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Interactions between β-lactam and aminoglycoside antibiotics

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Summary

Incubation of an aminoglycoside antibiotic (gentamicin or tobramycin) with a penicillin (carbenicillin or ticarcillin) at 20 or 37°C, but not at 5°C, resulted in considerable loss of antibacterial activity, possibly as a result of complexing, of the former as determined by plate assay against an aminoglycoside-sensitive, penicillin-resistant strain of *Klebsiella edwardsii*. The extent of this loss of aminoglycoside activity depended on the relative ratio of aminoglycoside:penicillin. Pretreatment of a penicillin with a broad-spectrum β-lactamase, or addition of the β-lactamase at the time of mixing an aminoglycoside with a penicillin, prevented this loss of activity. Reversal of preformed complex formation could be achieved to some extent by subsequent incubation with this β-lactamase. Incubation of other penicillins with gentamicin also resulted in loss of antibacterial activity of the latter antibiotic.

Introduction

Members of the aminoglycoside-aminocyclitol (AGAC) and β-lactam groups of antibiotics are important agents in the treatment of many bacterial infections. In some specific instances, a combination of an AGAC antibiotic, e.g. gentamicin or tobramycin with a β-lactam antibiotic such as carbenicillin or ticarcillin, has been employed clinically (McLaughlin and Reeves, 1971; Noone and Pattison, 1971) with the object of producing a synergistic response. However, interaction between

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gentamicin and carbenicillin has frequently been noted (Blair et al., 1982; Chow et al., 1982; Konishi et al., 1983; Lau et al., 1983). Such an interaction could limit the clinical usefulness of this type of combination. An inactivation interaction between AGAC and penicillins has been reported to occur under in vitro experimental conditions (McLaughlin and Reeves, 1971; Noone and Pattison, 1971; Riff and Jackson, 1972; Waitz et al., 1972; Pickering and Gearhart, 1979; White et al., 1979; Pieper et al., 1980; Edwards and Schentag, 1981; Farchione, 1981; Henderson et al., 1981; Pickering and Rutherford, 1981; O'Bey et al., 1982; Ebert and Clementi, 1983; Glew and Pavuk, 1983), and it is generally accepted that this inactivation occurs to a significant extent in patients with impaired renal function (Davies et al., 1975; Ervin et al., 1976; Blair et al., 1982; Chow et al., 1982; Thompson et al., 1982; Konishi et al., 1983; Lau et al., 1983). The monitoring of serum concentrations is an important part of AGAC therapy. It has been suggested that β-lactamase should be added to serum samples to prevent AGAC inactivation occurring prior to assay (Edwards and Schentag, 1981; Ebert and Clementi, 1983). However, in an earlier report it was noted that incubation of inactivated AGAC with β-lactamase may restore some of the lost activity (Noone and Pattison, 1971). Thus the inclusion of β-lactamase in serum samples may result in an overestimation of active AGAC. This would result in errors in subsequent dosage regimen adjustments since these are made assuming that the measured concentration is that of active AGAC in the patient.

The nature of this possible interaction has been studied in this report under various experimental conditions.

Materials and Methods

Antibiotics

Carbenicillin (Carb), ampicillin (Amp), cloxacillin (Clox), ticarcillin (Tic) and gentamicin sulphate (Gm) were purchased from Sigma Chemicals.

Tobramycin (Tob) as Tobramycin Sulphate Injection (Nebcin, 80 mg/ml) was obtained from Lilly Labs, and azlocillin (Az) and mezlocillin (Mez) from Bayer Leverkussen.

β-Lactamase preparation

This consisted of a broad-spectrum β-lactamase from Genzyme, Maidstone, Kent, U.K. The potency was 1000 units of activity per vial. One unit of activity is defined as that concentration hydrolyzing one μmole of penicillin per minute at 25°C. β-Lactamase solutions were prepared of potency 200 units/ml.

Horse serum

Horse serum (code SR 35) was purchased from Oxoid, London, U.K.

Test organisms

These consisted of *Bacillus pumilus* NCTC 8241, *Klebsiella pneumoniae* NCTC 10246, *K. edwardsii* NCTC 10896, *Pseudomonas aeruginosa* NCTC 10662 and *Ps.*

aeruginosa NCTC 10701. Disc sensitivity to carbenicillin (100 μg disc) and to gentamicin sulphate (10 μg disc) was tested in IST agar (Oxoid). Sensitivity was also investigated by removing cups from IST agar seeded with the test organism; the cups were filled with a penicillin (up to 500 μg/ml) or an AGAC antibiotic (10 μg/ml) and after pre-diffusion at 20°C, plates were incubated at 37°C for 24 h and inhibition zone diameters measured.

Assay techniques

Both large- and small-plate assay techniques were carried out. In the former, an overnight 37°C broth culture of *K. edwardsii* 10896 was diluted 1 : 2 with broth; 2.5 ml of the diluted culture was added to 250 ml of molten IST agar held at about 45°C. The mixture was poured into a 25 × 25 cm sterile glass plate. After the agar had set and hardened, cups were cut out (6 rows each with 6 cups) and filled randomly with 0.15 ml of samples. Plates were kept for 1 h at 20°C for pre-diffusion to occur, and zone sizes measured after incubation for 24 h at 37°C. The same general procedure was carried out in the small-plate assay, except that 0.2 ml of a 1 : 2 dilution of the *K. edwardsii* culture was inoculated into 20 ml of IST agar. Standard curves were plotted of inhibition zone diameter against log antibiotic concentration.

AGAC assay in presence of penicillin

All samples that contained active penicillin were pretreated with 0.04 ml β-lactamase (200 units/ml) for 1 h at 37°C prior to determining the residual AGAC concentration by the method described above.

Complex formation

A microbiologically inactive complex could result as a consequence of interaction between a penicillin and an AGAC antibiotic. This would be manifest as a reduction in the inhibitory zone diameter of an AGAC–penicillin mixture in comparison to a control AGAC solution. To assay the rate of complex formation, and on the assumption that the interaction requires the presence of an intact β-lactam ring (Waitz et al., 1972; Russo, 1980), then an AGAC–penicillin interaction must be quenched at the time of sampling. This was carried out by incubating a mixture of AGAC and penicillin at the required temperature (5, 20, 37°C) for the required period of time, removing an aliquot, adding sufficient β-lactamase to quench the remaining β-lactam and determining the residual concentration of AGAC antibiotic by plate assay.

Prevention of complex formation

The prevention of a complex between AGAC antibiotics and penicillins was investigated by mixing solutions of an AGAC antibiotic with β-lactamase-treated β-lactam antibiotic and recording the effect on *K. edwardsii* by the agar-plate diffusion assay described above.

Results

Sensitivity of test organisms

All strains tested, especially *B. pumilis* 8241 and *K. edwardsii* 10986, were sensitive to gentamicin sulphate. The most sensitive organism to carbenicillen was *Ps. aeruginosa* 10701, with *K. edwardsii* (a potent β-lactamase producer) being the most resistant. The last organism was suitable for assaying gentamicin sulphate or tobramycin over the range 3–15 μg/ml, in the presence or absence of carbenicillin or ticarcillin (see Fig. 1).

Effect of β-lactamase

Initial experiments were carried out to determine the effects of β-lactamase on AGAC antibiotics, and on a freshly prepared mixture of an AGAC with a penicillin. The β-lactamase chosen was shown in preliminary studies to be effective in hydrolyzing the penicillins used throughout this investigation. As can be seen from Tables 1 and 2, β-lactamase had no effect on the activity of gentamicin or tobramycin and in its presence no reduction in activity was suffered by gentamicin or tobramycin when incubated with carbenicillin or ticarcillin.

Complex formation

The following mixtures were prepared: (a) AGAC (10 μg/ml) + carbenicillin (500 μg/ml) in sterile distilled water; (b) as for (a), but the solvent was a phosphate-citrate buffer, pH 6.5, the pH of maximum stability of carbenicillin; (c) AGAC (10 μg/ml) + pretreated carbenicillin (500 μg/ml), i.e. carbenicillin pre-incubated for 2

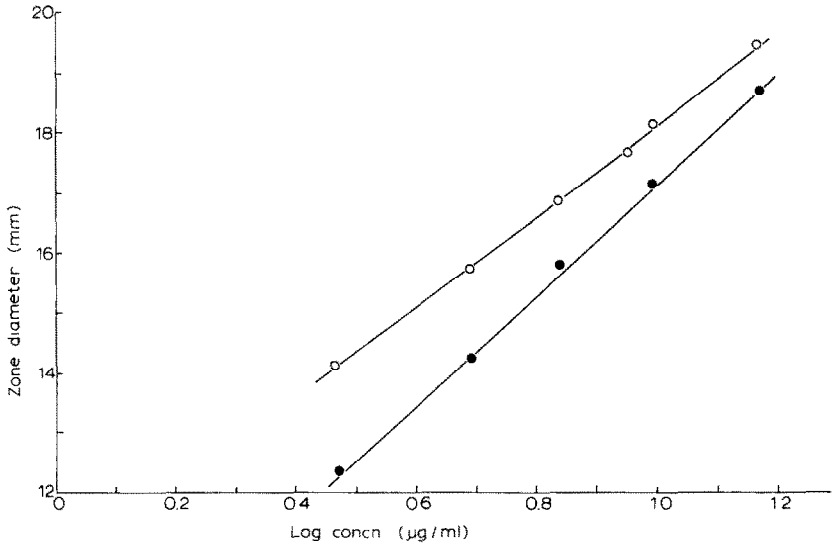

Fig. 1. Standard curves for the assay of gentamicin (●——●) and tobramycin (○——○).

TABLE 1
EFFECT OF β-LACTAMASE ON THE INACTIVATION OF GENTAMICIN (Gm) BY TICARCILLIN (Tic) OR CARBENICILLIN (Carb)

Mixture	Gm conc. (μg/ml)	Penicillin conc. (μg/ml)	Average zone diameter (mm)	Gm inactivation
Gm	5	0	14.3 ±0.3	– [a]
	10	0	16.9 ±0.2	–
Gm + β-lactamase [b]	5	0	14.4 ±0.3	–
	10	0	16.85 ±0.2	–
Gm + β-lactamase + Tic	5	500	14.35 ±0.2	–
	10	500	17.0 ±0	–
Gm + β-lactamase + Carb	5	500	14.4 ±0.2	–
	10	500	16.9 ±0.2	–

[a] No inactivation of Gm.
[b] β-Lactamase conc. = 8 units/ml.

h at 37°C with β-lactamase. Aliquots of each were placed in wells in IST agar seeded with *K. edwardsii* 10896, the plates allowed to remain at 20°C for 1 h and then incubated at 37°C for 18 h. Results are presented in Table 3, from which it may be inferred that an intact β-lactam ring is necessary for the alleged complexation to take place. A complete loss of activity of gentamicin and tobramycin occurred.

Assay of AGAC–penicillin interaction

Solutions in sterile water were made of the following: gentamicin (10 μg/ml) or tobramycin (10 μg/ml) with carbenicillin (500 μg/ml) or ticarcillin (500 μg/ml). The solutions were stored at 37°C, room temperature (20°C) or 5°C for 24 or 48 h

TABLE 2
EFFECT OF β-LACTAMASE ON THE INACTIVATION OF TOBRAMYCIN (Tob) BY TICARCILLIN (Tic) OR CARBENICILLIN (Carb)

Mixture	Tob conc. (μg/ml)	Penicillin conc. (μg/ml)	Average zone diameter (mm)	Tob inactivation
Tob	5	0	16.1 ±0.3	– [a]
	10	0	18.1 ±0.2	–
Tob + β-lactamase [b]	5	0	15.95 ±0.3	–
	10	0	18.1 ±0.2	–
Tob + β-lactamase [b] + Tic	5	500	15.95 ±0.2	–
	10	500	18.2 ±0.3	–
Tob + β-lactamase [b] + Carb	5	500	16.0 ±0.2	–
	10	500	18.0 ±0	–

[a] No inactivation of Tob.
[b] β-lactamase conc. = 8 units/ml.

TABLE 3
INACTIVATION OF GENTAMICIN (Gm) OR TOBRAMYCIN (Tob) BY CARBENICILLIN (Carb) UNDER DIFFERENT EXPERIMENTAL CONDITIONS

AGAC antibiotic	Average zone diameter (mm) ± S.D.			
	AGAC [a]	AGAC + Carb (1 : 50, water)	AGAC + Carb (1 : 50, buffer)	AGAC + Pretreated [b] Carb (1 : 50)
Gm	16.75	Small zone, no clear edge	No zone	16.66
Tob	18.65	11.0	No zone	18.45

[a] AGAC used at 10 μg/ml in all experiments ± penicillin.
[b] Pretreatment of Carb with β-lactamase: slight inactivation of Gm or Tob.

TABLE 4
EFFECT OF β-LACTAMASE ON AGAC–PENICILLIN MIXTURES WHICH HAD BEEN STORED AT 5°C FOR 48 h

Contact (h) with β-lactamase	Average zone diameter (mm) ± S.D.					
	Gm	Tob	Gm + Carb	Gent + Tic	Tob + Carb	Tob + Tic
0	16.25 ± 0.3	18.13 ± 0.3	16.25 ± 0.3	16.38 ± 0.2	18.0 ± 0	18.0 ± 0
2			16.25 ± 0.3	16.25 ± 0.3	18.25 ± 0.2	18.25 ± 0.2
4			16.25 ± 0.3	16.25 ± 0.3	18.0 ± 0	18.25 ± 0.3

and sufficient β-lactamase added to inactivate the penicillin. Samples were then removed at intervals and the inhibitory effects against *K. edwardsii* determined by the agar diffusion method used previously.

Storage of an AGAC–penicillin mixture at 5°C for 48 h produced no microbiological interaction between the two components (Table 4). In contrast, complex formation occurred when a mixture was stored at 20°C for 24 h (Table 5) or at 37°C for 24 h (Fig. 2) or 48 h (not shown). It was observed repeatedly that incubation of the complex with β-lactamase for between about 1.5 and 4 h increased the inhibition zones against *K. edwardsii* (Table 5, Fig. 2). This was also found after incubation of a gentamicin–carbenicillin mixture previously stored at 37°C for 24 h, with β-

TABLE 5
EFFECT OF β-LACTAMASE ON AGAC–PENICILLIN MIXTURES STORED FOR 24 h AT 20°C

Combination [a] (ratio 1 : 50)	% original AGAC activity after incubation with β-lactamase for (h)									
	0	0.5	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5
Gm:Carb	44.2	50.1	47.3	53.7	64.6	63.1	56.9	44.2	47.3	47.3
Gm:Tic	51.3	50.1	50.1	40.3	44.2	66.1	68.4	56.9	56.9	41.7
Tob:Carb	35.5	36.7	38.5	38.5	43.2	47.9	55.6	36.7	31.9	34.3
Tob:Tic	49.5	44.6	47.8	47.8	53.1	74.1	74.1	51.3	53.1	47.8

[a] AGACs at 10 μg/ml, penicillins at 500 μg/ml.

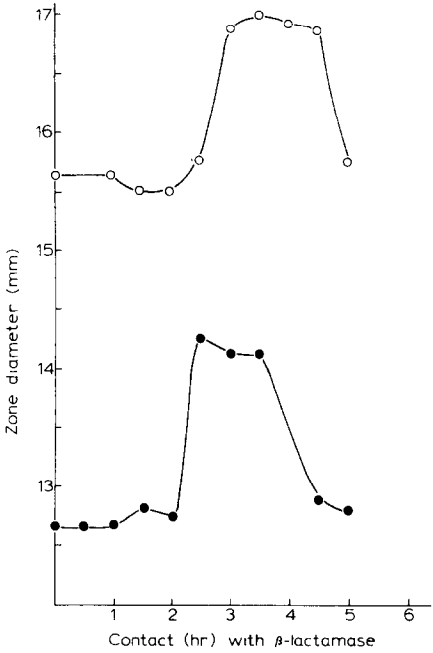

Fig. 2. Storage of AGAC–penicillin mixtures at 37°C followed by incubation of the complex with β-lactamase, and measurement of inhibition zones against an AGAC-sensitive penicillin-resistant strain of *K. edwardsii*. ●———●, gentamicin activity; ○———○, tobramycin activity.

lactamase: zone sizes after contact with enzyme for 0, 1 and 1.5 h were zero, after contact for 2, 2.5 and 3 h zones were 12 mm in each case (corresponding to 30% remaining gentamicin activity) and thereafter (3.5–5 h) no inhibition zones were

TABLE 6
EFFECT OF RELATIVE GENTAMICIN (Gm) AND CARBENICILLIN (Carb) CONCENTRATIONS ON Gm INACTIVATION

Concn. ratio [a] Gm:Carb	Average zone diameter (mm)±S.D.	% Gm remaining
1:0 [b]	17.0 ±0	100
1:10	16.3 ±0.2	85
1:20	15.7 ±0.2	71
1:30	15.5 ±0	67
1:40	14.5 ±0	53
1:50	14.25±0.2	50
1.50 (inactivated Carb) [c]	17.1 ±0.2	100

[a] Gm used at 10 μg/ml.
[b] Carb absent.
[c] Carbenicillin inactivated by β-lactamase.

TABLE 7
EFFECT OF VARIOUS PENICILLINS ON INACTIVATION OF GENTAMICIN (Gm) IN WATER OR SERUM

Gm + penicillin (1 : 50)	Water	Serum
	Av. zone diameter (mm) ± S.D.	Av. zone diameter (mm) ± S.D.
Amp	16.25 ± 0.3	14.0 ± 0
Amox	16.25 ± 0.3	16.86 ± 0.3
Az	16.75 ± 0.2	16.75 ± 0.3
Carb	14.25 ± 0.3	13.25 ± 0
Clox	16.0 ± 0	13.25 ± 0.2
Mez	16.75 ± 0.2	16.75 ± 0.3
Pen	14.0 ± 0.3	15.0 ± 0
Tic	14.25 ± 0.3	14.95 ± 0.2
None (Gm only)	16.95 ± 0.3	17.25 ± 0.2

observed. The observations have been presented as percentage of remaining AGAC activity.

Effect of carbenicillin concentration

All previous experiments employed a concentration ratio of AGAC : penicillin of 1 : 50. The effects of different concentration ratios were next studied, i.e. 1 : 40, 1 : 30, 1 : 20 and 1 : 10 and compared with the earlier findings. Solutions containing

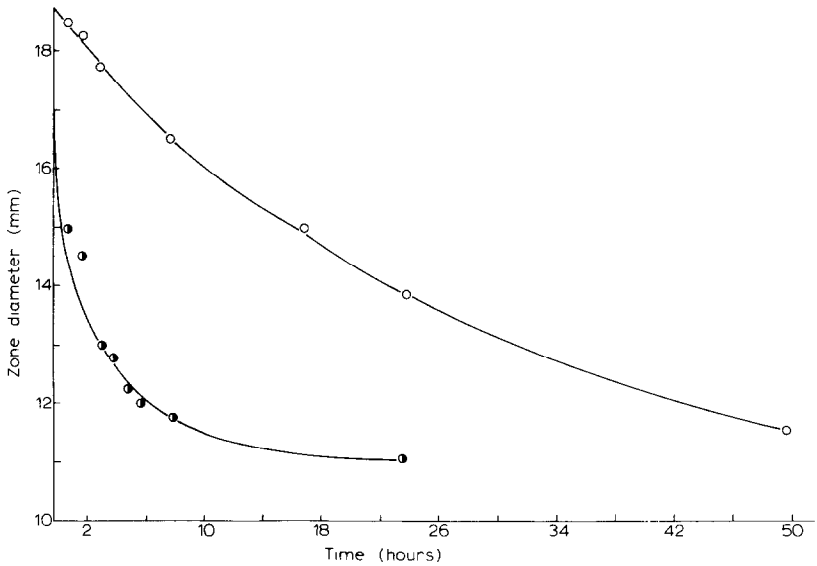

Fig. 3. Complex formation of an AGAC (gentamicin) and penicillin in water and serum. Gentamicin concentration in water (◑ ——— ◑) and in serum (○ ——— ○) was determined against an AGAC-sensitive, penicillin-resistant strain of *K. edwardsii*.

gentamicin (10 μg/ml) and the appropriate concentration of carbenicillin were stored for 24 h at 20°C and the gentamicin activity against *K. edwardsii* determined as before. The results (Table 6) demonstrate that gentamicin activity is lost progressively as the carbenicillin concentration is increased and that inactivated carbenicillin does not interfere with gentamicin activity.

Effect of other penicillins

Other penicillins were selected which were readily hydrolyzed by the *B. cereus* β-lactamases, and their effect on the stability of gentamicin in water (17 h at 37°C) or in serum (48 h at 37°C) was examined. Over the time intervals investigated, amoxycillin, azlocillin and mezlocillin did not interact significantly in water but some slight inactivation appeared to occur in serum (Table 7).

Effect of serum

An AGAC and a penicillin were allowed to react in serum and in water and at intervals the AGAC concentration was determined. As can be seen in Fig. 3, the AGAC is inactivated at a much slower rate in serum than in water.

Discussion

Inactivation of gentamicin by carbenicillin has been known for many years (Mclauglin and Reeves, 1971; Noone and Pattison, 1971; Riff and Jackson, 1972; Waitz et al., 1972). More recently, it has been shown that other AGAC antibiotics and penicillins may also form complexes (Davies et al., 1975; Ervin et al., 1976; Pickering and Gearhart, 1979; Farchione, 1981; Henderson et al., 1981; Pickering and Rutherford, 1981; Blair et al., 1982; Chow et al., 1982; Glew and Pavuk, 1983; Konishi et al., 1983). The nature of this complex formation is claimed to involve a nucleophilic opening of the β-lactam ring by an amino group of an AGAC antibiotic, producing a biologically inactive amide (Waitz et al., 1972; Russo, 1980; Henderson et al., 1981), although experimental evidence in support of this contention is lacking (see later, also).

Several factors influence the formation of the complex between an AGAC and a β-lactam. One of the most important of these is temperature; at 5°C over a period of 48 h, we were unable to demonstrate any loss in biologically active gentamicin (Table 4), whereas complex formation occurred at 20°C and 37°C (Table 5, Fig. 2). Our results at 5°C differ somewhat from other investigators (Edwards and Schentag, 1981) whilst agreeing with studies in which > 95% AGAC activity remained after 48 h at 4°C (O'Bey et al., 1982; Glew and Pavuk, 1983). It is, however, generally agreed that the rate of inactivation increases as the temperature rises.

Another important factor that influences complex formation is the relative AGAC:β-lactam concentration (Riff and Jackson, 1972). Table 6 shows that gentamicin activity is lost progressively as the carbenicillin concentration is increased. However, even at a concentration ratio as low as 10:1 we found a significant loss of gentamicin activity. Since the inactivation of gentamicin is clearly

dependent upon carbenicillin concentration, this indicates that carbenicillin is either associating or reacting with gentamicin and not merely catalyzing its degradation.

It is generally accepted that the inactivation of AGAC by penicillins involves nucleophilic attack in the β-lactam ring by an amino group of the AGAC molecule resulting in a biologically inactive amide. Although the isolation and identification of such a complex has not been reported, it is well documented that penicillins can react with amines to form this type of amide (Bungaard, 1976). Our results from incubation with β-lactamase clearly demonstrate a reactivation of AGAC (Fig. 2, Table 5). This suggests that inactivation does not result from amide formation since it would seem unlikely that β-lactamase could effect the hydrolysis of a stable amide. Moreover, the results suggest that the β-lactam ring remains intact during AGAC inactivation. The results presented in Tables 1 and 2 demonstrate that an intact β-lactam ring is necessary for the inactivation of AGAC to occur. Hence the inactivation of AGAC may result from a weak association with penicillin which involves the β-lactam ring but does not result in ring cleavage.

It has been suggested that β-lactamase should be added to serum samples to prevent AGAC inactivation occurring prior to assay (Edwards and Schentag, 1981; Ebert and Clementi, 1983). However, our results clearly indicate a reactivation of AGAC during incubation with β-lactamase. Thus the storage of samples for prolonged periods with added β-lactamase may result in an overestimation of active AGAC in vivo.

The likelihood that there could be continued inactivation of antibiotics during the assay procedure (Russo, 1980) has prompted us to add sufficient β-lactamase to inactivate completely the β-lactam component whenever an AGAC–β-lactam mixture has been assayed. From the results of our incubation studies it would seem conceivable that such a procedure could result in reactivation of AGAC during assay. However, we believe that this effect occurs to a minimal extent, if at all, since the high molecular weight of β-lactamase will result in a low diffusivity and hence much lower effective concentration in the plate during assay.

A third factor that can influence complex formation is the nature of the medium into which the antibiotics are placed. Thus, incubation of gentamicin and carbenicillin in a buffer of pH 6.5 at which there is maximum stability of carbenicillin produced total inactivation of gentamicin (Table 3), which suggests again that an intact β-lactam ring is necessary for complex formation. In contrast to the findings of Waitz et al. (1972) who studied inactivation in buffer at pH 7 and in water, our findings showed somewhat less inactivation of gentamicin and tobramycin incubated in water in the presence of carbenicillin. Our results from incubation in serum (Fig. 3) support those (Riff and Jackson, 1972) that have shown that inactivation of gentamicin by carbenicillin occurs less rapidly in the presence of this medium.

The assay method used throughout this work has involved a microbiological agar diffusion technique aimed at determining the concentration of biologically active AGAC antibiotic. Other investigators have demonstrated that a radioimmunoassay (RIA) gives higher gentamicin levels than a microbiological assay (Thompson et al., 1982). However, a brief report has appeared in which a comparison was made of EMIT and microbiological assays for determining biologically active gentamicin

remaining after an interaction between gentamicin and carbenicillin in serum (Ebert and Clementi, 1983). The basis of this technique was to distinguish biologically active gentamicin from what were termed 'gentamicin degradation products'. It was concluded that EMIT overestimated the biologically active gentamicin concentrations in vitro and that the microbiological assay was preferred over EMIT for clinical dose adjustments of gentamicin.

A preliminary study has been made of the possible interaction between gentamicin and other β-lactams that could be inactivated by the *B. cereus* β-lactamase used earlier. The results (Table 7) suggest that azlocillin, amoxycillin and mezlocillin did not interact significantly with gentamicin in water (see also White et al., 1979; Henderson et al., 1981). Others have shown that tobramycin and piperacillin do not produce a clinically significant interaction in patients with normal renal function (Mclaughlin and Reeves, 1971; Thompson et al., 1982; Lau et al., 1983); that amikacin is the most stable AGAC in vitro and in vivo in the presence of carbenicillin (Pieper et al., 1980; Blair et al., 1982); and that of a wide range of penicillins, cephalosporins and an oxacephem (moxalactam) only ticarcillin and carbenicillin caused more than a 10% loss of tobramycin over a 48-h period (Edwards and Schentag, 1981).

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