SYNERGISM BETWEEN β -LACTAM ANTIBIOTICS: A TEST OF THEORETICAL PREDICTIONS MADE WITH *STAPHYLOCOCCUS AUREUS*

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SYNERGISM between β -lactam antibiotics against Gram-negative bacteria is a well-recognised phenomenon (Hamilton-Miller, Smith and Knox, 1964; Sabath and Abraham, 1964; Sutherland and Batchelor, 1964), but little attention has been paid in this respect to Gram-positive bacteria. This may be because the effect is difficult to demonstrate because of the generally unfavourable kinetic characteristics of β -lactamases from Gram-positive organisms, and there seems to be little practical application for it. Kinetic considerations of a model of this type of synergism produced a means whereby the extent of synergism could be predicted (Hamilton-Miller, 1971*a*), and it is the purpose of the present paper to test practically the validity of the earlier theoretical analysis. In order to do this, it was decided to use a system involving synergism between cephalothin and various penicillins against *Staphylococcus aureus*; use of this system was suggested by the earlier findings (Hamilton-Miller, 1966 and 1970) that cephalothin acted as a competitive inhibitor of staphylococcal β -lactamase under both rigidly controlled (enzyme assay) and physiological (broth culture) conditions.

METHODS AND MATERIALS

The model. The naturally occurring lag phase in a culture of β -lactamase-producing organisms will be increased in the presence of a hydrolysable penicillin the initial concentration of which (S_0) is too high to allow growth to occur. The extent of this increase (t_-) can be expressed by the following equation (Hamilton-Miller, 1971*a*):

$$t_- = [K_m(\ln S_0 - \ln S_+) + S_0 - S_+] / V_{\max}. \quad (1)$$

where the symbols are as follows: K_m = Michaelis constant of penicillin for penicillinase; $V_{\max}$ = maximum rate of hydrolysis of penicillin by penicillinase; and S_+ = concentration of penicillin at which the organisms can start to grow. The parameter S_+ represents the intrinsic resistance of the strain (see Sabath and Abraham; Hamilton-Miller, 1965), and will be referred to hereafter as the maximum non-inhibitory concentration (MNIC).

The lag phase of the culture will be further prolonged if a competitive inhibitor of penicillinase is present, as the rate of penicillin destruction will be thereby decreased. This prolongation of lag phase (Δt) can be expressed (Hamilton-Miller, 1971*a*) by

$$\Delta t = iK_m(\ln S_0 - \ln S_+) / V_{\max} K_i \quad (2)$$

where i = concentration of competitive inhibitor and K_i = dissociation constant of penicillinase-inhibitor complex.

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In the experiments described below, prolongations of lag phase have been determined experimentally and compared with those predicted on the basis of equation 2.

Bacterial strains. *Staph. aureus* no. E3 (Knox, 1960) was used for most of the experiments; it is a multi-resistant, methicillin-sensitive strain of phage-type 77. Two penicillinase-less mutants of this strain, nos. E3T and E3/500, were as described by Knox and Smith (1961). *Staph. aureus* no. 13137 is a methicillin-resistant strain of phage-type 7/47/53/54/75/77, originally isolated by Jevons (1961).

Penicillins and cephalosporins. Benzylpenicillin (sodium salt) was purchased from Glaxo Ltd, Greenford, Middx; phenoxymethylpenicillin, phenethicillin, propicillin, sodium ampicillin, carbenicillin, methicillin, cloxacillin, oxacillin and dicloxacillin were generously given by Beecham Research Laboratories Ltd, Betchworth, Surrey; quinacillin by Boots Pure Drug Co. Ltd, Beeston, Notts; and cephaloridine and cephalothin by Glaxo Research Laboratories, Greenford, Middx.

Design of experiments. Cultural conditions were arranged so that t_{-} was as small as possible, i.e., in cultures growing in the presence of a hydrolysable antibiotic alone ("substrate controls"), the concentration of the latter fell from S_0 to S_+ as nearly as possible within the time covered by the natural lag phase. This was done by choosing a suitable value of S_0 (see below). Further, the magnitude of i was also chosen so that, in controls growing in the presence of inhibitor alone ("inhibitor control"), the lag phase was as nearly as possible of the same duration as that observed in broth alone ("broth control"). Thus, virtually all of any prolongation of lag phase observed in cultures growing in the presence of both hydrolysable antibiotic and inhibitor ("test") was attributable to the factor Δt in the above equation. The method by which Δt was measured (see below) in fact allows for the small contributions to the lag phase due to the presence of S_0 and i .

Culture media. Infusion broth and digest agar, both obtained from Southern Group Laboratories, London, S.E.13, were used throughout these experiments. All incubations were at 37°C.

Growth curve experiments. Staphylococci were grown overnight in broth containing 0.5 µg per ml of methicillin as inducing agent, and 0.4 ml of such cultures (which contained about 2×10^8 cocci per ml) were used to inoculate test-tubes containing 9.6 ml of broth, with and without the addition of various antibiotics, prewarmed to 37°C. The tubes were incubated at 37°C in a waterbath and read every 30 min. on an EEL nephelometer (Evans Electroselenium, Halstead, Essex) for periods up to 8 hr. In preliminary experiments, the maximum concentrations of substrate (S_0) and of inhibitor (i) which caused, separately, little or no increase in the naturally occurring lag phase of the culture were determined. Each experiment involved four tubes: broth control, substrate control (containing the hydrolysable penicillin at a concentration of S_0), inhibitor control (containing the penicillinase inhibitor at a concentration of i), and test (substrate at S_0 and inhibitor at i).

Measurements of Δt . The type of growth curve obtained in these experiments is represented schematically in fig. 1; the differences in lag phases between the curves are exaggerated for the sake of clarity. By the time a culture had reached a nephelometer reading of 10 (corresponding to 1.6×10^7 cocci per ml), growth was occurring at a maximal rate, which was very similar to that observed in the broth control (i.e., BF || CG || DH || EI). It was decided, therefore, to take the lag phase as the time taken for the culture to reach a nephelometer reading of 10; thus on fig. 1, the lag phases for the four cultures are A'B', A'C', A'D' and A'E'. Hence, the effect of the penicillin alone is to increase the lag phase from A'B' to A'C' (net prolongation = B'C'), the effect of the inhibitor alone is to cause a net prolongation of B'D', and the effect of the combination is to cause a net prolongation of B'E'.

If the effect of the combination is merely additive, it would be expected that the lag phase would be increased by the sum of the increases caused by each component separately, i.e., $B'E' = B'D' + B'C'$. If, however, $B'E' > (B'D' + B'C')$, this is an indication that the combination is synergistic, and we can use the value $B'E' - (B'D' + B'C')$ as a measure of the amount of synergism. This last expression, the prolongation of the lag phase due to the

presence of the combination, is clearly equivalent to the parameter Δt , under the experimental conditions described here.

Plate methods. Viable counts were carried out by making serial decimal dilutions in saline of the specimen to be counted, and spreading duplicate or triplicate 0.1-ml samples on nutrient agar. Plates were incubated for 18 hr at 37°C and colonies counted.

MIC determinations were carried out by the plate-dilution method with the following concentrations of penicillins in the medium: 500, 100, 50, 10, 5 and 1 μg per ml. One series of dilutions was made in agar, and the other in agar containing 0.2 μg per ml cephalothin. Each plate was spread with 0.1 ml of a large inoculum (undiluted overnight culture, or two serial ten-fold dilutions of it) of either induced or non-induced organisms, and was read after 9 and after 30 hours' incubation at 37°C.

FIG. 1.—Schematic representation of growth curves obtained in synergism experiments. Curve ABF = broth control; ACG = substrate control; ADH = inhibitor control; and AEI = test.

The MNIC, S_+ , was determined for each penicillin in a similar way, except that a small inoculum (about 60 cocci per plate) of induced organisms was used. Plates were read after 9 and after 30 hours' incubation at 37°C. The end-point in MNIC determinations was taken as the highest concentration which allowed 90 per cent. or more of the initial inoculum to grow.

Simultaneous determination of viable counts and growth curves. For these investigations, cultures were as for growth curves but scaled-up seven-fold (70 ml of culture in all, contained in a 100-ml bottle); 10-ml samples were removed at intervals, and their turbidity was read in the nephelometer and a viable count performed.

RESULTS

Patterns of growth curves. When a penicillinase-producing staphylococcus was grown in the presence of a series of sub-inhibitory concentrations of a hydrolysable penicillin, the family of growth curves obtained by nephelometry gave a characteristic pattern, called here "type A". A characteristic type-A

pattern is shown in fig. 2 (*Staph. aureus* strain E3 growing in the presence of benzylpenicillin). The characteristics of a type-A pattern are as follows: (1) the lag phase is proportional to the initial penicillin concentration (see fig. 3); (2) once growth starts, the rate is very similar to that of the control; and

FIG. 2.—Growth curves of *Staphylococcus aureus*, strain E3, cultured in the presence of different concentrations of benzylpenicillin (concentrations in μg per ml shown against the curves). "Type A" growth pattern (see Text).

(3) the yield of cells obtained eventually (judged by the nephelometer reading of tubes incubated overnight) is virtually the same for each tube in which growth occurred, irrespective of the initial concentration of penicillin. With the penicillinase-producing strains, *S. aureus* E3 and 13137, type-A patterns were obtained with benzylpenicillin, ampicillin, propicillin and phenethicillin.

Fig. 4 shows an example of the other type of growth curve ("type B"). This was observed when *Staph. aureus* strains E3 and 13137 were grown in the presence of non-hydrolysable β -lactam compounds, such as methicillin, quinacillin, the isoxazoly penicillins and cephalothin. Type-B patterns were also obtained when the non-penicillinase-producing strains, *Staph. aureus* nos.

FIG. 3.—The relationship between the prolongation of the lag phase of staphylococcal growth and the initial concentration of two penicillins; ○—○ with benzylpenicillin; ●—● with propicillin.

E3T and E3/500, were grown with either hydrolysable or non-hydrolysable β -lactam antibiotics.

The features of type-B growth curves are as follows: (1) there is little increase in lag phase with increasing concentrations of antibiotic; (2) the rate of growth is slower in the presence of the antibiotic; and (3) the yield of cells is substantially decreased when growth has occurred in the presence of the antibiotic. The following finding will illustrate this last point: overnight cultures of *Staph. aureus* strain E3 usually contained about 5×10^8 cocci per

ml, but cultures grown in the presence of $0.5 \mu\text{g}$ per ml of methicillin (about one-eighth of the MIC) contained from 1.5 to 2×10^8 cocci per ml.

An inspection of the growth curves obtained in a typical synergism experiment (fig. 5) shows that, in the presence of the combination, the lag phase is increased but the rate of growth is unaffected; further, observations on over-

FIG. 4.—Growth curves of *Staphylococcus aureus*, strain E3, cultured in the presence of different concentrations of methicillin (concentrations in μg per ml shown against curves). “Type B” growth pattern (see Text).

night cultures indicated that all tubes contained a comparable number of organisms. Thus, this pattern of growth curves conforms to type A.

Mean growth parameters were calculated from the growth curves of “broth control” tubes of the two *Staph. aureus* strains E3 and 13137. They were virtually identical for each strain ($P > 0.5$) the mean doubling time being 0.70 ± 0.03 hr (specific growth rate = 0.99 hr^{-1}) and the mean duration of the lag phase 1.27 ± 0.08 hr.

The correlation between calculated and observed prolongations in lag phase. Values of Δt were calculated from the formula given above, for systems involving 100 μg per ml of benzylpenicillin, phenoxymethylpenicillin, phenethicillin,

FIG. 5.—Staphylococcal growth curves showing synergism between ampicillin and cephalothin: (●) control; (○) cephalothin 0.1 μg per ml; (▲) ampicillin 100 μg per ml; and (□) cephalothin 0.1 μg per ml with ampicillin 100 μg per ml.

propicillin, and ampicillin, each in combination with 0.2 μg per ml of cephalothin, acting on *Staph. aureus* strain E3. Figures for K_m and $V_{\max}$ for each substrate, taken from Hamilton-Miller (1970), are given in table I, together with values of MNIC for each antibiotic, as determined by the plate assay;

K_t was taken as $0.056 \mu\text{M}$ (Hamilton-Miller, 1970). These values of Δt , together with those observed under experimental conditions, are given in table II.

TABLE I
Kinetic parameters of various substrates for penicillinase from Staphylococcus aureus, strain E3*

Penicillin	K_m (μM)	$V_{\max}$ (n moles per 1.6×10^7 cocci per ml per min.)	S_+ (μM)	S_0 (μM)
Benzylpenicillin	12.3	10.16	2.82	282
Phenoxymethylpenicillin	16.8	14.89	0.26	258
Phenethicillin	48.3	9.37	1.24	248
Propicillin	68.8	7.29	2.4	240
Ampicillin	130	25.7	2.7	270

* Values of K_m and $V_{\max}$ taken from Hamilton-Miller, 1970. S_+ is the maximum non-inhibitory concentration, S_0 is the initial penicillin concentration (see Text for details). The initial inoculum size was 1.6×10^7 cocci per ml.

It can be seen from table II that there is excellent agreement between the calculated and observed values of Δt for benzylpenicillin and phenoxymethylpenicillin, and agreement to within 10 per cent. in the case of phenethicillin.

TABLE II
Increases in lag phase (Δt) due to the presence of a competitive inhibitor (cephalothin or cephaloridine)

Penicillin	Values* of Δt (hr) in presence of		
	cephalothin ($0.2 \mu\text{g}$ per ml)		cephaloridine ($0.4 \mu\text{g}$ per ml)
	calculated	observed	observed
Benzylpenicillin	0.83	0.85	0.9
Phenoxymethylpenicillin	0.93	0.9	0.6
Phenethicillin	4.1	3.7	1
Propicillin	7.3	5.5	1.05
Ampicillin	3.5	2.9	1.1
Carbenicillin	...	3.8	...

* Values of Δt were calculated from equation 2 (see *Methods*), and observed in growth-curve experiments with *Staphylococcus aureus*, strain E3.

For ampicillin and propicillin, however, there is a greater discrepancy: 17 and 25 per cent. respectively. However, the absolute penicillinase activity of induced staphylococcal cultures is subject to rather wide and unpredictable day-to-day fluctuations (Dyke, Jevons and Parker, 1966), and thus $V_{\max}$ cannot be quantified as precisely as desirable. In calculating values of Δt , we used the mean value for absolute activity obtained in a previous study (Hamilton-Miller, 1970),

in which the coefficient of variation was 26 per cent., because it was not practicable to measure absolute activity for each inoculum used in these experiments. With this in mind, it can be seen that the observed correlation between observed and calculated values is as good as can be expected.

Substantial increases in lag phase were also observed when combinations were used of cephalothin (0.5 μ g per ml) with 200 μ g per ml of benzylpenicillin (Δt observed = 3.8 hr), phenethicillin (4.3 hr), propicillin (6.2 hr) and ampicillin (4.7 hr), acting against the methicillin-resistant strain *Staph. aureus* no. 13137. Values of Δt cannot be calculated in the case of this strain, because it possesses both endo- and exo-penicillinases, which have different kinetic properties (Hamilton-Miller, 1970).

TABLE III

Effect of cephalothin (0.2 μ g per ml) on the MIC of ampicillin, propicillin and carbenicillin for Staphylococcus aureus, strain E3

Penicillin	Inoculum size ($\times 10^5$)	Minimum inhibitory concentration (μ g per ml)			
		at 9 hr		at 30 hr	
		control	with cephalothin (0.2 μ g per ml)	control	with cephalothin (0.2 μ g per ml)
Ampicillin	550	>1000	>1000	>1000	>1000
	55	500	50	1000	500
	5.5	100	10	100	10
Propicillin	530	>500	100	>500	>500
	53	500	5	>500	50
	5.3	10	<1	10	5
Carbenicillin	530	>500	50	>500	>500
	53	50	5	500	50
	5.3	10	5	10	10

Not surprisingly, no synergism was observed between penicillins and cephalothin against the non-penicillinase-producing strains *Staph. aureus* nos. E3T and E3/500.

Of the β -lactam compounds tested, only cephalothin and cephaloridine were capable of acting as the enzyme-inhibiting member of synergistic pairs; this result was not unexpected in view of the low affinities of the penicillinase-stable penicillins for the staphylococcal enzyme (Hamilton-Miller, 1970). Cephaloridine, even when present at 0.4 μ g per ml was a much less effective enzyme inhibitor than was cephalothin (table II): the reason for this is outlined in the *Discussion*.

The effect of cephalothin on the MIC of penicillins for Staph. aureus strain E3. In the presence of 0.2 μ g per ml cephalothin there was a marked reduction in the MIC of ampicillin, propicillin and carbenicillin against large inocula (5×10^5 to 5×10^7 organisms) of induced *Staph. aureus* strain E3, when the plates were read after 9 hours' incubation (see table III). This effect was

reduced, but still present, after 30 hours' incubation. Propicillin was most affected by the presence of cephalothin, followed by carbenicillin, then ampicillin. It is significant that this is the same order as was observed in the experiments concerned with prolongation of lag phase (table II).

The original induction state of the organisms was found to be irrelevant, because, in a subsequent experiment with large inocula of uninduced cocci, virtually identical results to those shown in table III were obtained. This finding extends the observation of Novick (1962) that small inocula of induced and non-induced staphylococci have the same sensitivity to any one hydrolysable penicillin.

FIG. 6.—The changes in viable counts during the lag phase of a *Staphylococcus aureus* culture incubated in the presence of propicillin (100 μg per ml) and cephalothin (0.2 μg per ml); ●—● viable count; ○—○ nephelometer reading.

The correlation between changes in nephelometric reading and in viable count. These changes were investigated in cultures of *Staph. aureus* strain E3 in which lag phases had been prolonged by the presence of combinations of propicillin with cephalothin, ampicillin with cephalothin, and carbenicillin with cephalothin (penicillin at 100 μg per ml, cephalothin at 0.2 μg per ml on each occasion). Fig. 6 shows the results obtained with the first of these combinations; a very similar picture was observed with the other two. It can be seen that there was a bactericidal effect in the early part of the lag phase, during which viability was reduced to minimum values of 23 per cent., 43 per cent., and 8.3 per cent. of that of the initial figure by each of the three combinations respectively. These figures are the means of two separate experiments with each combination. Maximum bactericidal effect was found either 2 or 3 hr after inoculation. This bactericidal phase was followed by a period of net growth; when the viable count exceeded the initial inoculum size there was an increase

in nephelometer reading, and this represented the end of the lag phase. At this point the experiments were discontinued.

In control experiments (plain broth, penicillin only 100 μ g per ml, cephalothin only 0.2 μ g per ml) there was no bactericidal phase, and viable count and nephelometer reading followed approximately parallel paths.

DISCUSSION

The type of synergism investigated here involves a substrate and a competitive inhibitor of penicillinase acting together, the latter exerting a "penicillin-sparing effect" (Kasik *et al.*, 1967). The first aim of the experiments reported in this paper was to establish whether this type of synergism could be demonstrated, as it has been against many other penicillinase-producing bacteria, against *Staph. aureus*.

It is a curious coincidence that semi-synthetic penicillins such as methicillin and the isoxazolyl penicillins, which were designed to be resistant to staphylococcal penicillinase by virtue of a low affinity for the enzyme, a low dissociation constant for the enzyme-substrate complex, or both (Gourevitch *et al.*, 1963), should also possess a great stability to, and a high affinity for, the diverse β -lactamases produced by many other bacterial species. The cephalosporin analogues of such penicillins do not necessarily show these properties (Kasik and Peacham, 1968; O'Callaghan *et al.*, 1969).

It has been noticed previously that some penicillinase-stable penicillins seem to inactivate staphylococcal penicillinase under certain conditions. This phenomenon was observed for methicillin and oxacillin by Gourevitch *et al.* (1962), for cloxacillin by Smith *et al.* (1962) and for quinacillin by Dyke (1967), and was further investigated by Batchelor *et al.* (1963) and Dyke. The inhibition is not competitive, and rather high concentrations (10 mM or above) of the inhibitor are needed for the effect to be demonstrable. Gourevitch *et al.* (1964) were unable to demonstrate synergism due to this phenomenon in cultures of *Staph. aureus*.

There are two previous examples in the literature of synergism between β -lactam antibiotics against penicillinase-producing staphylococci: that between cephalosporin C and benzylpenicillin (Crawford and Abraham, 1957), and between D-phenethicillin and its diastereoisomer (Kraushaar and Schmid, 1961). In neither of these two reports can the synergism be attributed to the effect of competitive inhibition. Thus, the experiments described here are the first in which a definite penicillin-sparing effect has been shown in experiments in which synergistic pairs of β -lactam antibiotics acted against staphylococci.

For the other types of bacteria for which penicillin-sparing synergism can be shown, there has been a logical progression following the discovery of competitive inhibition by isolated enzyme systems (Hamilton-Miller and Smith, 1964; Sutherland and Batchelor, 1964; Sabath *et al.*, 1965; Kasik and Peacham, 1968). This progression has extended via the demonstration of antibacterial synergism (Hamilton-Miller *et al.*, 1964; Sabath and Abraham, 1964; Farrar *et al.*, 1967; Kasik *et al.*, 1967; O'Callaghan *et al.*, 1969;

Hamilton-Miller, 1971*b*) and the repetition of the phenomenon in in-vivo protection experiments (Kasik *et al.*, 1966; O'Callaghan and Muggleton, 1967) to clinical application in "combined therapy" (Shirley and Moore, 1965; Sabath *et al.*, 1967; Maddocks and May, 1969). For staphylococci, however, it seems very unlikely that the above progression will pass beyond the second stage, which, with the results described in the present paper, it has now reached. The vast majority of staphylococcal infections are caused by strains that are susceptible to a single antibiotic, and methicillin-resistant strains, still rare in this country but apparently on the increase (Parker and Hewitt, 1970), owe their resistance to an intrinsic rather than a β -lactamase-type mechanism (see review by Smith *et al.*, 1969), and thus would not be expected to be susceptible to a penicillin-sparing type of synergism.

The second object of these experiments was to investigate the application of equation (2), derived from a consideration of Michaelis-Menten kinetics, to the model system used for the demonstration of synergism. Equation (2) was derived from ideal theoretical concepts, and will be obeyed in practice only if (i) penicillinase in the intact growing staphylococcal cell follows ideal kinetics, and (ii) the assumptions made in the derivation of the equation (Hamilton-Miller, 1971*a*) are correct. The measure of agreement noted between the calculated and observed values of Δt (table III), bearing in mind the variations inherent in $V_{\max}$. (discussed in the *Results* section), allow the conclusion to be drawn that equation (2) can be used to predict the behaviour of cultures under certain conditions. Thus, we can further conclude that both conditions (i) and (ii) above are satisfactorily fulfilled. It is of interest that staphylococcal penicillinase, a particulate enzyme (see Richmond, 1963; strain E3 produces virtually no exoenzyme under these cultural conditions), obeys Michaelis-Menten kinetics under the conditions that obtain in the growing bacterium.

Staphylococcal cells were chosen to test the application of equation (2) because they have no permeability barriers to the β -lactam drugs (Hamilton-Miller, 1970); this form of the equation presupposes this fact, the derived expression in the presence of such barriers being much more complex (Hamilton-Miller, 1971*a*). The main disadvantage in the use of staphylococci lay in the inducibility of penicillinase in these organisms; induced inocula were used to minimise induction during the lag phase. As can be seen from table III, when cephaloridine was used as the competitive inhibitor, the observed and calculated values of Δt were very divergent; this can be explained by the fact that cephaloridine is noticeably more labile towards staphylococcal penicillinase than is cephalothin (Hamilton-Miller and Ramsay, 1967), and the derivation of equation (2) requires that i shall be constant. Hence, in the presence of an unstable competitive inhibitor, equation (2) does not apply.

The agreement found between the calculated and observed values of Δt in the presence of cephalothin also vindicates the somewhat arbitrary definition made here for the duration of the lag phase, namely the time taken for the culture to reach a turbidity corresponding to 10 nephelometer units.

The observation that two types of growth curve, A and B, were consistently

obtained, depending upon whether the drug in whose presence the bacteria were being cultured was destroyed or not, raises the question of the potential use of growth curves to determine whether a certain microbial strain destroys an antibiotic. A rapid answer to such a question would be of great preliminary assistance in investigations such as the mode of resistance of R-factor-containing bacteria (Okamoto and Suzuki, 1965). Further studies on drugs other than the β -lactam group are clearly indicated.

SUMMARY

The addition of sub-inhibitory concentrations of cephalothin, a competitive inhibitor of staphylococcal β -lactamase, to a culture of a penicillinase-producing *Staphylococcus aureus* containing a sub-inhibitory concentration of a hydrolysable penicillin (benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin, ampicillin or carbenicillin) was found to bring about an increase in the lag phase of the culture (Δt), during which there was a bactericidal effect. The extent of Δt depends upon certain enzymological parameters, of which a separate theoretical consideration has already been made (Hamilton-Miller, 1971a); the observed values of Δt were found to be in line with those predicted by means of the equation derived previously. Synergism between cephalothin and penicillins was also demonstrated by MIC determinations. The system used here to analyse synergism may have applications in other circumstances where antimicrobial agents are destroyed by resistant organisms.

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