample (%)	Enzyme activity remaining (%)
0	100	100
0.33	65	65
0.67	38	30
1	0	2.1
50	0	1.6

percentages of the control activity in Table 1. This coincidence, on the other hand, also indicates that there were no forms other than the native enzyme (pI 5.4) and the inactive protein with pI 5.15.

Discussion

AMPA, a new β -lactamase inhibitor, is chemically characterized by its 6-methylene double bond connected directly to the β -lactam ring, as well as a reactive keto group on the 6-substituent. The study of structure–activity relationships among 6-acylmethylenepenicillanic acid derivatives showed that these two structural features are the minimum requirement for exhibition of potent β -lactamase-inactivating activity (S. Adam, personal communication). The β -lactam ring of AMPA probably mirrors the β -lactam ring of the substrate, which gives AMPA its specificity for β -lactamase.

The following evidence, derived from the kinetic studies, supports the idea of a specific attack by AMPA on the TEM-1 β -lactamase. (1) The inactivation process is best explained by second-order kinetics with the very high rate constant of $0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$. This value is 2×10^6 times the second-order constant for degradation of AMPA by neutral hydroxylamine, and strongly suggests an enzymically catalysed interaction. (2) The inactivated enzyme consisted of only one species, although a transient intermediate compound was observed. An excess of AMPA (50-fold) over enzyme did not produce other forms of inactivated enzyme, as far as studied by isoelectric focusing. (3)

The apparent inactivation constant K_{app} decreased as the concentration of benzylpenicillin, a good substrate, was increased. Although this merely indicates that AMPA reacted at a specific site of the TEM-1 β -lactamase molecule that is essential for hydrolysis of benzylpenicillin, it seems reasonable to regard this specific site as the active site. This specificity, coupled with the chemical features, may explain the high activity and broad spectrum of AMPA. A second-order mode of inhibition does not necessarily deny the existence of a Michaelis-type of intermediate, but the dissociation of this complex into E and A should be negligible compared with the inactivation rate. However, an inactive intermediate, discernible by isoelectric focusing, was observed. On the other hand, the loss of enzyme activity during only short-time exposure to AMPA was not reversed by the addition of high concentration of benzylpenicillin.

From these facts, the simplest inactivation process could be drawn more precisely as follows:

where EA^* is the stable inactivated enzyme. k_{+1} should be $0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$, corresponding to the rate of loss of enzyme activity, since EA was practically inactive. k_{+2} , on the other hand, has not yet been determined. The value of $0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ is larger than that of other inhibitors, such as clavulanic acid (Charnas & Knowles, 1981b; Reading & Hepburn, 1979), penicillanic acid sulphone (Brenner & Knowles, 1981; Labia *et al.*, 1980) and 6 β -bromopenicillanic acid (Knott-Hunziker *et al.*, 1980). One olivanic acid derivative, compound MM 13902, is reported to have $k_{\text{inact.}}/K_1$ about $1 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (Charnas & Knowles, 1981a), but this compound is not an irreversible inhibitor of the RTEM β -lactamase. Interaction between AMPA and TEM-1 β -lactamase resembled that between the *Bacillus cereus* β -lactamase and 6 β -bromopenicillanic acid (Knott-Hunziker *et al.*, 1980) and that between the *Staphylococcus aureus* β -lactamase and clavulanic acid (Reading & Hepburn, 1979) with respect to inactivation kinetics. On the other hand, clavulanic acid is known to produce three different forms when TEM-2 enzyme is used (Charnas *et al.*, 1978). Such differences in the interaction of clavulanic acid with different enzymes are certainly of interest in the study of β -lactamases from different bacterial species. Since, however, clavulanic acid is a poor inhibitor of certain enzymes, AMPA, because of its broad spectrum of activity, seems to be a better probe for further studies on β -lactamases.

References

- Ambler, R. P. & Scott, G. K. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3732–3736

- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Brenner, D. G. & Knowles, J. R. (1981) *Biochemistry* **20**, 3680–3687
- Charnas, R. L. & Knowles, J. R. (1981a) *Biochemistry* **20**, 2732–2737
- Charnas, R. L. & Knowles, J. R. (1981b) *Biochemistry* **20**, 3214–3219
- Charnas, R. L., Fisher, J. & Knowles, J. R. (1978) *Biochemistry* **17**, 2185–2189
- Cole, M. (1981) *Drugs Future* **11**, 697–727
- Fisher, J., Belasco, J. G., Koshla, S. & Knowles, J. R. (1980) *Biochemistry* **19**, 2895–2901
- Hoffmann–La Roche (1982) *Eur. Patent Application* 50805
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249
- Knott-Hunziker, V., Orlek, B. S., Sammes, P. G. & Waley, S. G. (1980) *Biochem. J.* **187**, 797–802
- Labia, R., Lelievre, V. & Peduzzi, J. (1980) *Biochim. Biophys. Acta* **611**, 351–357
- Matthew, M., Hedges, R. W. & Smith, J. T. (1979) *J. Bacteriol.* **138**, 657–662
- Reading, C. & Hepburn, P. (1979) *Biochem. J.* **179**, 67–76
- Ross, G. W. & O'Callaghan, C. H. (1975) *Methods Enzymol.* **43**, 69–85
- Then, R., Angehrn, P., Adam, S. & Hofheinz, W. (1982) *Proc. Symp. 'Beta-Laktamasehemmer' Paul-Ehrlich-Soc. Chemother.* in the press
- Wrigley, C. W. (1971) *Methods Enzymol.* **22**, 559–564