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Inhibition of β-Lactamases by β-Lactam Antibiotics

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The inhibitory properties of a selected number of β-lactam antibiotics were studied, with the use of three distinct types of β-lactamases. The three enzymes were found to be distinguishable on the basis of their susceptibility to inhibition. Not one of the potential inhibitors tested was found to be a potent inhibitor of all three enzymes, but nafcillin possessed the broadest inhibitory activity. The enzyme isolated from *Enterobacter cloacae* was found to be the most susceptible. In some cases, the degree of inhibition varied with the time of incubation, and, depending upon the time chosen, widely different observations could be made. It is suggested that, in studies such as these, every consideration should be given to the period of incubation and to the concentration of inhibitor employed. Mixtures of inhibitor and cephaloridine did not always act synergistically against growing bacteria, and a number of reasons for failure are suggested.

The inhibition of β-lactamases by β-lactam antibiotics has been studied by a number of investigators (1, 4, 6, 11). These studies have proved useful for characterizing and identifying β-lactamases isolated from various bacteria, and have indicated the kind of modifications in structure which would be likely to give increased inhibitory activity. In addition, studies with assorted combinations of an inhibitor and a β-lactam antibiotic have been carried out to determine their effects on cultures of β-lactamase-producing bacteria (3, 5, 11). However, relatively little information is available concerning the comparative susceptibility of different β-lactamases from gram-negative bacteria to a selection of inhibitors and about the relative properties of the inhibitors themselves.

At least eight distinct types of β-lactamase are to be found among members of the *Enterobacteriaceae* (4), but these may be grouped into three main classes, (i) enzymes mainly active against cephalosporins, (ii) types mainly active against penicillins, and (iii) enzymes which are equally active against both types of β-lactam antibiotic.

For the present study, three enzymes have been used which are thought to be representative of these different types of β-lactamase. The inhibitory properties of cephalosporin- and penicillin-type inhibitors have been compared, and their activity in combination with cephaloridine against growing cultures has been examined.

MATERIALS AND METHODS

Organisms and growth media. *Enterobacter cloacae* P99 (P99) (6), *Escherichia coli* R^{+}_{TEM} (TEM), and *Klebsiella aerogenes* 1082E (K1) (4) were used throughout. All three organisms were routinely grown for 20 hr at 37 C in Oxoid nutrient broth and were maintained on nutrient agar slopes.

Growth determinations. Minimal inhibitory concentrations of antibiotics were determined by making serial dilutions in nutrient broth and inoculating with diluted, overnight (20-hr) cultures, to give approximately 5×10^5 organisms per ml. Minimal inhibitory concentrations of combinations of antibiotics were determined in either of two ways. The antibiotics were either serially diluted as above to give equal concentrations in each tube, or an isobologram was constructed by combining a range of concentrations of one antibiotic with a range of concentrations of the other antibiotic. The lowest concentrations which prevented growth were taken as end points. Curves were constructed for each combination tried, and a concave curve indicated synergism (9).

The effects of antibiotics on log-phase growth were determined with a Jouan biophotometer. This has a tungsten unfiltered lamp. None of the compounds interfered with this instrument.

Preparation of partially purified enzymes. The procedure adopted was similar to that described previously (6). For the purposes of improved enzyme production, *E. coli* R^{+}_{TEM} was grown in a casein-yeast medium (G. W. Ross, *personal communication*).

Determination of enzyme activity. Substrate profiles of several penicillins and cephalosporins were determined by use of the iodometric method (7). For

the cephalosporins only, an ultraviolet method was used (6) in which the change in optical density due to hydrolysis of the β-lactam ring was followed on a Unicam SP 800 recording spectrophotometer. Good agreement was obtained between the two methods.

The ultraviolet method was also used for the inhibition studies, with cephaloridine as the sensitive substrate. The standard concentration of cephaloridine in all experiments was 10^{-4} M (41.5 μg/ml); this gave an initial optical density reading of 1.4 at 255 nm, and after complete hydrolysis had occurred the reading fell to 0.6. In each test, 100 units of enzyme, as defined by Pollock and Torriani (8), was added to both the test and the control cuvettes, although the type of enzyme preparations used had no interfering ultraviolet (UV) absorption. All experiments were done at 37 C, and, except where stated, the period used for calculating enzyme activity was 5 min.

The inhibitors studied were all of the substrate analogue type; their structures and their λ_{max} associated with their β-lactam rings (where relevant) are given in Table 1. The compounds were used at various concentrations and, in all tests, were present in both the test and reference cuvettes. Thus, any absorbance, particularly of the cephalosporin analogues, was cancelled out. However, concentrations of more than 5×10^{-3} M interfered with the sensitivity of the instrument and could not be used. The results are quoted as either the fold increase in stability of cephaloridine in the presence of the compounds or else the concentrations of inhibitor required to reduce the rate of cephaloridine hydrolysis to 50% of that of cephaloridine alone.

RESULTS

Substrate profiles of the enzymes. The resistance of a selection of penicillins and cephalosporins to the enzymes was determined by the iodometric method. The rates of hydrolysis were all related to that of cephaloridine, which was given an arbitrary value of 100 (Table 2). The relative sensitivities of benzylpenicillin, ampicillin, and cephaloridine were in agreement with those previously published (4). The other penicillins and cephalosporins were chosen for test because they were considered likely to possess some resistance to β-lactamases.

It was observed that most of the compounds were highly resistant to P99 enzyme, in a pattern predictable from previous observations (5). However, no immediate pattern of structure-susceptibility relationship emerged from this study for K1 or TEM enzymes, either with respect to the acyl group or to the nucleus. All of the acyl groups were derived either from heavily substituted aromatic carboxylic acids or from an alpha-substituted aromatic acetic acid. Compounds which were derived from a simple aromatic acetic acid, as is cephaloridine, tended to be much more susceptible to all of the enzymes.

Inhibition profiles of the enzymes. Inhibition profiles were all measured by the UV method, and the degree of inhibition of enzyme activity, when each compound was incubated at equimolar concentration with cephaloridine and 100 units of enzyme, was calculated from the amount of cephaloridine which had been decomposed in 5 min, compared with a control in which cephaloridine was incubated with the enzyme in the absence of inhibitor. The results are expressed as the fold increase in stability of cephaloridine, which for this purpose has an arbitrarily assigned value of 1 (Table 3).

In addition to having different substrate profiles, the three enzymes also had markedly different inhibition profiles.

TABLE 1. *Names and structures of inhibitors*

Name or code no.[a]	6-Acylamido or 7-acylamido group	3 Substituent	UV λ_{max} for β-lactam ring
Cloxacillin (P)	3-(*o*-Chlorophenyl)-5-methylisoxazole-4-carboxamido-	—	—
291/1 (C)	3-(*o*-Chlorophenyl)-5-methylisoxazole-4-carboxamido-	Acetoxymethyl	265
Methicillin (P)	2,6-Dimethoxybenzamido-	—	—
5/1 (C)	2,6-Dimethoxybenzamido-	Acetoxymethyl	256
Nafcillin (P)	2-Ethoxy-1-naphthamido-	—	—
443/1 (C)	α-Naphthamido-	Acetoxymethyl	270
443/68 (C)	α-Naphthamido-	Methyl	272

[a] P = penicillin; C = cephalosporin.

TABLE 2. *Substrate profiles of the three enzymes*

Substrate	Susceptibilities of analogues to enzyme		
	P99	K1	TEM
Cephaloridine	100	100	100
5/1	0	10	2
Methicillin	0	30	0
291/1	0	5	2
Cloxacillin	0	25	1
443/1	8	10	10
443/68	8	2	2
Nafcillin	0	5	2
Benzylpenicillin	1	170	100
Ampicillin	0	170	100
Cephaloglycin	30	80	40
Cephalexin	10	15	5

It was observed that all of the compounds tested inhibited P99, but were generally much less effective against K1 and TEM. These two enzymes could be easily distinguished from P99 on this basis and, to a lesser extent, from each other. There was no apparent relationship between the extent of the inhibition obtained and the structure of the inhibitor. The results obtained with 443/1 and 443/68 indicated significant differences between P99 and K1 enzymes.

Comparison of inhibitors. The inhibition experiments were repeated (Table 4). A wide concentration range was used to determine the concentrations necessary to achieve 50% inhibition of the enzyme over 5 min, with cephaloridine as substrate at 10^{-4} M. Table 4 shows the relative activities of the inhibitors against each of the enzymes and also clearly shows the differences in susceptibility of the different enzymes. Whereas P99 was susceptible to very low concentrations of all of the inhibitors apart from 443/68, with which much more was needed, the other two enzymes were not inhibited by all of the compounds, and, when inhibition was obtained, higher concentrations were usually needed. Of the compounds tested, nafcillin possessed the broadest activity.

TABLE 3. *Increase in stability of cephaloridine in the presence of equimolar concentration of the inhibitors*

Inhibitor	Fold increase in stability of cephaloridine to enzyme		
	P99	K1	TEM
Cloxacillin	>50	1.0	7.5
291/1	>50	1.0	1.0
Methicillin	>50	1.0	>50.0
5/1	>50	1.0	1.0
Nafcillin	>50	>50.0	>50.0
443/1	>50	2.7	16.2
443/68	13	47.0	>50.0
Nil	1.0	1.0	1.0

TABLE 4. *Concentrations of inhibitors necessary to achieve 50% inhibition of enzyme activity against cephaloridine*

Inhibitor	Enzyme[a]		
	K1	P99	TEM
Cloxacillin	—	3.2×10^{-9}	1.6×10^{-5}
291/1	—	4.0×10^{-9}	—
Methicillin	—	8.0×10^{-9}	3.0×10^{-6}
5/1	—	8.0×10^{-9}	—
Nafcillin	4.1×10^{-6}	3.2×10^{-8}	2.0×10^{-8}
443/1	8.0×10^{-5}	2.6×10^{-7}	5.0×10^{-6}
443/68	2.6×10^{-6}	8.0×10^{-5}	8.0×10^{-7}

[a] Numbers represent molar concentrations; — = no inhibition obtained at 10^{-4} M or lower.

Duration and extent of inhibition. The course of inhibition of the β-lactamases was then followed over 2 hr, which is much longer than the 5 min or so conventionally used in enzyme inhibition studies. Prolonging the time of observation revealed two distinct patterns of behavior. The first (type I) was exemplified by P99, in the presence of various concentrations of cloxacillin. With a constant concentration of the susceptible substrate, cephaloridine (10^{-4} M), in the presence of cloxacillin, complete inhibition was obtained until the cloxacillin added was less than 10^{-8} M. At 3.2×10^{-9} M, the slope of the decrease in optical density was half that of the control and, as the concentration of cloxacillin was decreased further, the slope became steeper until ultimately it became similar to that of the control. Decomposition of the susceptible substrate occurred immediately in the presence of inadequate amounts of cloxacillin, giving a set of curves which were approximately radial, starting at an optical density of 1.4 and zero time (Fig. 1). Complete inhibition occurred with concentrations in excess of 10^{-7} M, and this was permanent over the 2 hr of the experiment. Inhibition of P99 by methicillin and of TEM by cloxacillin produced a similar pattern over a 2-hr period, although the amount of cloxacillin needed to produce 50% inhibition of TEM was 1,000-fold greater than that required for P99.

Inhibition of TEM by methicillin showed a different pattern, type II (Fig. 2). In the first place,

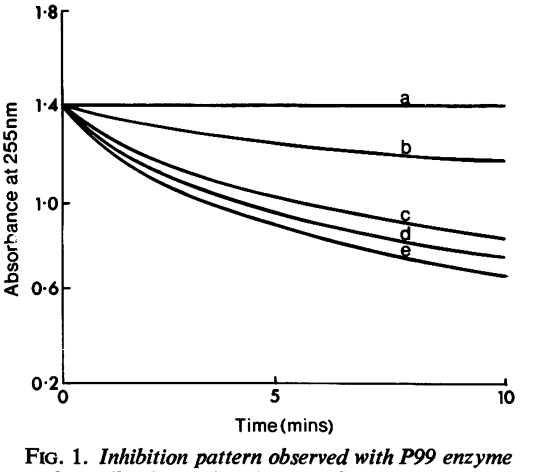

FIG. 1. *Inhibition pattern observed with P99 enzyme and cloxacillin (type I). The cephaloridine concentration was 10^{-4} M. The cloxacillin concentrations were as follows: a, 10^{-7} M; b, 10^{-8} M; c, 3×10^{-9} M; d, 10^{-10} M; e, none.*

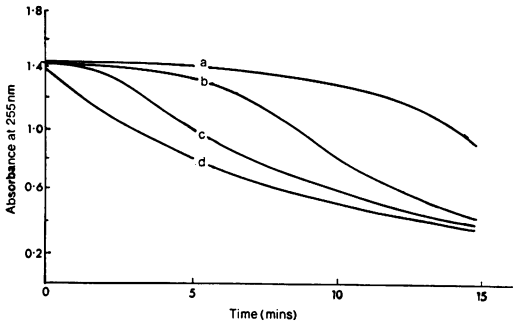

FIG. 2. *Inhibition pattern observed with TEM enzyme and methicillin (type II). The cephaloridine concentration was 10^{-4} M. The methicillin concentrations were as follows: a, 5.0 × 10^{-6} M; b, 2.5 × 10^{-6} M; c, 1.0 × 10^{-6} M; d, none.*

the concentration necessary to achieve 50% inhibition was three orders of magnitude larger than with P99 and cloxacillin. Secondly, it was difficult to decide how to calculate rate of destruction of the susceptible substrate because the degree of inhibition observed varied markedly with time. Initially, the optical density trace showing cephaloridine decomposition was virtually horizontal, indicating negligible hydrolysis. After a period of time which depended upon the concentration of methicillin present, the optical density trace inflected sharply and then showed a rate of fall equivalent to that seen with cephaloridine and TEM when no methicillin was present. The interval from zero time to the point of inflection was so closely related to the concentration that this time could be used as a means of estimating very low concentrations of methicillin. If the abrupt transition in slope of the curve could be interpreted as meaning that more than 90% of the methicillin had decomposed before the cephaloridine was affected, it could also be used to determine the rate of decomposition of methicillin by TEM. It is interesting to observe that the type of inhibition seen with TEM apparently depends on both the nature of the acyl group, as the responses obtained with cloxacillin and methicillin were different, and the nature of the nucleus. The corresponding cephalosporin, 5/1, gave the same type of response with TEM as cloxacillin did with P99. Other instances of this TEM/methicillin type of inhibition occurred with P99 when 443/68 was used as inhibitor and, to a lesser extent, with 443/1. It occurred with K1 and either nafcillin or 443/1 (Table 5).

Further study of K1 showed that the transient inhibition effected by 443/1 was attributable, at least in part, to destruction of 443/1 itself. Figure 3A shows that only when most of the 443/1 had been destroyed was the cephaloridine hydrolyzed. With 443/68, there was an apparently constant inhibition but, in this case, both cephaloridine and 443/68 were hydrolyzed slowly and simultaneously (Fig. 3B).

TABLE 5. *Types of inhibition observed*

Type I		Type II	
Inhibitor	Enzyme	Inhibitor	Enzyme
Cloxacillin	P99	Methicillin	TEM
Methicillin	P99	443/1	K1
Nafcillin	P99	443/1	P99
Nafcillin	TEM	443/68	P99
Cloxacillin	TEM	Nafcillin	K1
5/1	TEM		

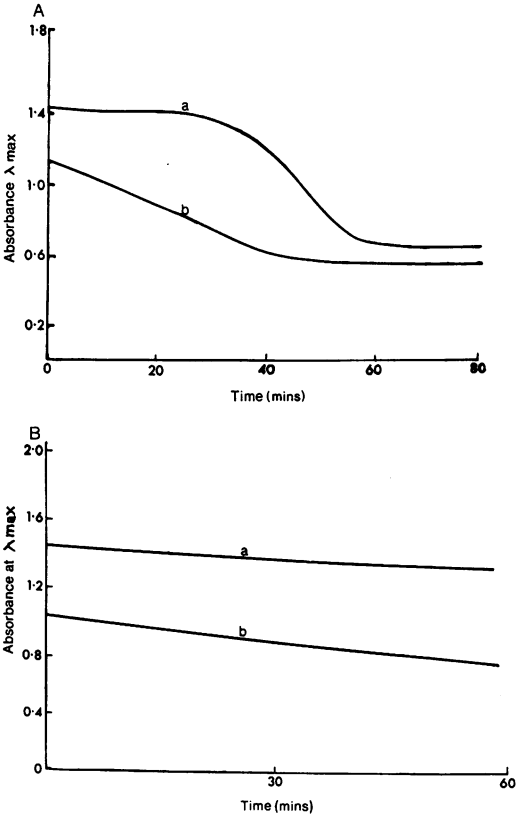

FIG. 3A. *Effect of 443/1 on K1 enzyme. (a) Hydrolysis of cephaloridine (10^{-4} M, 255 nm) in the presence of 443/1 (10^{-4} M). (b) Hydrolysis of 443/1 (10^{-4} M, 270 nm).*

FIG. 3B. *Effect of 443/68 on K1 enzyme. (a) Hydrolysis of cephaloridine (10^{-4} M, 255 nm) in the presence of 443/68 (10^{-4} M). (b) Hydrolysis of 443/68 alone (10^{-4} M, 272 nm).*

Effect of the inhibitors and cephaloridine on bacterial growth. The organisms producing the enzymes were tested for their susceptibility to cephaloridine and the inhibitors in broth dilution tests. With an inoculum of approximately 5×10^5 organisms/ml, and equal amounts of cephaloridine and inhibitor (w/w), the degree of synergism seen was less than what might have been predicted (Table 6). In those cases where it did occur, the broth dilution tests were repeated, with the use of variable proportions of cephaloridine and an inhibitor. From the minimal inhibitory concentrations obtained, isobolograms were constructed which confirmed the synergism, or lack of it, seen in the first test. This effect was further confirmed when the compounds were added to cultures growing in the Jouan biophotometer, where the inoculum was ca. 10^7 organisms/ml.

With *E. cloacae* P99, most inhibitors showed synergy; this is exemplified by the isobologram of cephaloridine and 291/1 shown in Fig. 4 and is confirmed by the effect on the growing culture when followed by optical density measurements (Fig. 5). This had an immediate effect on the optical density of the culture, and lysis rapidly ensued. Nafcillin, methicillin, and particularly 443/1 had a synergistic effect with cephaloridine against *E. coli* TEM, but no synergy was obtained with *K. aerogenes* K1.

DISCUSSION

Three aspects of β-lactamase inhibition have been investigated in the present study. The enzymes chosen were distinct types, based on their substrate profiles and their relative susceptibilities to inhibition by cloxacillin and *p*-chlormercuribenzoate (4).

The inhibitory compounds investigated here were chosen because earlier studies had shown them either to be inhibitors of P99 or to be resistant to either or both TEM and K1. This was taken to mean that they might also be inhibitors of those enzymes, although β-lactamase-resistant cephalosporins and penicillins are not necessarily inhibitory to the enzymes (2, 6).

This study has shown that the three enzyme types can be readily distinguished by their inhibition profiles, based on their initial susceptibilities to the inhibitors studied. In addition, the enzymes also differed in their type of response to an inhibitor and in the duration and degree of inhibition being characteristic of the enzyme/inhibitor system being examined.

The resistance of some of the compounds, e.g., cloxacillin or 5/1, to P99 is probably complete, and they very effectively inhibited this enzyme. Incomplete hydrolysis of the susceptible substrate by P99 in the presence of this kind of compound is probably due to an inadequate concentration of the inhibitor, which would give incomplete saturation of the enzyme.

In this situation, the rate of hydrolysis of the susceptible substrate will decrease with increase in concentration of the inhibitor and can be illustrated by the radial UV traces obtained under these conditions with P99 and cloxacillin (Fig. 1). A different picture was seen with TEM and methicillin, which cannot be explained in this way.

From the comparatively abrupt change in the UV traces which showed that, after a variable period of stability in the presence of methicillin, cephaloridine was hydrolyzed at the same rate as the control, it was inferred that TEM could slowly hydrolyze methicillin, although this substance seemed to be completely stable to TEM when tested by conventional methods of assay. The inflected UV traces obtained with different methicillin concentrations are probably due to methicillin's having a much greater affinity for the enzyme and a much higher resistance to it than does cephaloridine. Thus, no cephaloridine would

TABLE 6. *Minimal inhibitory concentrations of combinations of inhibitors and cephaloridine*

Antibiotic	Minimal inhibitory concn (μg/ml) against		
	E. cloacae P99	*K. aerogenes* K1	*E. coli* TEM
Cephaloridine (CER)	2,000	>1,000	100
Cloxacillin	>1,000	>1,000	>500
CER + cloxacillin	50 + 50	> (1,000 + 1,000)	100 + 100
Methicillin	>1,000	>500	>500
CER + methicillin	100 + 100	> (500 + 500)	25 + 25
443/1	>500	>500	500
CER + 443/1	> (500 + 500)	> (500 + 500)	12.5 + 12.5
Nafcillin	>500	>500	>500
CER + nafcillin	250 + 250	> (500 + 500)	25 + 25

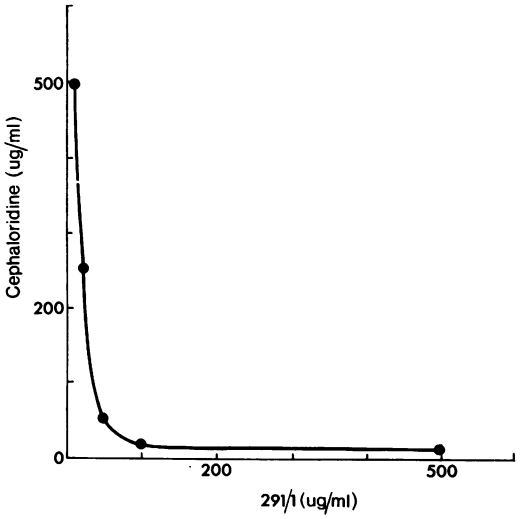

FIG. 4. *Isobologram of cephaloridine and 291/1 against E. cloacae P99.*

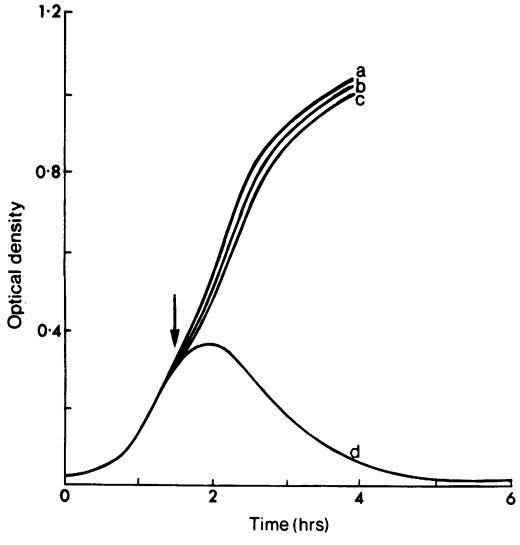

FIG. 5. *Effect of a combination of cephaloridine and 291/1 on the growth of E. cloacae P99. (a) Control, no antibiotic; (b) cephaloridine, 50 μg/ml; (c) 291/1, 50 μg/ml; (d) cephaloridine, 50 μg/ml, +291/1, 50 μg/ml.*

be hydrolyzed until almost all of the methicillin had disappeared from the system (Fig. 2), when it would be hydrolyzed at the normal rate, as if no inhibitor had been present. It is unlikely that this effect would be shown by other methods of estimation because they are less sensitive or are unable to distinguish between the inhibitor and the susceptible substrate after hydrolysis, or both.

These different types of optical density trace for cephaloridine with various enzymes in the presence of various inhibitors probably represent extreme examples of the two types of inhibition which can occur. It is very likely that an entire range could exist between the two, depending on choice of enzyme, inhibitor, and substrate. This makes it very difficult to make meaningful extrapolations from inhibition studies with isolated enzymes to the desired practical applications of inhibiting the growth of organisms producing such enzymes.

The type of substitution of the 7-aminocephalosporanic acid nucleus needed to inhibit major classes of β-lactamase has also been investigated. The inhibitors chosen for this study were found to be the most active among a large number of cephalosporin analogues examined in preliminary studies (*unpublished data*). Previous studies (6) have shown that P99 is more susceptible to cephalosporin inhibitors with a leaving group at position −3 and that, among these, the most active are those with bulky acyl groups, derived from aromatic carboxylic acids. The determining structure is the acyl group, and for P99 it appears immaterial whether it is attached to a penicillin or a cephalosporin nucleus. These observations have been confirmed for P99, but the situation with TEM and K1 is far more complex. These enzymes are much more difficult to inhibit than P99; in addition, the effectiveness of inhibitory compounds was diminished, not increased, by bulky multiplanar substituents on the acyl group. For inhibition of all three enzymes, it was essential to maintain the acyl group derived from an aromatic carboxylic acid, but the most effective inhibitors for K1 and TEM were those where the acyl group was planar. With cephalosporin inhibitors, inhibition of K1 was greater when there was no leaving group at position 3; this is the opposite of the situation observed with P99. In addition to the presence of a suitable acyl group, the nature of the nucleus was also important for inhibition of TEM. In this limited study, penicillins appeared to be better inhibitors of this enzyme than the analogous cephalosporins. Thus, these three types of enzyme can also be differentiated on the basis of the structure/activity relationship of inhibitors of the substrate analogue kind.

It is obvious that very different amounts of any one inhibitor could be required to inhibit different enzymes. P99 and TEM were bracketed together as being inhibited by cloxacillin (4), although they were different in other respects. This study has shown that the two enzymes required very different amounts of cloxacillin to effect the same degree of inhibition. Organisms such as *E. coli* may have any one of several β-lactamases, some of them mediated by R factors or, alternatively,

organisms of quite different type may have the same enzyme, e.g., both *Pseudomonas aeruginosa* and *E. coli* can have an enzyme similar to that mediated by R_{TEM} (12). It becomes increasingly necessary in this field for not only the organism but also the type of enzyme it produces to be clearly defined so that results from different laboratories can be correlated. Definition by reference to an inhibitor needs the degree of inhibition and possibly also the type of inhibition to be stated.

Whereas an analogue might inhibit an isolated β-lactamase, it did not necessarily follow that such a compound would exert a synergistic effect when mixed with a compound such as cephaloridine, which has high antibacterial activity but also considerable susceptibility to β-lactamases. Synergism was observed with some but not all combinations where it might have been expected. The reasons for this are not known, but it is likely that some compounds cannot penetrate bacterial cells rapidly and so do not reach the location of the enzyme in adequate concentration. It can be shown that inhibitors are less effective in preventing hydrolysis of cephaloridine by whole cells than by isolated enzyme. In addition, the compounds are exposed to enzyme action for a much longer period of time in growth tests and a slight susceptibility may have a much more obvious effect under these conditions. It follows, therefore, that the degree of inhibition of the isolated enzyme is not the only criterion by which the likely merits of an inhibitor can be assessed, but the more powerful and prolonged the inhibition of isolated enzyme, the more likelihood there is of achieving a synergistic response in growth tests.

However, in some bacteria at least, the amount of enzyme produced is small, and complete inhibition of the enzyme is not essential for achieving synergy (*unpublished data*); any compound which can significantly inhibit isolated enzyme should be tested against growing bacteria.

No one inhibitor has emerged which could be used successfully against a range of β-lactamase-producing bacteria. Of the compounds tested here, nafcillin possessed the broadest spectrum of inhibition, but, overall, it was disappointing when tested in growing cell systems. Thus, at present, the clinical use of combinations of this nature will necessitate a thorough study of the infecting organisms prior to use (3, 10).

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