THE IN-VITRO SENSITIVITY TESTING OF BETA-LACTAM ANTIBIOTIC COMBINATIONS

Philip John Turner

A Thesis submitted for the Degree of

Doctor of Philosophy

"The University of Aston in Birmingham"

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SUMMARY

Clavulanic acid is a beta-lactam that has a high affinity for beta-lactamase enzymes, but is also inherently stable to the majority of them. Combination of clavulanic acid with amoxycillin protects the latter from attack by beta-lactamase enzymes and allows it to achieve its antibacterial effect.

The effect of clavulanic acid on the activity of amoxycillin was studied, initially against well characterised strains of enterobacteria. Chessboard titrations, disc sensitivity testing and minimum inhibitory concentration (MIC) determination were used. A turbidimetric system was also used which allowed some MIC effects of the combination to be studied. From the results it became apparent that apart from its role as a beta-lactamase inhibitor, clavulanic acid affects the activity of other beta-lactam antibiotics in a different manner. This interaction was seen with beta-lactamase negative organisms and took the form of a complimentation effect due to the fact that different penicillin binding proteins (PBPs) were being bound by clavulanic acid and its partner. Clavulanic acid binds to PBP2 and from the studies carried out greatest complimentation occurred when it was combined with an antibiotic that bound to PBP1.

It also became apparent that there were problems with testing the sensitivity of organisms to the combination of amoxycillin and clavulanic acid. In a second part of the study, the methods for determining the susceptibility of strains of bacteria to amoxycillin in the presence of clavulanic acid were examined. Conventional disc sensitivity testing resulted in a majority of bacterial strains being classified as of intermediate sensitivities, even when the MIC of amoxycillin in the presence of clavulanic acid suggested that the strains were sensitive. Breakpoint sensitivity testing overcame this problem and a recommendation is made that this should be the method of choice for testing this combination.

Key words.

Clavulanic Acid
Sensitivity Testing
Penicillin-binding Proteins

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INTRODUCTION

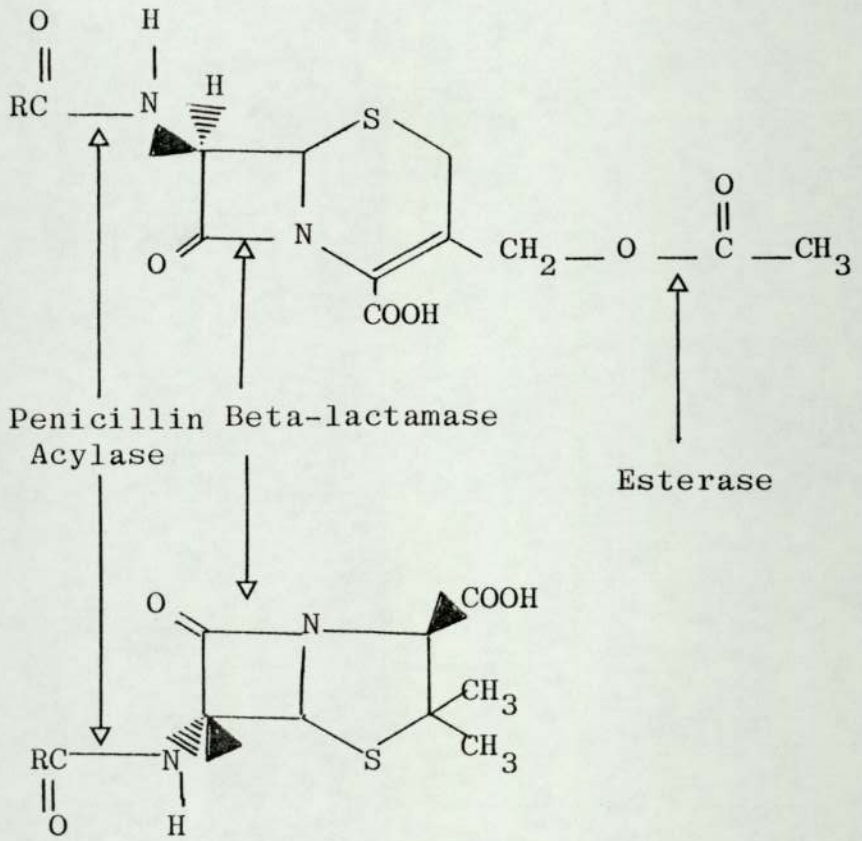
1.1. Beta-lactamases.

In 1940 an extract from disrupted cells of a strain of Escherichia coli was noted to have the property of destroying penicillin (Abraham and Chain 1940). The active component of the extract was an enzyme and was termed penicillinase. The introduction of other beta-lactam antibiotics into clinical practice such as the cephalosporins, has led to the recognition of a series of enzymes which will inactivate these antibiotics and to the replacement of the term penicillinase by that of beta-lactamase.

As the name suggests this group of enzymes acts by hydrolysing the beta-lactam ring and producing penicilloic or cephalosporoic acid which has no antibacterial activity (see Fig. 1). This is not the only mechanism of resistance to beta-lactam antibiotics, other methods include diminished cell wall permeability (Zimmermann and Rosselet 1977) and changes in the affinity of the target sites for the antibiotic (Hakenbeck et al. 1980). However, in the majority of bacteria of clinical significance the most important mechanism of resistance to the penicillins and cephalosporins is the production of beta-lactamase.

It is probable that all bacteria and most fungi produce beta-lactamases of one sort or another (Citri and Pollock 1966; Richmond and Sykes 1973; Sykes and Matthew 1976) and that they were being produced by bacteria long before the antibiotic era (Pollock 1967). Although in most instances,

FIGURE 1. BACTERIAL ENZYMES HYDROLYSING BETA-LACTAM ANTIBIOTICS



particularly in the gram positive bacteria, there appears to be a causal relationship between beta-lactamase and beta-lactam antibiotic resistance, there is evidence to suggest that the enzyme possesses a physiological function rather than just a protective one (Saz and Lowery 1979). In experiments with Bacillus cereus Ozer et al. (1970) showed that beta-lactamase possessed a function in cellular metabolism, most probably in cell-wall spore metabolism. Mutant strains of B. cereus which were impaired in sporulation showed no defect in sporulation pattern when beta-lactamase was added exogenously. This implies that with B. cereus the beta-lactamase is involved in certain aspects of cell-wall and spore-coat metabolism. In families of bacteria not of the Bacillaceae it is conceivable that similar reactions occur, but obviously not concerned with spore formation. Leggate and Holms (1967) reported that a semisynthetic penicillin with little or no antibiotic activity induced marked beta-lactamase activity in Staphylococcus aureus, but only at the end of the exponential phase of growth.

Sachithanandam et al. (1978) reported the isolation of a potent beta-lactamase from a fully susceptible strain of Staph. aureus. The beta-lactamase hydrolysed benzyl-penicillin, methicillin and cephaloridine, the activity was not inducible and was cell-bound, being liberated only by disruption of the producing cell. The enzyme was found in significant levels only during the very early exponential phase of growth.

These findings are consistent with the hypothesis stated by Sykes and Matthew (1976) that the normal function

of beta-lactamase may be to break a beta-lactam structure which is a transitory cell-wall intermediate.

In gram-positive organisms the beta-lactamases are usually excreted into the surrounding medium, whilst in gram-negative bacteria they are located in the periplasmic space (Curtis, Richmond and Sykes 1972). Synthesis of a beta-lactamase can be either chromosomally or plasmid mediated and either constitutive or inducible (Richmond and Sykes 1973).

Plasmid mediated beta-lactamases are produced in much larger amounts than are the chromosomal beta-lactamases. The amount of beta-lactamase produced is related to the number of plasmid copies and to the effect of regulatory genes (Neu 1980). Attempts to classify beta-lactamases have been applied primarily to those of gram-negative origin (Richmond and Sykes 1973; Sykes and Matthew 1976). The first of these schemes attempted to classify the beta-lactamases on the basis of biochemical parameters such as substrate profiles (relative ability to hydrolyse penicillin and cephalosporin substrates) and inhibition profiles.

Inhibition profiles are obtained by testing to see whether a particular beta-lactamase is inhibited by cloxacillin or p-chloromercuribenzoate (PCMB); this is because in the first case beta-lactamases showing preferential activity against the cephalosporins are more readily inhibited by the isoxazolyl penicillins than by methicillin, whereas with beta-lactamases showing preferential activity against penicillins the reverse is true. PCMB, used at a concentration of 0.5-1 mM inhibits the activity of cysteine-containing enzymes.

TABLE 1.

RICHMOND AND SYKES (1973) CLASSIFICATION OF BETA-LACTAMASES PRODUCED BY GRAM-NEGATIVE BACTERIA

Class	Relative activity against		Inducible	Inhibition	
	Pen.	Ceph.		Cloxacillin	PCMB
Ia	-	+++	I	S	R
Ib	-	+	C	S	R
Ic	-	++	I	S	R
Id	-	+	I	S	R
II	++	-	C	S	R
III	+++	+	C	S	R
IV	+	+	C	R	S
V	++	+	C	R	S

I: Inducible

C: Constitutive

S: Sensitive

R: Resistant

Richmond and Sykes (1973) succeeded in grouping the beta-lactamases into five broad classes using these criteria (see Table 1).

This scheme initially proved very useful but with the introduction of increasing numbers of beta-lactam antibiotics as substrates and with knowledge gained from different types of bacteria it became evident that classification based on substrate profiles was inadequate. Accordingly, Sykes and Matthew (1976) prepared a novel classification in which the enzymes were grouped on a genetic basis, namely, chromosomally-mediated beta-lactamases and plasmid-mediated beta-lactamases. In these two main groups, the enzymes were further subdivided according to the Richmond and Sykes scheme (see Table 2).

1.1.1 Chromosomally-mediated beta-lactamase.

The majority of chromosomally-mediated beta-lactamases hydrolyse cephalosporins more readily than penicillins and they are usually described as Class 1 cephalosporinases. Class 1 enzymes are produced by a wide range of pathogenic bacteria. Most are inducible and are produced by organisms such as *Citrobacter spp* (Sawai et al. 1968), *Enterobacter spp* (Farrar and Krause 1970), Indole-positive *Proteus spp* (Hamilton-Miller, Smith and Knox 1965), *Pseudomonas spp* (Sabath et al. 1965) and *Serratia spp* (Farrar and Newsome 1973). Examples of non-inducible Class 1 enzymes are much less common and include some of the beta-lactamases produced by *E. coli* (Dale and Smith 1971), *Shigella spp* (Sykes and Matthew 1976), *Salmonella spp* (Sykes and Matthew 1976), and *Enterobacter cloacae* (Fleming et al. 1967).

TABLE 2.

DIFFERENTIATION OF BETA-LACTAMASES FROM GRAM-NEGATIVE ORGANISMS AFTER SYKES AND MATTHEW (1976)

Mediation	Type	Class	Inducibility
Chromosomal	Cephalosporinase	I	+
		I	-
	Penicillinase	II	-
	Broad Spectrum	IV	-
Plasmid	TEM	III	-
	OXA	V	-
	PSE		-

Only a small number of bacterial species produce beta-lactamases that are chromosomally mediated and more active against penicillins than cephalosporins - the Class II enzymes. The most important example being that produced by ampicillin-resistant Proteus mirabilis (Sawai et al. 1968).

The Class IV chromosomally-mediated beta-lactamases possess broad spectrum activity and are exemplified by enzymes produced by Klebsiella (Hamilton-Miller et al. 1965) and some strains of Bacteroides (Salyers et al. 1977).

1.1.2 Plasmid-mediated beta-lactamases.

The first report of a beta-lactamase mediated by a plasmid that could be transferred between bacteria was by Datta and Kontomichalou in 1965. Since then many more examples have been found; these enzymes fall into three classes, namely TEM-type enzymes, OXA-type and the PSE group.

The TEM-type group of beta-lactamases have similar substrate profiles, hydrolysing ampicillin and cephaloradine but not the isoxazolyl penicillins or methicillin (Matthew 1971). The group consists of four types, TEM-1, TEM-2, SHV-1 and HMS the last being very rare (Sutherland 1982). The TEM-type beta-lactamases are by far the more frequently isolated of the plasmid mediated enzymes (Matthew and Harris 1976). This high incidence of isolation is attributed to their carriage on translocatable elements (transposons) which can migrate rapidly between chromosome and plasmid (Heffron et al. 1975). The TEM-type group of beta-lactamases are widely distributed amongst enteric

bacteria including Pseudomonas (Furth 1979) and are being increasingly found among strains of Haemophilus influenzae (Sykes et al. 1975), and Neisseria gonorrhoea (Sykes and Percival 1978).

The OXA class of plasmid-mediated beta-lactamases are so named (oxacillin hydrolysing) because of their ability to hydrolyse isoxazolyl penicillins and methicillin at a faster rate than that at which they hydrolyse ampicillin (Matthew 1979). Three OXA-type beta-lactamases are recognised, the OXA-1 hydrolysing methicillin about ten times more rapidly than the other two. They are found amongst a wide range of bacteria, OXA-1 being the commonest isolated.

The third class of plasmid-mediated beta-lactamases are referred to as the PSE (Pseudomonas specific enzymes). Until recently, as the name suggests, they were found only amongst strains of Pseudomonas spp (Slocombe 1980), but they have now been described in a small number of isolates of Esch. coli (Medeiros et al. 1980).

1.2. Beta-lactamase Inhibitors.

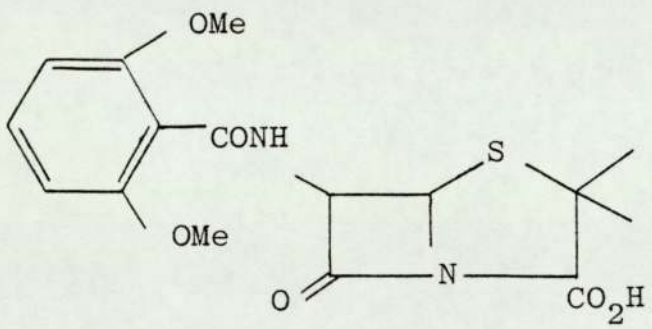
Structural modifications of the basic penicillin and cephalosporin nuclei have produced compounds which are resistant to beta-lactamase hydrolysis to varying degrees. The antistaphylococcal penicillins, e.g. methicillin (Figure 2) are effective inhibitors of the chromosomally mediated beta-lactamases produced by Staph. aureus and Bacillus sp. (Rolinson et al. 1960). However, although it was possible to demonstrate a synergistic effect between these compounds and ampicillin or benzylpenicillin, the degree of synergy was insufficient for clinical application (Sutherland and Batchelor 1964).

The 2-isopropoxy-1-naphthyl penicillin BRL 1437 was shown to be a good beta-lactamase inhibitor (Cole et al. 1972), but its therapeutic usefulness was limited due to poor absorption following oral administration (Greenwood and O'Grady 1975).

Carbenicillin has also been reported to be a competitive inhibitor of beta-lactamases and the kinetics of the inhibition of several cephalosporinases by this antibiotic have been studied by Labia and Fabre (1976).

The problem in providing effective synergy between these compounds and beta-lactamase susceptible compounds such as ampicillin, is that they are all competitive inhibitors of beta-lactamases and hence must be available in a fairly high concentration to protect the substrate. Unfortunately, high concentrations of these compounds in the vicinity of their beta-lactamase targets are difficult to achieve because most of them have difficulty in penetrating

FIGURE 2. METHICILLIN



the outer membrane and cell-wall of gram-negative bacteria.

The possibility that beta-lactamase inhibitors might exist as naturally occurring substances produced by microorganisms caused Brown et al (1976) to develop an agar plate test for inhibition of the beta-lactamase of Klebsiella aerogenes NCTC 418. The test involved the incorporation of benzylpenicillin in agar seeded with a strain of Kleb. aerogenes. Samples of fermentation broths were placed in wells cut in the agar, and the plate incubated at 37°C overnight. As a result of beta-lactamase production by the Kleb. aerogenes the penicillin in the agar was inactivated thus allowing bacterial growth over the general area of the plate. However, if the test sample contained an inhibitor of beta-lactamase this diffused into the agar surrounding the well, inactivated the beta-lactamase in that area and thus allowed a zone of inhibition due to action of the penicillin to be formed around the well.

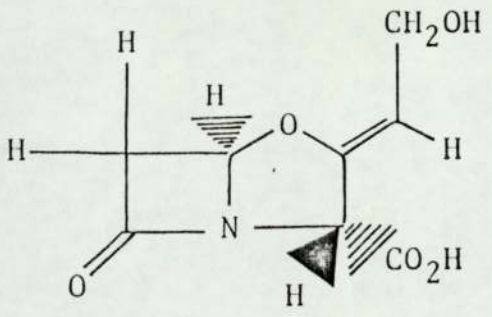
During an investigation (Reading and Cole 1977) of metabolites produced by Streptomyces clavuligerus, a pronounced beta-lactamase inhibiting activity was detected in culture filtrates using the agar plate test as described above. High dilutions of the culture filtrate also inhibited the TEM type beta-lactamase of E.coli. Although S. clavuligerus was known to produce various antibiotics structurally related to cephalosprin C, chromatography suggested that these were not responsible for the inhibitory activity. This was confirmed by the isolation of a beta-lactamase inhibiting compound which was named clavulanic acid. Structure determination (Howarth et al.

1976) revealed that clavulanic acid possessed a novel bicyclic structure containing a beta-lactam ring (Figure 3).

Clavulanic acid has good inhibitory properties against a wide range of beta-lactamases from both gram-positive and gram-negative bacteria (Reading 1982). It lacks significant inhibitory activity only against the chromosomally-mediated cephalosporinases. Its antibacterial activity is low, an M.I.C. range of 25-125 mg/L being common for most bacteria, greater activity is shown against Neisseriae gonorrhoea (M.I.C. 1.25-5.0 mg/L) (Comber et al 1980).

The compound appears to be sufficiently similar in structure to the penicillins and cephalosporins to fit into the catalytic centre of the beta-lactamase enzymes. Initially this active site is occupied without involving any chemical reactions and the clavulanic acid functions solely as a competitive inhibitor (Cole 1982). This is followed by a second phase in which a reaction occurs resulting in acylation of the beta-lactamase through the beta-lactam carbonyl of the clavulanic acid molecule (Charnas et al. 1978; Fisher et al. 1980). The acyl enzyme so formed is no longer active but is capable of being slowly hydrolysed by water and thus resulting in the restoration of active beta-lactamase and degradation products of clavulanic acid; because of this the acyl enzyme is said to be 'transiently inactivated' (Rolinson 1980). If the presence of the inhibitor is maintained, however, a second reaction occurs between beta-lactamase and inhibitor which is irreversible, and remains so even after removal of excess clavulanic acid

FIGURE 3.



STRUCTURE OF CLAVULANIC ACID

(Reading 1981).

The protective effect that clavulanic acid has on beta-lactamase labile substrates has now been reported for a variety of penicillins and cephalosporins against a range of organisms including anaerobic bacteria (Hunter et al. 1978; Jackson et al. 1978; Wise et al. 1978).

The broad spectrum, orally absorbed penicillin, amoxycillin is unfortunately liable to hydrolysis by many of the clinically important beta-lactamases, at rates similar to, or even greater than those obtained with benzylpenicillin (Reading 1982). It is, however, relatively stable to the chromosomally-mediated cephalosporinases - the group of enzymes that clavulanic acid lacks significant activity against. It can be seen, therefore, that amoxycillin and clavulanic acid are ideal partners for a synergistic formulation.

Sabath (1968) stated four requirements for synergy by competitive inhibition of beta-lactamase:-

(i) that beta-lactamase must be an important factor in the organisms resistance,

(ii) that the inhibitor must have very much greater affinity for the beta-lactamase than the hydrolysable antibiotic to be protected,

(iii) that the inhibitor must be stable to beta-lactamase action and,

(iv) that the inhibitor must have little or no antibacterial activity at the concentrations to be used.

Bobrowski et al (1973) suggested a fifth requirement, namely that the inhibitor should be able to penetrate the cell wall at least as well as the labile antibiotic in view

of the permeability barrier which exists to the site of action of cell-bound beta-lactamase.

Reading (1982) showed that at a ratio of 2:1 (amoxycillin:clavulanic acid) virtually complete protection of the amoxycillin was seen against both cell-free staphylococcal beta-lactamase and TEM-1 beta-lactamase. He also demonstrated that clavulanic acid can readily penetrate the cell wall in E. coli and reach its beta-lactamase target.

Bioavailability studies in animals (Mizen et al. 1980), and later in human volunteers (Ball et al. 1980), showed the inhibitor to be well absorbed by the oral route and that its pharmacokinetics were comparable with those of amoxycillin. On the basis of these studies, a formulation known as Augmentin, containing 250 mg of amoxycillin (as the acid trihydrate) and 125 mg of clavulanic acid (as the potassium salt) was selected for clinical investigation. After administration of Augmentin to man, the serum concentrations of amoxycillin are about twice those of clavulanic acid for up to six hours after dosing (Jackson et al. 1980). That is, the ratio of amoxycillin to clavulanic acid in serum approximates the ratio of the components in the formulation administered.

1.3. Mode of Action of Beta-lactam Antibiotics.

The early work on the mechanism of action of penicillin resulted in the discovery that it inhibited the final crosslinking of the peptide sidechains of nascent peptidoglycan and led to the suggestion that it acted as an analogue of acyl-D-alanyl-D-alanine (Tipper and Strominger 1965). In 1966, Isaki et al. demonstrated penicillin-sensitive enzymatic reactions : peptidoglycan transpeptidase and D-alanine carboxypeptidase, in E.coli membrane. By the mid 1970s it was clear that bacteria possessed multiple D-alanine carboxypeptidases and peptidoglycan transpeptidases, but the precise role of these enzymes in cell growth and the mechanism of action of penicillin was unknown (Blumberg and Strominger 1974). The demonstration that penicillin bound tightly to the enzymes that it inhibited (Blumberg and Strominger 1974) provided a method for the detection of penicillin-sensitive enzymes as penicillin-binding proteins (PBPs). Spratt and Pardee (1975) developed an autoradiographic method for the detection and study of PBPs. Using this method Spratt (1975) was able to study all of the penicillin-sensitive enzymes of bacterial cells regardless of which reactions of peptidoglycan synthesis they catalysed and opened the way to the identification of the killing targets for beta-lactam antibiotics.

The number of PBPs varies from species to species. E.coli contains seven PBPs in the cytoplasmic membrane and each of these corresponds to a distinct penicillin-sensitive enzyme (Table 3). The role of the PBPs of E.coli in growth,

TABLE 3.

PROPERTIES OF THE PENICILLIN-BINDING PROTEINS OF ESCHERICHIA COLI. (after Spratt 1983)

PBP	Mol.Wt.	Consequence of Inactivation of PBP	Enzymic Activity of PBP
1A 1B	92,000 90,000	Rapid cell lysis occurs if both PBP 1A and PBP 1B are inactivated	Both PBP 1A & PBP 1B are transglycosylase/ transpeptidase involved in cell elongation.
2	66,000	Growth as spherical cells	A transpeptidase that may initiate peptidoglycan insertion at new growth sites.
3	60,000	Inhibition of cell division leading to filamentous growth	A transglycosylase/ transpeptidase required for formation of the cross wall at cell division
4	49,000	No obvious growth defect	A D-alanine carboxy-peptidase that may function in vivo as a secondary transpeptidase in the maturation of peptidoglycan.
5	42,000	No obvious growth defect	A D-alanine carboxypeptidase
6	40,000	No obvious growth defect	D-alanine carboxypeptidase

morphogenesis, and the killing action of beta-lactam antibiotics has been elucidated in two main ways (Spratt 1980). Firstly, beta-lactam antibiotics produce at least three morphological effects on E.coli (filamentation, rapid cell lysis, and growth into spherical cells) and the degree to which beta-lactams produce these responses has been correlated with their binding to particular PBPs (Spratt and Pardee 1975; Spratt 1975). Secondly the consequences of the inactivation of each of the PBPs has been determined by the isolation of mutants that lack or produce thermo-labile forms of these proteins (Spratt 1975; Spratt et al 1977; Spratt 1980).

Rapid lysis of E.coli occurs when both PBP-1A and 1B are inhibited (Spratt 1975, 1977a). By purifying E.coli PBP-1B Nakagawa et al (1979) finally demonstrated that two enzyme activities of the formation of crosslinked peptidoglycan from the lipid-linked precursor GlcNaC-MurNaC (-pentapeptide) - diphosphate-un-decaprenol, i.e. activities of peptidoglycan transglycosylase and penicillin-sensitive transpeptidase, were residing in the preparation of PBP-1B. More recently, Ishino et al (1980) have succeeded in demonstrating similar peptidoglycan synthetase activities in purified PBP-1A of E.coli. Inactivation of PBP3, either with a beta-lactam antibiotic, or by transferring a mutant that produces a thermolabile form of PBP3 to the restricitive temperature, results in the inhibition of cell division and the growth of E.coli into long filamentous cells, and eventually cell death (Spratt 1975, 1977B). The inactivation of PBP2 results in the growth of E.coli as spherical cells and leads to eventual cell lysis (Spratt

1977c).

Mutants that lack detectable levels of PBP4, PBP5, or PBP6 are viable and do not show significant morphological abnormalities. This implies that these three PBPs are non-essential at least under laboratory conditions (Spratt 1980; Broome-Smith and Spratt 1982).

The study of mutants shows that inactivation of PBP 1A/1B or PBP2, or PBP3, is sufficient for the death of E.coli and beta-lactam antibiotics exist that kill by each of these three routes. The beta-lactams can therefore kill by three completely different mechanisms (rapid lysis, production of spherical cells, or filamentation) and effective derivatives are known that kill at or above their M.I.C. exclusively by rapid lysis (e.g. cefsulodin), by production of spherical cells (mecillinam) or by filamentation (aztreonam). More typical beta-lactams (e.g. amoxycillin), at the concentration achieved in serum, kill by two or all three of these mechanisms (Spratt 1980; Curtis et al 1979).

Clavulanic acid shows a good affinity for penicillin-binding protein 2, a moderate affinity for proteins 1,4,5 and 6, and low affinity for protein 3 (Spratt et al. 1977d).

1.4. Antibiotic Sensitivity Testing.

Antibiotic sensitivity testing can be performed by a number of methods; Braude et al (1955) suggested the use of gradient plates:- antibiotic containing agar was poured into a Petri dish with one edge of the dish elevated, resulting in the agar solidifying as a slant. Over this first agar layer another was poured - this time not containing antibiotic - and allowed to harden in a horizontal position. Diffusion of the antibiotic from the lower agar into the upper layer resulted in a concentration gradient being produced. Bacteria were streaked onto the gradient plate from low towards high concentration, the distance from the beginning of the gradient gave a mathematical expression of the concentration of antibiotic at a given point. This method obviously required a careful technique and a lot of expertise in interpreting the results.

The commonest methods of testing antibiotic sensitivity are based on the establishment of concentration gradients from predetermined diffusion centres towards the periphery of the plate. The diffusion centre can be either cylindrical holes punched into the agar and filled with the antibiotic to be tested (Erlanson 1951), or small glass cylinders applied to the surface of the agar filled with the antibiotic (Grove and Randall 1955). For routine measurements of bacterial sensitivity, however, most laboratories now use filter paper discs impregnated with a stated amount of antibiotic (Ericsson 1960). Variations in the actual method used and how to control and interpret the

results have resulted in four main methods.

(i) The Kirby-Bauer method (Bauer et al. 1966) is widely used in the U.S.A. It specifies that only Muller-Hinton medium can be used. The inoculum is standardised by adjusting the density of a suspension of the organism under test to that of a barium-sulphate standard. The plates are inoculated by dipping a sterile cotton-wool swab into the standardised suspension removing the excess and streaking the swab across the surface of the agar in three directions.

Discs are applied and the plates incubated at 35 to 37° C for 16 to 18 hours The zones of inhibition produced are measured to the nearest millimetre by using calipers, each zone size is interpreted by reference to a table (National Committee for Clinical Laboratory Standards, Sub-Committee on Antimicirobial Susceptibility Testing 1975), into one of three categories:

Sensitive: Infection treatable with normal dosage,

Intermediate: Infection that may respond to therapy with a higher dosage or if the infection is in a situation where the agent is concentrated,

Resistant: Not treatable with this agent.

Controls are tested daily using the same technique and the zone sizes recorded, the mean and standard deviation of five successive observations are compared to limits given for each antimicrobial in Tables (National Committee for Clinical Laboratory Standards Sub-Committee on Antimicrobial Susceptibility Testing 1975).

(ii) The Ericsson method is commonly used in Sweden and is a development of the method recommended by the W.H.O.

sponsored International Collaborative Study of Sensitivity Testing (Ericsson and Sherris 1971). It specifies that either Muller-Hinton or PDM medium is used, the inoculum is standardised to give a semi-confluent growth after incubation for 15-18 hours. The plate is inoculated by flooding, excess inoculum is removed and the plates are then dried for 30 minutes at 37°C before discs are applied. The antibiotics are allowed to diffuse for 30 minutes at room temperature before the plates are incubated at 37° C overnight.

The diameters of the zones of inhibition are measured with calipers, and the resulting figure is interpreted by reference to tables into one of four categories:

Sensitive: General infections treatable with normal dosage,

Fairly sensitive: General infections treatable with higher dosage,

Slightly sensitive: For infections in sites where the agent may be concentrated,

Resistant: Not treatable with this agent.

Controls are again tested daily using the described procedure and the zone sizes obtained compared to tables.

(iii) The Comparative method (Stokes and Waterworth 1972) is commonly used in British Laboratories, any medium designed for sensitivity testing can be used, the inoculum is prepared from a fully grown nutrient-broth culture or from a suspension of several colonies emulsified in saline so that a semi-confluent growth of colonies is produced on the plates after overnight incubation.

The plates are inoculated by using a wire loop with an

internal diameter of 4 mm to place the inoculum onto the surface of the agar and spreading this evenly with a sterile dry swab in three directions. Discs are applied with forceps or a sharp needle and pressed gently to ensure even contact. The plates are incubated at 35-37°C overnight. Control organisms are inoculated on separate plates by the same technique, for organisms isolated from urine the control is E.coli NCTC 10418; from other material Staph.aureus NCTC 6571; and for pseudomonas Ps.aeruginosa NCTC 10662.

If the test zones are obviously larger than the control or give no zone at all it is not necessary to make any measurements. If there is any doubt zones should be measured with calipers. Each zone size is interpreted as follows:

Sensitive: Zone diameter equal to, wider than, or not more than 6 mm smaller than the control;

Intermediate: Zone diameter greater than 12 mm but smaller than the control by more than 6 mm,

Resistant: Zone diameter 12 mm or less.

(iv) The Stokes method (Stokes and Waterworth 1972) has the advantage, like the comparative method, that any medium designed for sensitivity testing can be used. The inoculum is prepared in the same manner as that for the comparative method, so that a semiconfluent growth results after overnight incubation. The same three control organisms that are used in the comparative method are used in this method, whichever control is appropriate is inoculated as two bands on either side of the plate leaving a central band uninoculated. This is best achieved by using a cotton wool swab dipped into the inoculum. The test organism is seeded

evenly in the band across the centre of the plate, and antibiotic discs are applied on the line between the test and control organisms and the plate incubated at 35-37° C overnight.

The advantage of this method is that having the control and test organisms adjacent on the same plate the difference between the respective zone sizes can be directly measured. Interpretation is as follows:

Sensitive: Zone size equal to, wider than, or not more than 3 mm smaller than the control,

Intermediate: Zone size greater than 3 mm but smaller than the control by more than 3mm,

Resistant: Zone size 3 mm or less.

The four methods described have been compared (Brown and Blowers 1978) and little difference was found, the Ericsson and Kirby-Bauer techniques were more time consuming to set up than the other two methods, Ericsson because of his recommendation of 30 mins drying and prediffusion periods, and Kirby-Bauer because of the standardisation of inoculum. By all methods very obviously sensitive or resistant strains do not require measurements of their zones of inhibition; where there is a doubt the Stokes method is easiest to read because of the control being adjacent to the organism under test.

There are several factors that can effect the results of diffusion tests regardless of which method is actually used. These factors must, as far as is possible, be controlled in order that reliable results can be obtained.

Amongst these factors are the rate of diffusion of the antibiotic; penicillin, for example, has a low molecular

weight and diffuses rapidly in agar whereas the polymyxins have high molecular weights and so diffuse only slowly (Garrod 1958). Very slow diffusion results in small zones and poor discrimination between sensitive and resistant strains.

The type of culture medium used for the test is very important, the effects of medium constituents, e.g. thymidine may cause zones to appear unclear. The pH of the medium affects the activity of antibiotics to varying degrees (Abraham and Duthie 1946; Garrod et al. 1973). Sensitivity tests are best carried out as close to the normal body pH of 7.3 and most media have a pH value close to this.

Minerals and salts can have considerable effects, free divalent cations, for example, chelate tetracycline and reduce zone sizes (Ericsson and Sherris 1971). These two workers listed the ideal characteristics of a sensitivity testing medium pointing out such things as it should support, without enrichment, the growth of the majority of rapidly growing pathogens; it should not be subject to marked pH shifts; its contents should be defined and test results with reference strains and methods should be indentical for batches prepared by different manufacturers.

Comparisons done on the common sensitivity testing agars (Brown and Kothari 1978) showed that they all had advantages and disadvantages and that none were outstandingly good or bad. The choice of medium to be used tended to be a preference of each individual laboratory.

The density of inoculum affects the size of zones of inhibition, very light inocula resulting in large zones and

heavy inocula in small zones. For most disc methods, semi-confluent growth of colonies is considered ideal and the means of obtaining this depend on the method of inoculation and the individual performing the test.

Paper discs as reservoirs of antibiotics for sensitivity testing were introduced in the 1940s (Vincent and Vincent 1940). Nowadays the majority are produced by commercial firms and therefore the standards of disc production are in the hands of the manufacturers. In the U.S.A,. there is statutory control of antibiotic discs by the Food and Drug Administration of the Department of Health Education and Welfare (Federal Register 1961). This states that the discs must contain between 67 and 150 per cent of their stated content. Another advantage of the Stokes method of antibiotic sensitivity testing is that each individual disc is controlled and any substandard ones will be picked out.

The disc sensitivity result can only be considered as semi-quantitative, because of the considerable differences between organisms in different clinical situations. The minimum inhibitory concentration (MIC) of the drug is a more immediate indicator of sensitivity and so the relationship between the MIC and zone size is important. This relationship is the foundation of interpretation in the Ericsson and Kirby-Bauer methods. A number of workers have shown that the relationship of zone size to log MIC is approximately linear and that regression lines expressing this relationship can be produced by performing MICs and disc test simultaneously on a large number of strains (Ericsson 1960; Kanazawa 1966; Sherris et al. 1967).

Ideally the regression line would pass through a continuous series of points all of which were exactly on the line. Ericsson and Sherris (1971) showed that this was not the case in practice due to a number of reasons:

(i) The ideal assumes continuous MIC measurements whereas in fact log2 dilution steps are normally used. Assuming no technical error occurred, this would result in a spread of points of 2 to 4 mm for each MIC increment;

(ii) There is experimental error in MIC estimations and diffusion tests. An error of $\pm$ 2 mm in diffusion tests is not unusual;

(iii) The ideal assumes that all organisms grow at exactly the same rate. This is not so and slow-growing organisms have larger zones for particular MIC values;

(iv) Inherent differences of behaviour in diffusion tests of organisms against which the drug has the same MIC.

The relationship of the MIC to the expected concentration of the antibiotic in the blood or urine is also important, in fact, the concentration of active agent at the site of infection is the significant value and this will depend on how well the agent penetrates to the site of infection, how much of the agent is protein bound and how quickly it is excreted from the body. Generally a value of two to four times the MIC will ensure effective therapy.

In summary, when properly performed, disc diffusion tests have a high degree of reproducibility and are a reliable guide to the therapeutic use of antibiotics, however, they are affected by the factors mentioned above and the result may not always be clear cut, so alternatives have been sought. It is obviously not feasible to perform

a complete MIC determination for each antibiotic against every isolate. However, a number of laboratories have now simplified this test by inoculating one or two critical concentrations of each drug (Waterworth 1978, 1981), the so called break-point technique. The advent of the multiple inoculator means that solid agar can be inoculated with as many as fifty isolates and the interpretation of the result, as growth or no growth, is much simpler than the measuring and interpretation of zones of inhibition.

Both disc diffusion techniques and breakpoint methods need time for the bacteria being tested to become visible to the naked eye, consequently results are not usually available until after overnight incubation. Many laboratory workers have been looking for ways in which the time taken to obtain a sensitivity result can be shortened. One of the methods that has been developed is photometry.

Photometry is based on the theory that a suspension of cells will attenuate a beam of light in a manner proportional to its cell density. Suspended particles such as bacterial cells, which are nearly transparent, owe most of their light extinction properties to scattering. Scattering is defined as the deviation of a light beam from its original path (Carlberg 1980). If the undeviated light is measured to determine the degree of scattering that is turbidometry; if the scattered light is assessed directly, the method is nephelometry.

Under ideal conditions, there should be a linear relationship between light activation of a cell suspension and cell density in a similar manner to that expressed in the Beer-Lambert law for true solutes.

Mathematically the Beer-Lambert Law is stated as:

$$I = I_o e^{-klc}$$

where I = the intensity of a beam of light,

I_o = its intensity after passing through a solution,

k = constant characteristic of the solute, including the wavelength of light,

l = pathlength of light through the solution

c = concentration of the absorbing solute.

The expression can be rewritten to convert the expression from the natural log base to base 10 as follows:

$$\text{Log}_{10} \ (I^O/I) \quad = alc$$

a is a new constant covering the change in log base. The term $\log_{10} (I^O/I)$ is absorbance or optical density. Thus for a specific wavelength there is a straight-line relationship between the absorbance of a solution and the concentration of the solute.

This relationship has formed the basis of the Abbott MS-2 system. Changes in optical density due to growth of organisms are monitored at 5 min intervals. The readings obtained are stored in a computer and growth curves constructed. By comparison of the growth curves produced in the presence of antibiotic to one produced as a control (no antibiotic), a decision can be made as to the susceptibility of the organism. The time taken for this result is between four and six hours. The MS-2 system has been evaluated in the United States (McCarty et al. 1978), and good correlation was found with the disc diffusion test and the microdilution test.

1.5 Testing Combinations of Antibiotics.

There has been much discussion over the use of a single disc to determine the sensitivity of an organism to a combination of antibiotics. Ericsson and Sherris (1971) and Acar (1980) all state that when two antimicrobials are being tested they should not be tested with one disc. Grace et al. (1975) stated that cotrimoxazole (a combination of trimethoprim and sulphonamides) could be an exception to this because the two compounds diffused through agar at similar rates, maintaining approximately a 1:20 concentration ratio which is approximately the ratio of trimethoprim to sulphamethoxazole observed in plasma during treatment.

However, it should be borne in mind that although the combined disc can detect susceptibility and the assumed synergistic effect on strains that are sensitive to both trimethoprim and sulphamethoxazole, when a strain is only sensitive to one of the drugs, the diameter of the zone of inhibition, although decreased, may still remain within the susceptibility range. Resistance to either drug in this case would not be detected unless each drug were tested separately (Waterworth 1969).

In the case of Augmentin, a unique situation occurs in that although a combination is being tested, for all general purposes the clavulanic acid moiety will not be exhibiting an antimicrobial effect in the accepted sense. Comber et al. (1980) have suggested that a single disc containing 20 µg amoxycillin + 10 µg clavulanic acid gave the best results. Both compounds were shown to diffuse through agar at

approximately similar rates with the diffusion of amoxycillin being similar to that from a 20 µg amoxycillin disc and that of clavulanic acid similar to that from a 10 µg clavulanic acid disc.

1.6. Objects of the Study.

Since the early 1970s infection caused by beta-lactamase producing strains of gram-negative bacilli has increased, as a result of the prevalence of bacteria with plasmid-mediated beta-lactamases in the general population (Matthew 1979). This trend has been particularly notable among isolates of E. coli from urinary tract infections in general practice. E. coli is the commonest urinary pathogen isolated from specimens submitted from both hospital and general practice. Farrell and Turner (1982) showed that during a three month survey in general practice E.coli accounted for 75 per cent of urinary tract infections. Only 70 per cent of these strains were fully sensitive to amoxycillin. McAllister (1982) in a similar survey of sensitivities for E. coli from urinary tract infections in the Royal Hospital for Sick Chilren, Glasgow, found only 53.6 per cent of strains susceptible to amoxycillin.

Apart from developing new antibiotics with more resistance to beta-lactamases another way of countering this problem is to produce beta-lactamase inhibitors such as clavulanic acid and incorporate them with amoxycillin in order to protect it.

This study sought to examine the effect of clavulanic acid on the activity of amoxycillin against, initially, various selected strains of enterobacteria which either had no beta-lactamase present or, if beta-lactamase was present, had been well characterised. This would give an insight into the interaction between amoxycillin and clavulanic acid

when beta-lactamase was present. A number of workers (Neu and Fu 1978; Wise et al. 1978; Greenwood et al. 1979) have also suggested evidence of an interaction between amoxycillin and clavulanic acid against non-beta-lactamase producing bacteria. Comber et al. (1980) stated that a more specific test than conventional serial dilution may be required to reveal this. For this reason continuous turbidimetric recording of bacterial growth in the presence and absence of antibiotics was undertaken in an attempt to understand this interaction more fully.

A second part of the study sought to investigate the methods of determining the susceptibility of strains of bacteria to amoxycillin in the presence of clavulanic acid. This was undertaken because although Comber et al. (1980) had found a good correlation between MICs and inhibition zone diameter on testing Augmentin, this had not been the experience on preliminary testing at Birmingham (Farrell and Turner 1982). Usually sensitivity testing of strains of enterobacteria such as E. coli to amoxycillin results in the strains being placed into one of two distinct categories of either resistant or sensitive. Consequently with this bimodal distribution of sensitivity only a small number of strains fall into an intermediate category of moderately sensitive. With Augmentin, although many strains were fully sensitive many of the remainder could only be assigned to the intermediate category of moderately sensitive, even though they were susceptible to concentrations of Augmentin which would be regarded as sensitive. This could be a problem of Augmentin sensitivity discs so alternative methods of incorporating predetermined concentrations of

antibiotic into solid media and automated turbidimetric monitoring of liquid medium containing antibiotic were examined to see if any recommendation as to which method proved most reliable could be made.

2.1. MATERIALS.

2.1.1. Bacterial strains.

Seven groups of bacterial strains were used in these studies.

(i) Ten enterobacteria comprising a collection of strains which have been extensively studied by various investigators (Table 4), their beta-lactamases when present are well characterised. These strains were used to examine the effect of clavulanic acid on the activity of amoxycillin.

(ii) One hundred and twenty-six strains of various species of bacteria (Table 5) were used to determine the relation between the size of the zone of inhibition and the minimum inhibiting concentration for a number of antibiotics studied. These strains were provided by Professor Hans Ericsson.

(iii) One hundred Escherichia coli strains were isolated from urine specimens submitted to the Bacteriology Department of East Birmingham Hospital by General Practitioners for routine bacteriological examination.

(iv) Fifty-two Staphylococcus aureus strains were isolated from soft-tissue infections seen at the Accident and Emergency Department of East Birmingham Hospital.

(v) Fifty-seven organisms, which had proved to be resistant to amoxycillin on disc diffusion testing in the routine bacteriology laboratory. These fifty-seven organisms were originally isolated from urine specimens

TABLE 4. TEN WELL CHARACTERISED ENTEROBACTERIA

Ref. Number	Organism	Beta-lactamase	Reference
J.53. 2	Escherichia coli	Absent	Hedges et al. 1974
J.53. R6K	Escherichia coli	TEM 1	Egawa et al. 1967 Hedges et al. 1974
J.53 R1010	Escherichia coli	SHV 1	Matthew et al. 1979
J.53 R455	Escherichia coli	OXA 1	Hedges et al. 1973
J.53 R46-TS	Escherichia coli	OXA 2	Datta and Kontomichalou 1965. Dale and Smith 1974 Hedges et al. 1974
J.53. R57B	Escherichia coli	OXA3	Datta and Hedges 1972 Dale and Smith 1974 Hedges et al. 1974
1522E	Klebsiella aerogenes	Absent	Curtis et al. 1979
1082E	Klebsiella aerogenes	K1/4C	Marshall et al.1972
-	Enterobacter cloacae	Absent	Goldner et al. 1969
1841OP	Enterobacter cloacae	P99	Fleming et al. 1963

TABLE 5.

ERICSSON ORGANISMS

Code	API-profile	Species	Code	API-profile	Species
1		Enterococcus	51	3305572	E. cloacae
2		"	52	5044542	E. coli
3		"	53	5144552	"
4		"	54	3305573	E. cloacae
5		"	55	5044552	E. coli
6		"	56	3104573	E. cloacae
7		"	57	3305573	"
8		"	58	1255773	Klebs.pneum.
9		"	59	5144572	E. coli
10		"	60	5215773	Klebs.pneum.
11		"	61		Pseudomonas
12		"	62		"
13		"	63		"
14		"	64		"
15		"	65		"
16		"	66		"
17		"	67		"
18		"	68		"
19		"	69		"
20		"	70		"
21	5144532	E. coli	71	0734000	Prot. mirabilis
22	5144551	"	72	0334000	"
23	5044502	"	73	0334000	"
24	1044552	"	74	0334000	"
25	5144572	"	75	0736000	"
26	5144572	"	76	0736000	"
27	5144152	"	77	0734000	"
28	5144552	"	78	0334000	"
29	5144552	"	79	0734000	"
30	5044562	"	80	0334000	"
31	5144572	"	81		Staph. aureus
32	5144552	"	82		"
33	5144552	"	83		"
34	5144512	"	84		"
35	5144572	"	85		"
36	5144572	"	86		"
37	5215773	Klebs.pneum	87		"
38	5144572	E. coli	88		"
39	5144172	"	89		"
40	5044542	"	90		"
41	5215773	Klebs.pneum	91		"
42	5144532	E. coli	92		Staph. albus
43	5144532	"	93		Staph. aureus
44	3305573	E. cloacae	94		"
45	3344513	C. diversus	95		"
46	1104533	C. freundii	96		"
47	3305573	E. cloacae	97		"
48	5144512	E. coli	98		"
49	5144512	"	99		"
50	5144172	"	100		Staph. albus

cont.....

TABLE 5. contd/

Code	API-profile	Species
101		Pseudomonas
102		"
103		"
104		"
105		"
106		"
107		"
108		"
109		"
110		"
111	5307721	Serratia marc.
112	5307721	"
113	5307761	Serratia liq.
114	5307761	Serratia marc.
115	5307761	"
116	5307761	"
117	5307761	"
118	5307761	"
119	5307761	"
120	5307761	"
121	5307523	Serratia liq.
122	5317361	Serratia marc.
123	5307761	"
124	5307721	"
125	5307761	"
126	5307761	"

submitted by General Practitioners' patients. They identified as follows: E.coli (34), Kleb. aerogenes (9), Kleb. oxytoca (3), P. mirabilis (3), P. vulgaris (3), C. freundii (2) P. morgani (2) and Ent. cloacae (1) .

All isolates were identified by conventional methods as described by Cowan and Steel (1974).

(vi) The Oxford strain of Staph. aureus (N.C.T.C. 6571) and E. coli (N.C.T.C. 10418) were used as control strains for all determinations of antibiotic sensitivity by disc diffusion. Both of these organisms were obtained as freeze-dried cultures from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London.

(vii) In some experiments it was necessary to determine the concentration of amoxycillin and clavulanic acid present. A bioassay method was used with Bacillus subtilis (N.C.T.C. 8236) for the amoxycillin concentrations and Kleb. aerogenes (B.R.L. 1003) for the clavulanic acid concentrations. The first of these was obtained from the National Collection of Type Cultures and the second from Beechams Research

All the organisms were maintained on nutrient agar slopes (Oxoid Code No. CM3) at room temperature. Fresh nutrient agar slope cultures were prepared from single colony isolates at approximately one month intervals.

Prior to experiments a sample from an agar slope was streaked onto a plate containing Columbia agar (Oxoid Code No. CM331), with five per cent horse blood (Tissue Culture Services) added. The plate was then incubated overnight at

37°C and a single colony picked for experimentation.

2.1.2. Media.

Agar media used throughout the studies were prepared from dehydrated powders supplied by Oxoid Limited, Basingstoke, Hampshire, England.

Blood agar plates were prepared from Columbia agar (Oxoid Code No. CM331) with five per cent horse blood added.

Sensitest agar plates, for use in disc sensitivity testing, were prepared from Oxoid Sensitest agar (CM409) with five per cent lysed horse blood added. Lysis was achieved by the addition of saponin.

Antibiotic assay medium No. 2 (CM335) was used for assays to determine the amount of amoxycillin and clavulanic acid present. Assay broth (CM287) was used for all optical density studies.

All agars were prepared according to the manufacturers instruction and sterilised before dispensing into petri dishes.

2.1.3. Antibiotics.

Antibiotics were provided by the manufacturers, as powders of known potency; amoxycillin and ticarcillin, as the sodium salts, and clavulanic acid by Beecham Pharmaceuticals Research Division, Betchworth, Surrey, England; cefsulodin, as free acid, by Ciba-Geigy A.G.; Basel, Switzerland; aztreonam, as free acid, by E.R.Squibb and Sons Ltd., Hounslow, England; mecillinam, as the hydrochloride dihydrate, by Leo Laboratories Ltd.,

Aylesbury, Buckinghamshire, England and tetracycline, as the hydrochloride, by Lederle Laboratories, Gosport, Hampshire, England.

All antibiotic powders were stored at room temperature in a dessicator under vacuum. Amounts required were weighed out, after correction for potency, on an electronic balance and dissolved in sterile distilled water.

2.1.4. Chemicals.

The water used throughout these experiments was first deionised, and then distilled in a glass still before sterilisation by autoclaving.

Normal saline was prepared by dissolving 0.85 g of Sodium Chloride in 100 ml of distilled water. The resulting solution was dispensed in 5 ml amounts in bijoux bottles and sterilised by autoclaving.

2.1.5. Antibiotic discs

Amoxycillin (20 µg), penicillin (1 unit) and Augmentin (30 µg) discs were obtained from Beecham Research Laboratories and tetracycline (10 µg) discs from Diamed Diagnostics, Bootle, Lancashire, England.

All discs were stored at -40°C. When required for use they were removed from the freezer and allowed to reach room temperature before the containers were opened.

2.1.6. Apparatus.

The incubator used was a free-standing bench type (Leec Ltd., Nottingham, England (set at 37°C.). All weighings were carried out on an electronic balance (Unimatic Balances,

Avery Co., Smethwick, Warley, England). All zone sizes around antibiotic discs used for sensitivity testing, and antibiotic assay plate zone sizes were measured using a zone reader (Luckham Ltd., Burgess Hill, West Sussex, England).

A rotary plater (Denley Instruments, Billingshurst, Sussex, England) was used in the inoculation of plate sensitivity tests. A multipoint inoculator (Denley Instruments) was used for spot inoculation of antibiotic containing agar plates in the breakpoint method of sensitivity testing.

Optical density studies were performed using an Abbott MS-2 Research System (Abbott Laboratories, Basingstoke, Hampshire, England). This instrument is an automated nephelometer; it measures the optical density (O.D.) of a solution at 670 nm wavelength by using paired light emitting diodes and detectors.

Research cuvettes (Fig. 4) are supplied by the manufacturer. These consist of 11 chambers which can have varying dilutions of antibiotic in medium added to them. The organism whose drug susceptibility is under test can then be added and a tight fitting lid applied.

The cuvette is then placed in the analysis module (Fig. 5). This is a controlled temperature incubator and shaker. The temperature is set at 35 C. Up to eight may be placed in the analysis module and each chamber of each cuvette will be monitored by light emitting diodes and detectors at five minute intervals. During the short time it takes for reading the O.D. (approximately 20 seconds) the shaker stops agitating.

The O.D. readings are stored on a magnetic tape housed

FIGURE 4. RESEARCH CUVETTES FOR USE IN THE ABBOTT MS-2

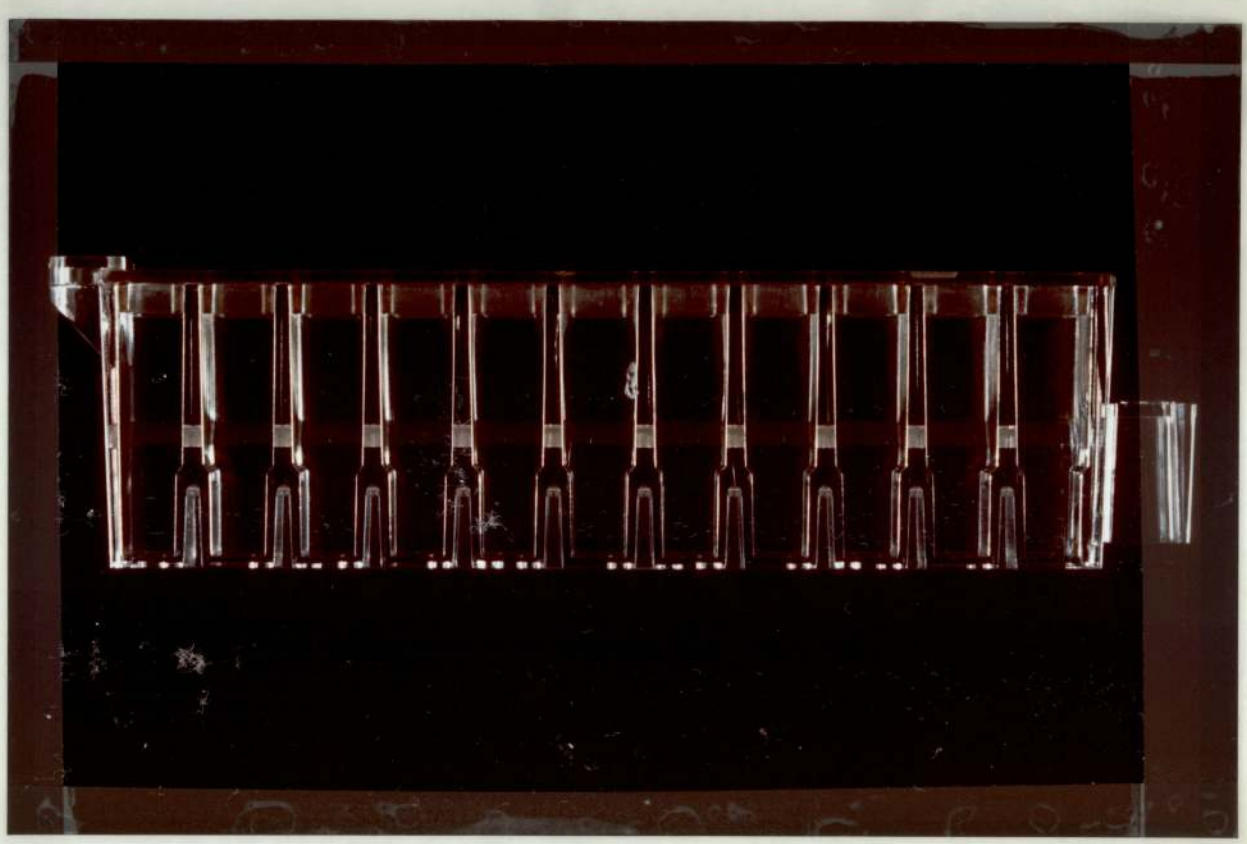
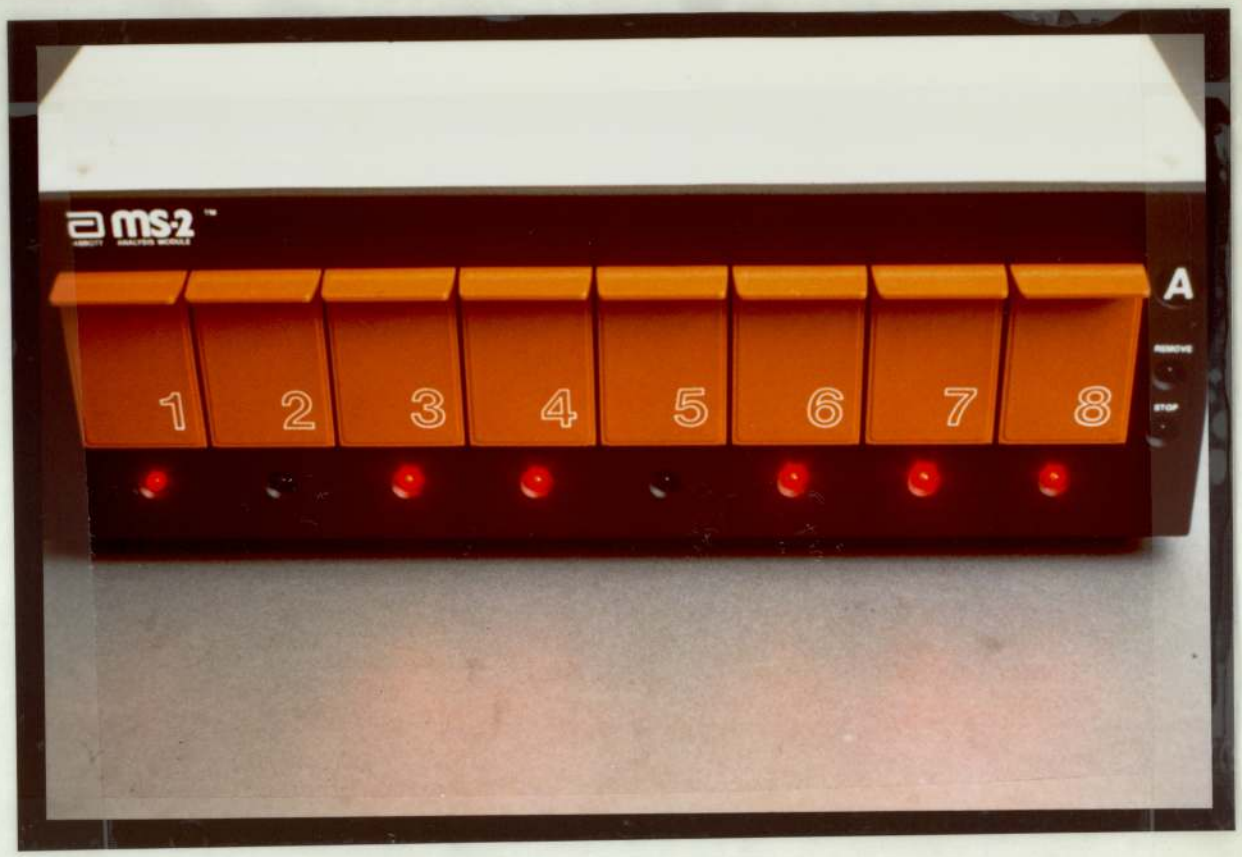


FIGURE 5. ABBOTT MS-2 ANALYSIS MODULE



in the control module (Fig. 6). This also has a keyboard to permit instructions to be given to the microprocessor. The magnetic tape will store O.D. information from up to 18h of readings and can then be instructed, via the keyboard, to display this information in the form of a set of growth curves of O.D. v time (Fig. 7) on a visual display unit.

The growth curves show, on the y axis, the O.D. for the media in the 11 chambers of the cuvette. The origins are offset for better visualization. In the majority of experiments described in this thesis, the curve designated CNTL represents the data from the control chamber containing medium only, i.e. with no antibiotic present. Curves numbered 10 to 1 represent the data from chambers containing medium with doubling dilutions of the antibiotic from 256 mg/L down to 0.5 mg/L. The x axis gives the time plot frequency, the smaller bars corresponding to 30 mins and the larger to 60 mins.

Hard copies of the growth curves were taken from the visual display unit and are used where appropriate in the results section of this thesis.

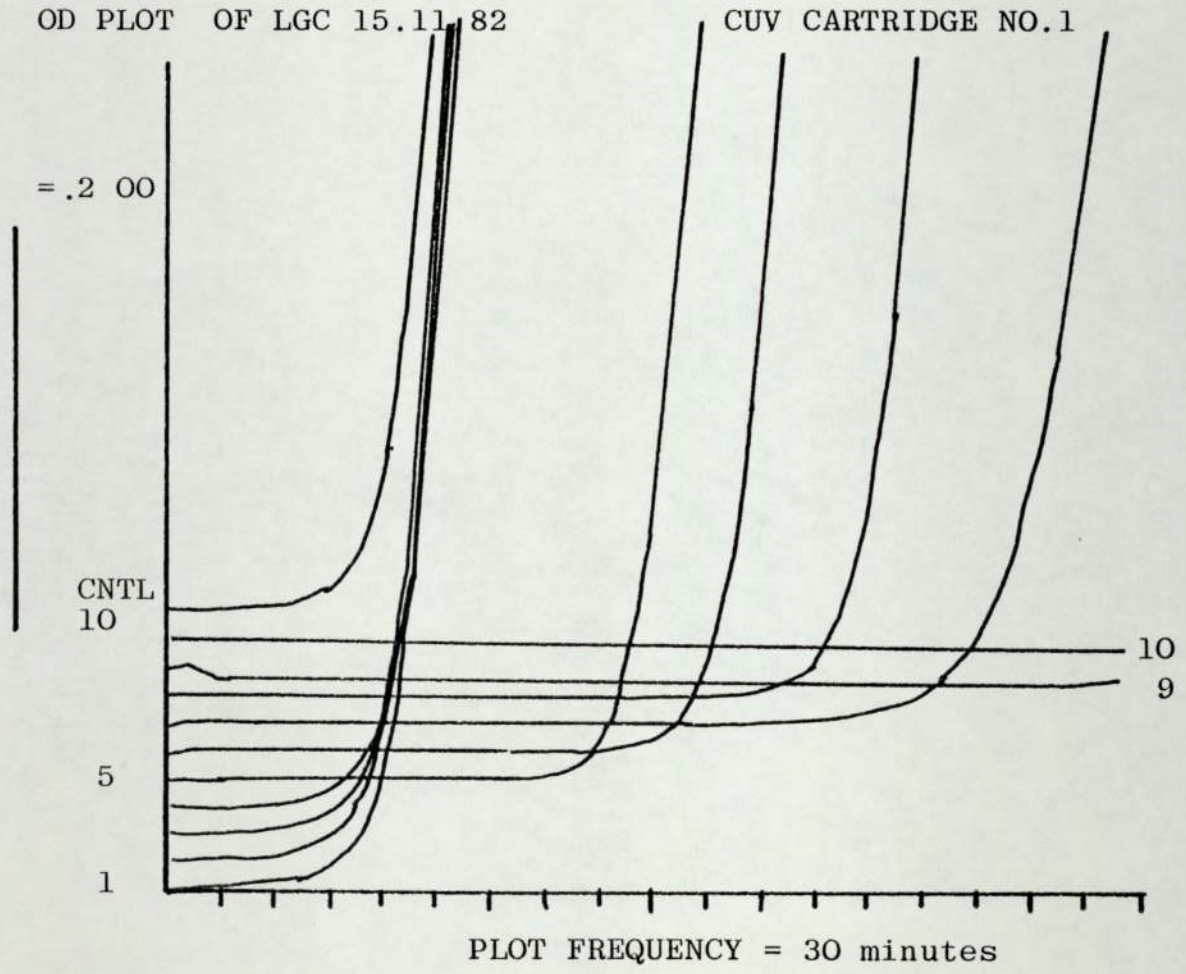
Bacterial morphology was studied using a light microscope (Leitz Instruments Ltd., Luton, Bedfordshire, England).

Large size assay plates and microtitre plates were supplied by Nunc Ltd., Kamstrup, Roskilde, Denmark.

Petri dishes (85 mm) were obtained from Sterilin Ltd., Teddington, Middlesex, England.

FIGURE 6. ABBOTT MS-2 CONTROL MODULE

FIGURE 7. ABBOTT MS-2 GROWTH CURVES



2.2 Experimental methods.

The work described in this thesis may be divided into a number of phases. Initially the effect of clavulanic acid on the activity of amoxycillin was examined. The organisms involved in this part were enterobacteria that have been extensively studied in the past. Any beta-lactamases that were present were well characterised, whereas if wild strains had been used for this initial part of the investigation, characterisation of their beta-lactamases would have been very difficult with the techniques available.

The knowledge gained from investigating these well characterised strains was then used to study the various methods of determining the susceptibility of clinical isolates to amoxycillin in the presence of clavulanic acid with a view towards making possible recommendations as to which techniques would give the most appropriate result.

2.2.1. Disc sensitivity tests.

Sensitest agar with five per cent lysed horse blood added was poured into Petri dishes on a flat, horizontal surface, 13.5 ml of agar to each 85 mm dish. Poured plates were stored at 4°C and used within one week of preparation.

Before inoculation the plates were dried in an incubator at 37°C, with the lids ajar so that there were no droplets of moisture on the agar surface. This usually took 40 mins.

The inoculum was prepared by emulsifying a single colony in 5 ml of saline. This gives a semi-confluent growth of colonies after overnight incubation.

Pearson and Whitehead's (1974) modification of the Stokes (1972) method was used for setting up the test; a petri dish containing sensitest agar was placed on a rotary plater revolving at 150 rpm. A control culture of E.coli (N.C.T.C.10418) or Staph. aureus (N.C.T.C.6571) - on an impregnated swab - was spread in the central area of the plate. The organism under test was then inoculated, using a separate swab, as a peripheral ring.

Antibiotic discs were applied using a sharp needle on the line between the test and control organisms, and pressed down gently to ensure an even contact with the medium. The plates were then incubated overnight at 37°C.

Zones of inhibition were measured using a zone reader and interpreted as follows:

Sensitive: zone size equal to, wider than, or not more than 3 mm smaller than the control,

Intermediate: zone size greater than 3 mm, but smaller than the control by more than 3 mm

Resistant: zone size 3 mm or less.

2.2.2. Minimum Inhibitory Concentration - Agar Dilution Method.

Waterworth's (1978) method was used to prepare the antibiotic containing agar plates. Antibiotic was weighed out and dissolved in sterile distilled water to give a concentration of 2560 mg/L. Doubling dilutions from this solution were made in sterile distilled water down to a concentration of 5 mg/L. One volume (1.5 ml) of each of the ten solutions resulting from this procedure was placed into one of ten petri dishes and nine volumes (13.5 ml) of melted sensitest agar, cooled to 55°C, was added to each plate and mixed thoroughly. This gave ten plates containing doubling dilutions of antibiotic from 256 mg/L to 0.5 mg/L. After the plates had set they were dried at 37°C, with their lids ajar, for 40 minutes.

The plates were then spot-inoculated using a multipoint inoculator, this delivered 10 organisms per spot from a 1:1000 dilution of an overnight broth culture.

A control plate, containing 15 ml of sensitest agar only (no antibiotic), was always included with each set of M.I.C. plates in order to ensure that the organisms under test were viable.

The plates were incubated overnight at 37° C, the minimum inhibitory concentrations (M.I.C.s) were read by eye as the lowest concentration of antibiotic that prevented visible growth.

2.2.3. Regression line analysis.

Using a pool of reference strains which were known to have M.I.C.s in the upper, middle and lower ranges of clinically useful concentrations of most antibiotics (Ericsson and Sherris 1971) regression lines were calculated relating the M.I.C. determined for each strain to the corresponding zone of inhibition. The method of least squares (Colton 1974) was used.

2.2.4. Tests of combined antibacterial action.

When two drugs are together their combined effect may be:

i. Indifferent, when the activity of both drugs is unaffected by the presence of the other;

ii. Synergic, when the activity of both drugs is significantly greater than that of either acting alone in the same concentration. Small increases in activity are generally considered to be additive;

iii. Antagonistic, when the activity of one drug is reduced by the presence of the second.

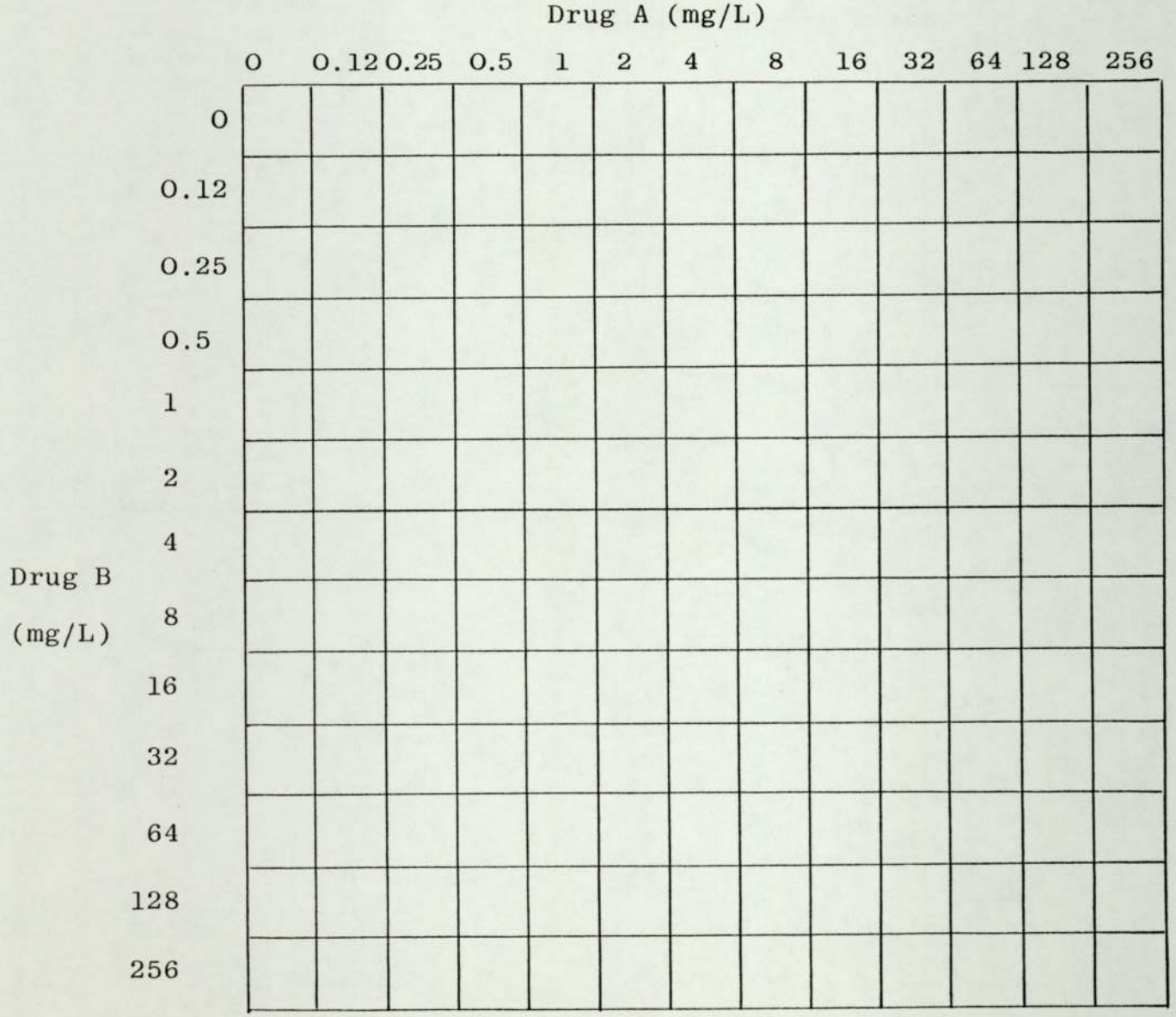
There are various means of demonstrating antimicrobial interactions (Elek and Hilson 1954; Garrod and Waterworth 1962), the chessboard titration has the advantage that many different combinations and concentrations can be tested.

2.2.4.1. Chessboard Titrations.

Serial twofold dilutions of antibiotic in water were prepared ranging from 5120 mg/L to 2.5 mg/L, for both antibiotics under test. To a petri dish were added 0.75 ml amounts of each antibiotic and 13.5 ml of melted agar, cooled to 55°C, the resulting solution was well mixed. This gave a set of 169 plates encompassing all combinations of the two drugs from 256 mg/L to 0.12 mg/L (Fig. 8). Where one drug was present alone 0.75 ml of sterile distilled water was added to maintain the correct dilutions. The control plate had 15 ml of agar only in the petri dish.

After the plates had set they were allowed to dry at 37° C for 40 mins., before being inoculated with cultures using

FIGURE 8. PATTERN USED FOR CHESSBOARD TITRATIONS



a multipoint inoculator as described in section 2.2.2.

Plates were incubated at 37° C overnight and then read with the naked eye to record the absence or presence of growth.

Synergism was quantified by using the method of fractional inhibitory concentration (F.I.C.) index suggested by Elion et al. (1954). This makes use of the following formula:

$$\text{F.I.C. Index} = \frac{\text{M.I.C.of drug A in the present of Drug B.}}{\text{M.I.C.of Drug A alone}} + \frac{\text{M.I.C.of Drug B in the presence of Drug A.}}{\text{M.I.C. of Drug B alone}}$$

An F.I.C. index of 1 was indicative of an additive effect only. Those between 0.7 and 1 were indicative of some synergy. Below 0.5 indicated substantial synergy and F.I.C. index of 2 and above were indicative of antagonism (Berenbaum 1978).

2.2.5. Optical Density Studies.

These studies were conducted using the Abbott MS-2 as described in section 2.1.6.

Doubling dilutions of antibiotic in assay broth (Oxoid CM287) in either the presence, or absence, of 8 mg/L of Clavulanic Acid were made in ten of the chambers in a cuvette. These dilutions covered the range 256-0.5 mg/L of antibiotic. The remaining chamber was used as a control with no antibiotic present. Assay broth was chosen because it supported the growth of the organisms involved in the study without the need for additives and it is a clear solution initially which readily allows changes in optical density to be recorded.

Each chamber contained 1 ml of assay broth with, or without, antibiotic. One drop from a bacterial suspension equivalent to 10^6 organisms/ml was added to each chamber to give an initial concentration of 5×10^4 orgs/ml.

The cuvette was placed in the analysis module at 35° C ± 0.5° C and by use of the keyboard on the control module optical density measurements were initiated.

Optical density measurements were continued overnight and the results stored in the microprocessor; the following morning the growth curves were displayed on the visual display unit and a hard copy made.

2.2.6. Tests for Beta-lactamase Production.

2.2.6.1. Microbiological Method (McGhie et al. 1977).

An agar plate containing sensitest agar was flooded with a culture of the Oxford Staph. aureus (N.C.T.C.6571) to obtain confluent bacterial growth. An ampicillin disc (25 ug) was placed in the centre of the plate and a heavy inoculum of the isolates under examination streaked radially from the disc (4 to a plate). The plate was incubated at 37° C overnight. Organisms positive for beta-lactamase production gave rise to a deep indentation of growth in an otherwise circular zone of inhibition of staphylococcal growth.

2.2.6.2. The Chromogenic Cephalosporin Method (O'Callaghan et al. 1972).

Nitrocefin (Glaxo Laboratories Ltd., Greenford, Middlesex, England), is a cephalosporin which is normally yellow in solution. If, however, it undergoes hydrolysis due to the action of beta-lactamase, a red solution is produced.

A working solution of nitrocefin was prepared by adding 0.5 ml of dimethylsulphoxide to 5 mg of solid nitrocefin; once the compound had dissolved 9.5 ml of 0.1M phosphate buffer pH7.0 was added. The resulting solution was stored in the dark in a refrigerator.

Bacterial cell suspensions were prepared by picking several colonies of the organism under test from an overnight plate and emulsifying in 0.5 ml of sterile saline. 50 µl aliquots of the cell suspension were placed in a microtitre plate containing 50 µl of nitrocefin solution.

The development of a red colour within 30 minutes was indicative of the presence of beta-lactamase.

A negative result in the above method may reflect either a low concentration of beta-lactamase or the inability of nitrocefin to reach the enzyme and interact. To differentiate between these two possibilities the cell suspensions were sonicated in order to break open the cells and the test repeated on the resulting cell lysates.

2.2.7. Antibiotic Assay.

2.2.7.1. Amoxycillin Assay.

Medium: 150 ml Oxoid Antibiotic Medium No.2.

Organism: Spore suspension of Bacillus subtilis (N.C.T.C.8236).

Method: The spore suspension was prepared by adding 300 ml of heart infusion agar to a sterile three litre Roux flask and allowing it to solidify along one of the large surfaces at the bottom. Five millilitres of an overnight brain heart infusion broth culture of the B. subtilis was added to the surface of the heart infusion agar and the flask incubated at 37 °C for seven days. After seven days the spores were harvested with 10-20 glass beads in a few millilitres of sterile saline being poured over the surface. The liquid was poured off into a universal container and the resulting spore suspension was heated to 65°C for 30 mins., washed with 50 ml sterile distilled water, then centrifuged at 3000 x g. The spores were resuspended in 25 ml of sterile distilled water to give a stable solution which kept for at least six months at 4°C. 0.75 ml of the spore suspension was added to the autoclaved agar after cooling to 56 ° C. The agar was then poured into a large plastic assay plate, placed on a level surface, and allowed to set.

Standards of 8,4,2,1 and 0.5 mg/L amoxycillin were prepared in horse serum and the specimens were diluted with horse serum if necessary.

Thirty six 5 mm wells were bored in the plate with a sterile cork-borer and the agar plugs removed with a needle.

Using a quasi-latin-square pattern the wells were filled with 75 µl of either standard or test and the plate incubated at 37 ° C overnight. The following morning zones sizes were measures using a zone reader and the mean inhibition zone diameters of the standard plotted against the logarithm of the concentration of antibiotic in the standards using semi-logarithmic graph paper. The line of best fit was constructed through the points, and the concentration of antibiotic present in the test specimens calculated by obtaining the mean inhibition zone diameter of the tests and extrapolating.

2.2.7.2. Clavulanic Acid Assay.

Medium: 150 ml Oxoid Antibiotic Medium No. 2.

Organism: Klebsiella aerogenes (B.R.L.1003).

Method: The agar was autoclaved and allowed to cool to 56° C. To this was added 1.8 ml of an overnight broth culture of Kleb. aerogenes (B.R.L.1003) and 4.5 ml of a 120 mg/L solution of benzyl penicillin. The agar was then poured into a large assay plate, placed on a level surface and allowed to set.

Standards of 10,5,2.5,1.25,0.62 and 0.31 mg/L clavulanic acid were prepared in horse serum.

Thirty-six 5 mm wells were bored in the plate with a sterile cork-borer and the agar plugs removed with a needle.

Using a quasi-latin-square pattern the wells were filled with 75 µl of either standard or test specimen. If dilution of the tests was deemed necessary, horse serum was used as the diluent. The plate was then incubated at 37 ° C overnight. the following morning zones sizes were measured

using a zone reader. The mean inhibition zone size of the standards was plotted against the logarithm of the concentration of the antibiotic in the standards using semi-logarithmic graph paper. The best fit line was drawn through the points and the concentration of clavulanic acid in the test specimens obtained by extrapolation.

Clavulanic acid is a weak antibacterial agent but a potent inhibitor of certain beta-lactamases. This property is utilised in the above assay. A subinhibitory concentration (120 mg/L) of benzylpenicillin is added to a nutrient agar containing the beta-lactamase producing organism Kleb. aerogenes (B.R.L.1003). At the concentration tested, clavulanic acid has no inhibitory effect on the growth of the assay organism, but it does inhibit the beta-lactamase activity of the Kleb. aerogenes. This prevents the destruction of the benzylpenicillin incorporated into the agar, and as a consequence, inhibition zones are produced by the penicillin, the diameters of which are proportional to the concentration of clavulanic acid in the standards or tests.

2.2.8. Morphological Studies.

Antibiotic-induced changes in morphology of the bacteria were observed in methylene blue stained slide preparations examined by light microscopy after various lengths of time of exposure to antibiotic.

A heavy suspension of the organism under test (approx. 10^8) was added to doubling dilutions of the antibiotic prepared in 1 ml quantities in assay broth, in the chambers of the research cuvette. A heavy inoculum was used to facilitate the ease of examination of the slides prepared from the cuvette chambers especially after only a few hours exposure.

The cuvette was placed into the Abbott MS-2 (see section 2.1.6. for description) at 35°C. After intervals of 1 hour, 2 hours and 4 hours, samples of the bacterial suspensions at various concentrations of antibiotic, and one from a suspension containing no antibiotic, were taken and placed in sterile glass tubes. The tubes were centrifuged at 5,000 r.p.m. for 5 mins. and the supernatant poured off into stericol. The button of bacterial cells was resuspended in a small amount of saline and slides prepared by taking a loopful of this suspension. Slides were fixed by heat then stained with a 1% solution of methylene blue and examined by light microscopy.

3. RESULTS AND DISCUSSION

3.1. The effect of clavulanic acid on the activity of amoxycillin against various selected strains of enterobacteria.

3.1.1. Strains of *E.coli*, *Kleb. aerogenes* and *Ent. cloacae* which produced well characterised beta-lactamases (lac +ve) and ones that did not produce beta-lactamase (lac -ve) were used to investigate the effect of clavulanic acid on the activity of amoxycillin. The strains used are listed in Table 4.)

3.1.2.1. Chessboard Titrations.

Chessboard titrations of amoxycillin cross-titrated against clavulanic acid were set up as described in section 2.2.4.1. for each of the strains. Two examples of the results sheets obtained are shown in Figures 9 and 10. The first of these showing the pattern of growth and no growth of lac -ve E.coli J.53.2 and the latter the pattern with the TEM1 lac +ve E.coli J.53.R6K.

Fractional Inhibitory Concentrations (F.I.C.s) were calculated for each of the ten strains using the formula of Berenbaum (1978) as described in section 2.2.4.1. The F.I.C. index values obtained when each strain was used as the indicator oganism are shown in Table 6 together with the described beta-lactamase activity of each strain. When the E.coli strains were used as indicator organisms the F.I.C. index was <0.7 in every case. A similar result was obtained when the two strains of Kleb. aerogenes were used. An F.I.C. index of 1 was produced with the lac -ve Ent. cloacae and one of 2 with the lac +ve Ent. cloacae.

3.1.2.2. Discussion.

An F.I.C. index of 2 was produced when the P99 lac +ve strain of Ent. cloacae was used as an indicator organism; an index of this value is indicative of antagonism. The beta-lactamase produced by this particular organism is chromosomally mediated and belongs to Class I. Therefore, as Slocombe (1980) pointed out, there is a relatively poor level of inhibition of the lactamase by clavulanic acid, so one would not expect to see any evidence of synergy. The

FIGURE 9. CHESSBOARD TITRATION OF AMOXYCILLIN AND CLAVULANIC ACID . E.COLI (lac -ve) J.53.2 USED AS ORGANISM

Escherichia coli Blase Neg. 10^4 Orgs/ml.

Amoxycillin (mg/L) \ Clavulanic Acid (mg/L)	0	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256
0	+++	+++	+++	+++	+++	+++	+++	+++	++	±	-	-	-
0.12	+++	+++	+++	+++	+++	+++	+++	+++	++	-	-	-	-
0.25	+++	+++	+++	+++	+++	+++	+++	+++	++	-	-	-	-
0.5	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-
1	+++	+++	+++	+++	+++	++	++	±	-	-	-	-	-
2	+++	+++	+++	+++	++	++	++	-	-	-	-	-	-
4	++	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-	-	-	-	-	-	-
128	-	-	-	-	-	-	-	-	-	-	-	-	-
256	-	-	-	-	-	-	-	-	-	-	-	-	-

FIGURE 10. CHESSBOARD TITRATION OF AMOXYCILLIN AND CLAVULANIC ACID. E.COLI (Lac +ve) J.53.R6K USED AS ORGANISM

Escherichia coli Blase Tem (1) 10^4 orgs/ml

Amoxycillin (mg/L) \ Clavulanic Acid (mg/L)	0	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256
0	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-
0.12	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-
0.25	+++	+++	+++	+++	+++	+++	+++	+++	±	-	-	-	-
0.5	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-
1	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-
2	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-
4	+++	+++	+++	+++	+++	+++	+++	++	-	-	-	-	-
8	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
16	+++	+++	+++	+++	+++	+	-	-	-	-	-	-	-
32	+++	+++	+++	+++	++	-	-	-	-	-	-	-	-
64	+++	+++	+++	±	-	-	-	-	-	-	-	-	-
128	+++	+++	±	-	-	-	-	-	-	-	-	-	-
256	+++	±	-	-	-	-	-	-	-	-	-	-	-

TABLE 6.

FRACTIONAL INHIBITORY CONCENTRATION INDEX RESULTS

Organism	Beta-lactamase	F.I.C. Index
Escherichia coli	Absent	0.625
Escherichia coli	TEM 1	0.02
Escherichia coli	SHV 1	0.01
Escherichia coli	OXA 1	0.09
Escherichia coli	OXA 2	0.13
Escherichia coli	OXA 3	0.02
Enterobacter cloacae	Absent	1.0
Enterobacter cloacae	P99	2.0
Klebsiella aerogenes	Absent	0.25
Klebsiella aerogenes	K1/4C	0.1

F.I.C. index produced when the lac -ve Ent. cloacae was used as indicator organism was 1.0; this is indicative of an additive effect only.

The F.I.C. index values obtained with the remaining strains were all <0.7; this is indicative of synergy. This was not a surprising result in the case of the lac +ve strains as their beta-lactamases are all either plasmid mediated or class IV chromosomally and therefore susceptible to the action of clavulanic acid (Slocombe 1980; Reading 1982).

It was, however, interesting to observe that the lac -ve strains of E.coli and Kleb. aerogenes also produced F.I.C. index values indicative of synergy. This cannot be due to the clavulanic acid inhibiting a beta-lactamase as there is none there to inhibit and so it must reflect a real mutual enhancement of antibacterial potency by the amoxycillin and the clavulanic acid.

3.1.3.1. Disc diffusion susceptibility tests.

The criteria for placing a zone size result into the sensitive, intermediate or resistant category were those given in section 2.2.1. Table 7 lists the results obtained from the Stokes method of sensitivity testing together with the M.I.C.s for amoxycillin and Augmentin obtained by agar incorporation techniques. These tests were performed as stated in sections 2.2.1. and 2.2.2.

The disc results for amoxycillin placed four of the strains in the sensitive category, the three lac -ve strains and the E.coli containing the plasmid coding for OXA 2 beta-lactamase. The remaining six strains, all lac +ve, were resistant. This contrasts with the results obtained with Augmentin where although the same four strains were classified as sensitive, the remaining six were classified as resistant in the case of two and intermediate in the other four. The two strains classified as resistant to Augmentin were the Enterobacter cloacae and the Klebsiella aerogenes. Both of these organisms have beta-lactamases that are chromosomally mediated, P99 in the case of the Enterobacter and K1/4C in that of the Klebsiella. P99 is a member of the Class I Cephalosporinase chromosomal beta-lactamases and as such is not inhibited by clavulanic acid. This was reflected in the fact that the M.I.C. of both amoxycillin and Augmentin was >256 mg/L.

K1/4C is a member of the Class IV broad spectrum chromosomal beta-lactamases. Clavulanic acid can exhibit some inhibition with this class, as shown by an Augmentin M.I.C. of 8 mg/L as compared to the amoxycillin M.I.C. of

TABLE 7.

DISC SENSITIVITY (STOKES CRITERIA) AND M.I.C. RESULT FOR TEN ENTEROBACTERIA

Organism	Amoxycillin		Augmentin	
	Disc result	M.I.C. (mg/L)	Disc result	M.I.C. (mg/L)*
Escherichia coli	S	4.0	S	2.0
Escherichia coli (TEM1)	R	> 256	I	1.0
Escherichia coli (SHV1)	R	> 256	I	2.0
Escherichia coli (OXA1)	R	> 256	I	4.0
Escherichia coli (OXA2)	S	4.0	S	1.0
Escherichia coli (OXA3)	R	64	I	2.0
Klebsiella aerogenes	S	1.0	S	< 0.5
Klebsiella aerogenes (K1/4C)	R	> 256	R	8.0
Enterobacter cloacae	S	< 0.5	S	< 0.5
Enterobacter cloacae (P99)	R	> 256	R	> 256

* Clavulanic acid present ∂ϰ a constant concentration of 8 mg/L.

>256 mg/L. However, it is the plasmid mediated beta-lactamases of class III and V which respond most to clavulanic acid and this is shown by the reduction in Augmentin M.I.C. with the TEM, SHV and OXA containing E.coli.

It is of interest to note that when the M.I.C.s of amoxycillin alone against the various lac +ve strains of bacteria are examined, the two strains containing the OXA 2 and OXA 3 plasmid mediated beta-lactamases have much lower values than those containing the TEM1, SHV 1 and OXA 1 plasmid mediated beta-lactamases. This is probably a reflection of the fact that OXA 2 and OXA 3 beta-lactamases hydrolyse amoxycillin at only about a tenth of the rate of the TEM 1, OXA 1 and SHV 1 beta-lactamases (Matthew 1979).

The four strains of E.coli which were classified as of intermediate sensitivity on disc testing all had an Augmentin M.I.C. < 4.0 mg/L and F.I.C. index values (see Table 5) < 0.1. Therefore, one would have expected them to be sensitive on disc testing.

The Augmentin disc used for sensitivity testing was a 30 ug disc containing 20 ug amoxycillin and 10 ug clavulanic acid. Comber et al. (1980) have shown that the two compounds diffused from the disc throughout the agar at approximately similar rates. The control strain for the Stokes method was E.coli N.C.T.C. 10418. This is a non-beta-lactamase producer. It is also highly sensitive to amoxycillin so the zone size around the disc is large. The fact that the Augmentin disc contains twice as much amoxycillin as clavulanic acid means that the antibacterial component will extend into the agar further than the

beta-lactamase inhibitory component. Because zone sizes of sensitive beta-lactamase-producing organisms are determined by the clavulanic acid concentration then a smaller zone size compared to the control will be produced. As a consequence the strains are classified, with conventional Stokes criteria, as of intermediate susceptibility.

One of the problems with both chessboard titrations and disc sensitivity testing is that only the end result of a series of events, which have taken place, is recorded. Clearly antibiotics can exert an effect at sub M.I.C. concentrations (Lorian 1980). The effects produced by antibiotics or sub MIC levels are not milder than those seen at M.I.C. but are different in kind. They can be evaluated by observing changes in a number of parameters, e.g. bacterial morphology, ultrastructure, biochemistry and multiplication rate. Growth of bacteria can be conveniently, continuously studied using turbidimetry (Watson et al. 1968). The term 'minimum antibacterial concentration' (M.A.C.) has been suggested to indicate the lowest concentration of an antibiotic causing an observable antibacterial effect (Greenwood 1979). When this term is applied to turbidimetric methods, it signifies a deviation from the normal growth curve produced by the organism in the absence of any antibiotic.

3.1.4. Turbidimetric studies.

Unless stated otherwise the experiments in this section were set up as described in section 2.2.5. The lac -ve strain of *E. coli* and the lac +ve strain containing the TEM 1 plasmid were used as the two test strains in these experiments.

3.1.4.1. Studies with Amoxycillin

Figure 11 shows the growth curves produced by the lac -ve strain of E. coli in the presence of doubling dilution of amoxycillin (256-0.5 mg/L) in assay broth. A tubidimetric M.I.C. of 2 mg/L of amoxycillin is illustrated and it can be seen that concentrations of amoxycillin equal to or greater than 2 mg/L produced no increase in turbidity for 19 hours. This contrasts with the control containing no antibiotic which after two hours incubation undergoes a considerable increase in optical density.

The chamber containing 0.5 mg/L of amoxycillin behaved in an identical fashion to the control and identical growth curves were produced. Growth in the chamber containing 1 mg/L of amoxycillin was delayed until five hours and this is reflected in a shift of the growth curve to the right. This means that the M.A.C. is 1 mg/L.

Figure 12 shows the same lac -ve strain of E. coli exposed to amoxycillin (256-0.5 mg/L) in the presence of a constant concentration of 8 mg/L clavulanic acid. The control curve showed an increase in optical density after 2 hours but none of the other curves showed an increase in optical density until 15 hours had elapsed when the lowest curve, corresponding to 0.5 mg/L of amoxycillin, began to rise. This corresponded to an M.A.C. of <0.5 mg/L and a turbidimetric M.I.C. of 1 mg/L.

The response of the lac +ve strain of E. coli to amoxycillin alone is shown in Figure 13. Due to the antibiotic being destroyed by the beta-lactamase all the growth curves produced by the chambers containing

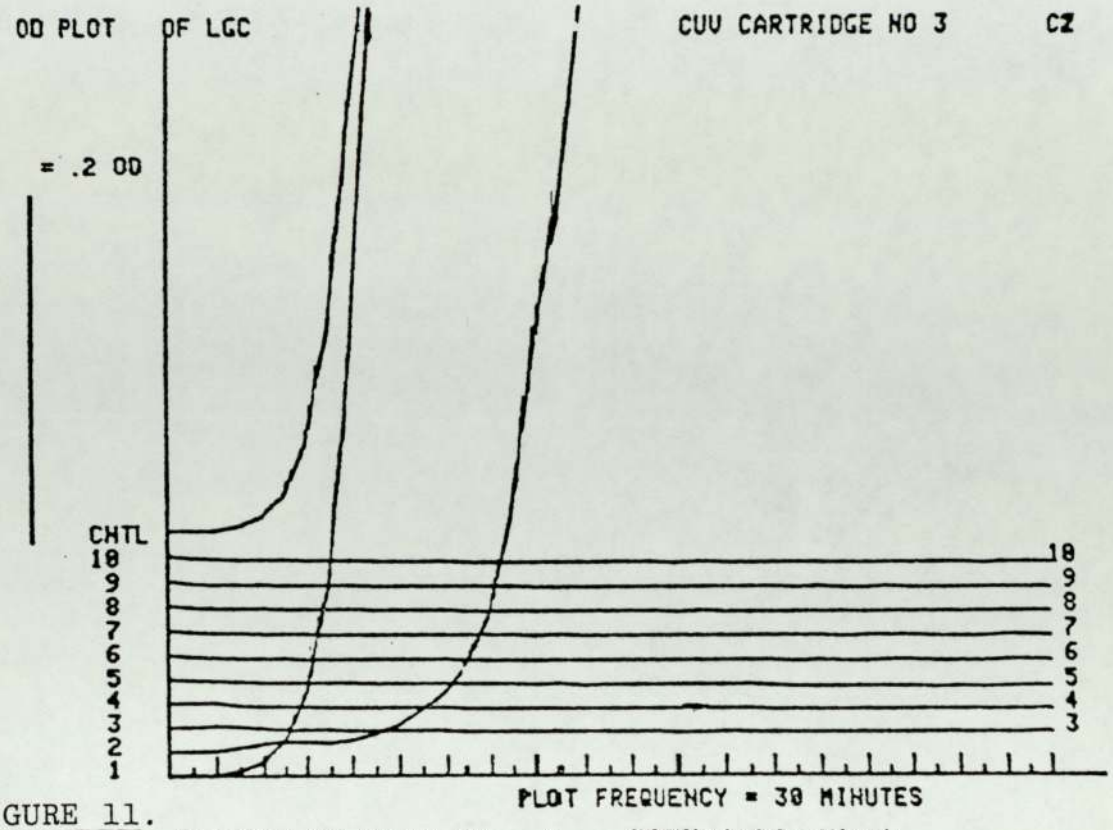


FIGURE 11.

ESCHERICHIA COLI (β-LASE NEG) V AMOXYCILLIN 0.5 - 256 mg/L

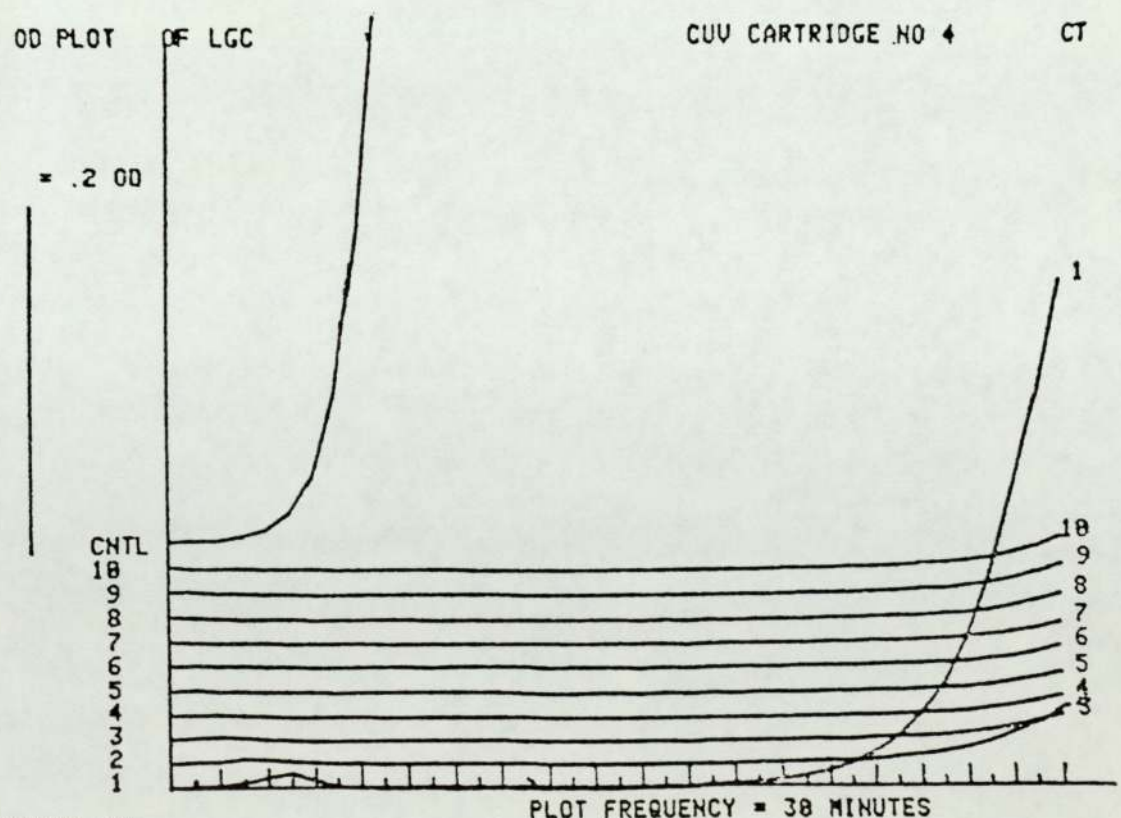


FIGURE 12

ESCHERICHIA COLI (β-LASE NEG) V AMOXYCILLIN 0.5 - 256 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

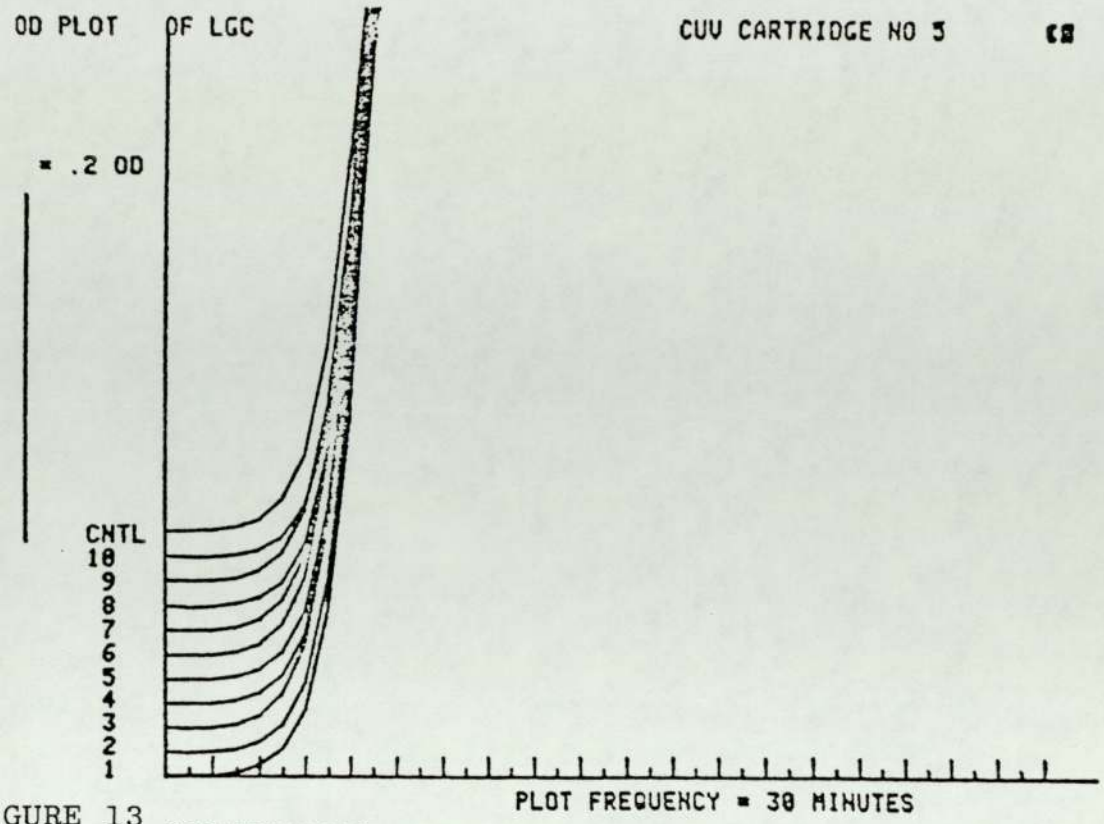


FIGURE 13 ESCHERICHIA COLI (β-LASE TEM 1) V AMOXYCILLIN 0.5 -256 mg/L

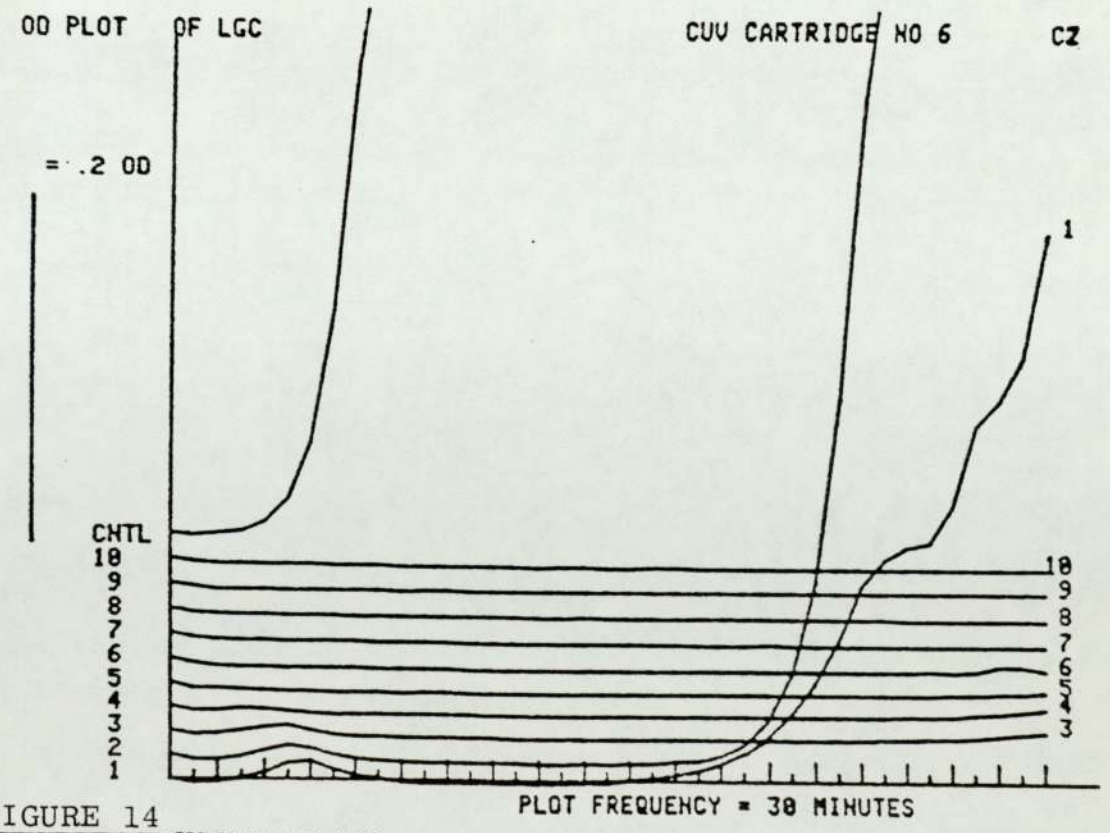


FIGURE 14 ESCHERICHIA COLI (β-LASE TEM 1) V AMOXYCILLIN 0.5 - 256 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

amoxycillin produce identical growth curves to that of the control chamber with no antibiotic present. Both M.A.C. and tubidimetric M.I.C. are >256 mg/L.

Figure 14 shows the results obtained with the lac +ve strain when clavulanic acid (8 mg/L) was also present in the chambers. The activity of amoxycillin was markedly potentiated by clavulanic acid in that even concentrations of amoxycillin of 0.5 and 1 mg/L had an antibacterial effect (M.A.C. = <0.5 mg/L) and concentrations of >2 mg/L inhibited the growth of the lac +ve strain completely (turbidimetric M.I.C. = 2 mg/L).

3.1.4.2. Discussion.

In the case of the lac +ve strain the addition of clavulanic acid to amoxycillin enabled the latter drug to remain potent due to the clavulanic acid inhibiting the beta-lactamase. Turbidimetric M.I.C. was reduced from >256 mg/L to 2 mg/L. However, even when no beta-lactamase was present the addition of clavulanic acid caused a response delaying the onset of growth of the lac-ve strain for fifteen hours.

An explanation of this synergy could be that because amoxycillin binds to penicillin binding protein 1 and 3 (Curtis et al. 1979) and clavulanic acid binds primarily to Penicillin-binding protein 2 (Spratt et al. 1977d) then a similar process to that seen when a gram negative organism is exposed to a combination of cephalexin and mecillinam (Greenwood and O'Grady 1973) is occurring.

In the case of cephalexin and mecillinam the first of these binds to PBP 3 (Curtis et al. 1979) and mecillinam

binds to PBP 2 (Spratt 1977a), that is to say cephalexin would normally cause long filamentous cells to be produced by the bacteria whilst mecillinam would cause the formation of large spherical cells. When the two antibiotics are present together, because they attack different PBP's, spheroplasts are produced, and because these are osmotically fragile they burst - thus the two antibiotics present together are much more rapidly bactericidal than are the individual compounds.

In order to test this theory a number of experiments were performed:-

(1) The assay of amoxycillin and clavulanic acid after overnight incubation in the Abbott MS-2 system, in order to test the stability of these two drugs.

(2) The use of what could be termed "Penicillin-binding protein probes", that is to say combining clavulanic acid with a number of different beta-lactam compounds that bound to specific PBPs; the following were used:

Ticarcillin, this antibiotic binds to PBPs 1 and 3 (Curtis et al. 1979) like amoxycillin. If a similar result to that obtained with amoxycillin and clavulanic acid can be obtained with ticarcillin and clavulanic acid it would prove that the result was not peculiar to the augmentin combination and lend more support to the 'PBP synergy' hypothesis.

Cefsulodin binds specifically to PBP 1 (Curtis et al. 1979).

Aztreonam has highest affinity amongst the PBPs for PBP 3 (Curtis- personal communication).

Mecillinam binds specifically to PBP 2 (Spratt 1977).

If the 'PBP synergy' hypothesis is correct one would expect the following results when these antibiotics were combined with clavulanic acid (remembering that clavulanic acid binds to PBP 2).

Antibiotic	P.B.P.Bound	Expected Result When Combined With Clavulanic Acid.
Amoxycillin	1 and 3	Synergy
Ticarcillin	1 and 3	Synergy
Cefsulodin	1	Synergy or Indifference
Aztreonam	3	Synergy or Indifference
Mecillinam	2	Indifference or Antagonism

Cefsulodin or aztreonam would show indifference if the synergy was between either PBPs 2 and 3 or 2 and 1 respectively. Mecillinam would possibly show antagonism as both compounds would be competing for the same PBP.

Results of these experiments were as follows:

3.1.5.1. Assay of Amoxycillin and Clavulanic Acid concentrations.

The lac -ve strain of *E. coli* was used in these experiments. The amounts of amoxycillin and clavulanic acid remaining in the assay broth in the chambers of the cuvettes were determined as described in sections 2.2.7.

Figures 15 and 16 show the graphs obtained from the standard concentrations of amoxycillin and clavulanic acid respectively. Amounts of the drugs present in the samples taken were calculated by determining the average zone size produced by the samples and reading off direct from the graph and multiplying by dilution factor.

Results are given in full below:

Amoxycillin assay.

Standards (mg/L)	Zone sizes (mm)	Average (mm)
0.5	154, 153, 154, 150	152.75
1.0	179, 177, 179, 177	178.00
2.0	200, 201, 198, 197	199.00
4.0	218, 222, 218, 217	218.75

Tests	Zone sizes (mm)	Average (mm)	Conc(Mg/L)
Amoxycillin 128	183,184,182,183	183	1.20
Amoxycillin 1	No zone x 4	No zone	0
Amoxycillin 128/ Clavulanic acid 8	181,183,182,180	181.5	1.15
Amoxycillin 1/ Clavulanic Acid 8	No zone x 4	No zone	0

The two large concentrations were diluted 1/100 so using this factor results of :- 120 mg/L and 115 mg/L were obtained.

FIGURE 15. GRAPH OF AMOXYCILLIN CONCENTRATION v ZONE SIZE

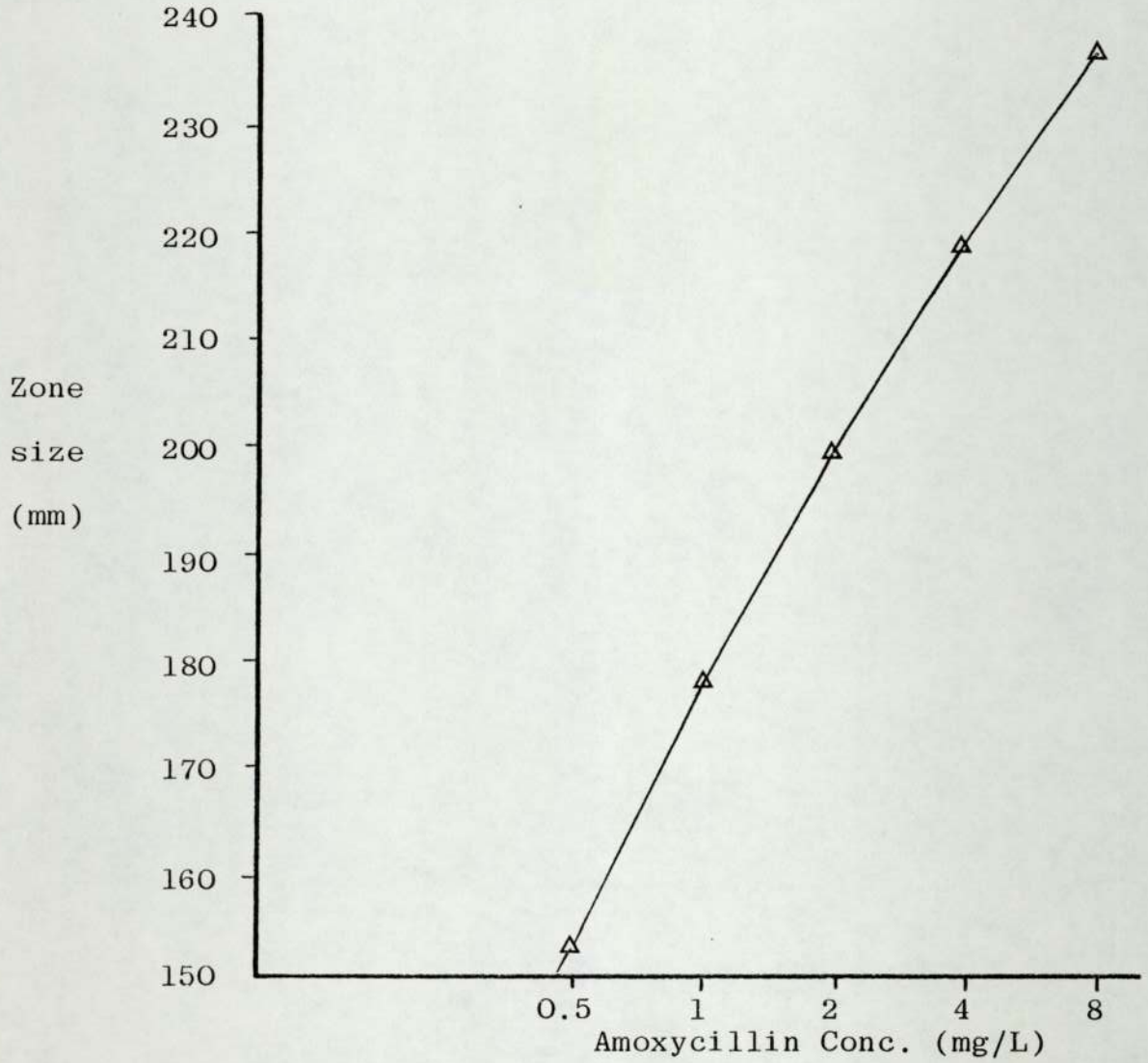
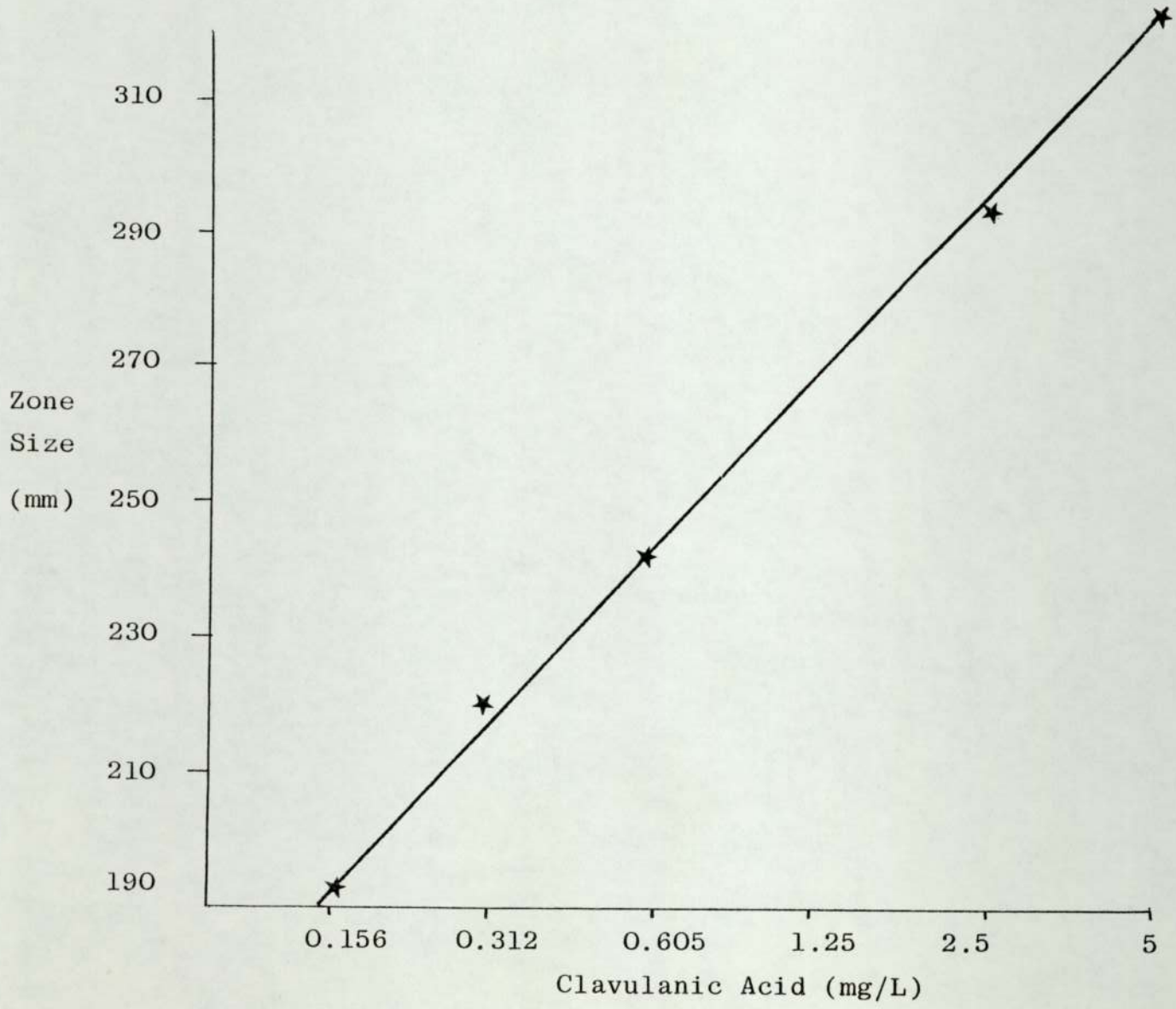


FIGURE 16. GRAPH OF CLAVULANIC ACID CONCENTRATION v ZONE SIZE



Clavulanic Acid Assay.

Standard (mg/L)	Zone size (mm)	Average (mm)
0.156	190, 193, 193	192
0.312	220, 221, 225	222
0.625	243, 241, 241	241.6
1.25	272, 265, 260	265.6
2.5	291, 296, 288	291.6
5.0	322, 324, 320	322.

Tests	Zone size (mm)	Average (mm)	Conc(mg/L)
Amoxycillin 128			
Clavulanic Acid 8	285, 280, 280	281.6	1.85
Amoxycillin 1/			
Clavulanic Acid 8	288, 286, 287	287	2.10

Both tests were diluted 1/4 so using this factor results of:- 7.4 mg/L and 8.4 mg/L were obtained.

3.1.5.2. Discussion

In assay broth at 37°C after overnight incubation the high concentrations of amoxycillin virtually lost no activity, the small losses probably being accounted for by dilution factors. At low concentration, however, there was no detectable antibiotic present regardless of whether clavulanic acid was present or not.

Clavulanic acid, like the high conentration of amoxycillin, was virtually unaffected by overnight incubation at 37°C. Again the small differences can probably be accounted for by dilution factors.

In summary it is possible that the chambers of the cuvette containing low concentrations of amoxycillin initially could show regrowth of bacteria due to the amoxycillin decaying and the presence of bacterial persisters. This is possibly why the lac -ve strain of E. coli exposed to amoxycillin and clavulanic acid eventually grew in the chamber that had contained 1 mg/L amoxycillin after 15 hrs (see Fig 12), because the clavulanic acid remaining at 8 mg/L would not be antibacterial.

3.1.6. Tubidimetric Monitoring of "PBP probes".

Experiments were set up as described in section 2.2.5 using the lac -ve *E.coli* and the lac +ve TEM 1 *E.coli* as bacterial strains.

3.1.6.1. Clavulanic Acid Alone.

Figure 17 shows the growth curves produced when the lac -ve strain of *E.coli* was exposed to assay broth containing doubling dilutions of clavulanic acid 256-0.5 mg/L. A turbidimetric M.I.C. of 32 mg/L is produced and a M.A.C. of 16 mg/L.

Figure 18 shows the growth curves produced when the lac +ve strain of *E.coli* was exposed to the same series of concentrations of clavulanic acid. An identical result for turbidimetric M.I.C. and M.A.C. as that given with the lac -ve strain was produced.

Discussion.

These two results confirm what was shown in the chessboard titration (see section 3.1.2.1., Figs. 9 and 10) that the antibacterial action of clavulanic acid alone is very weak. This has also been shown by Comber et al. (1980).

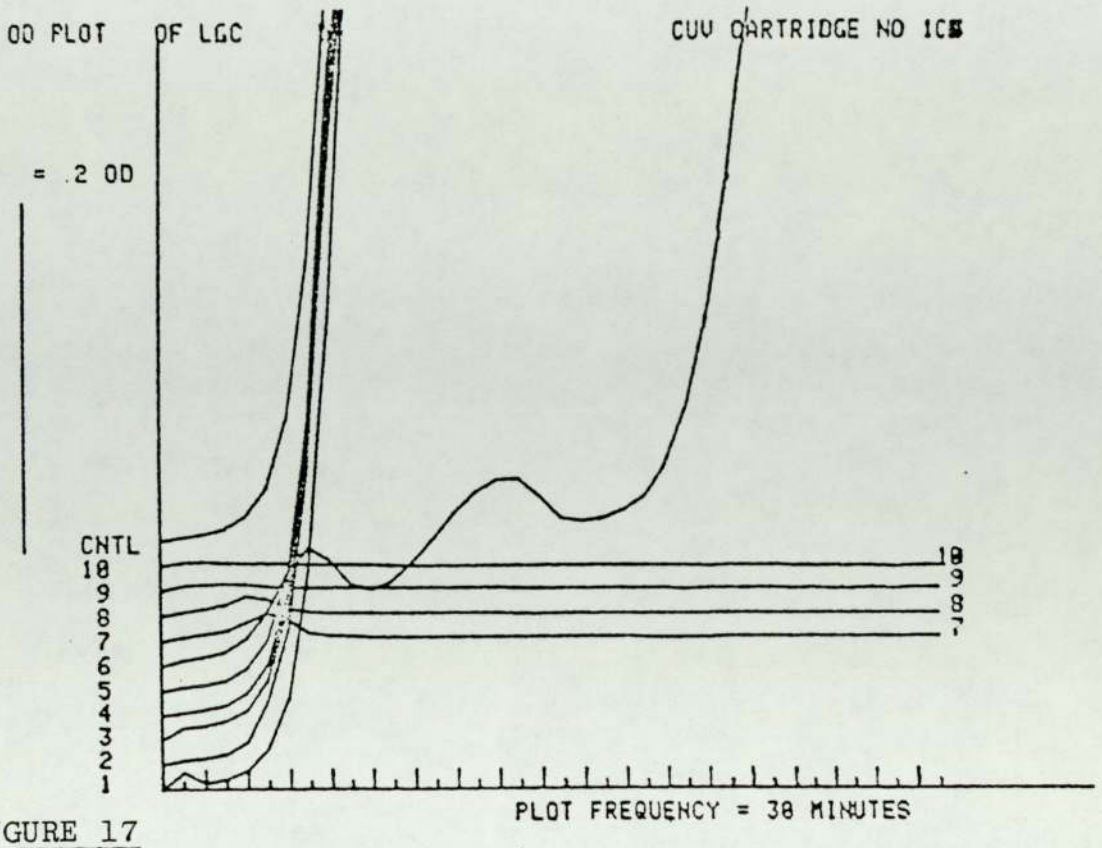


FIGURE 17

ESCHERICHIA COLI (B-LASE - NEG) 10^4orgs/ML V CLAVULANIC ACID 256 - 0.5 mg/L

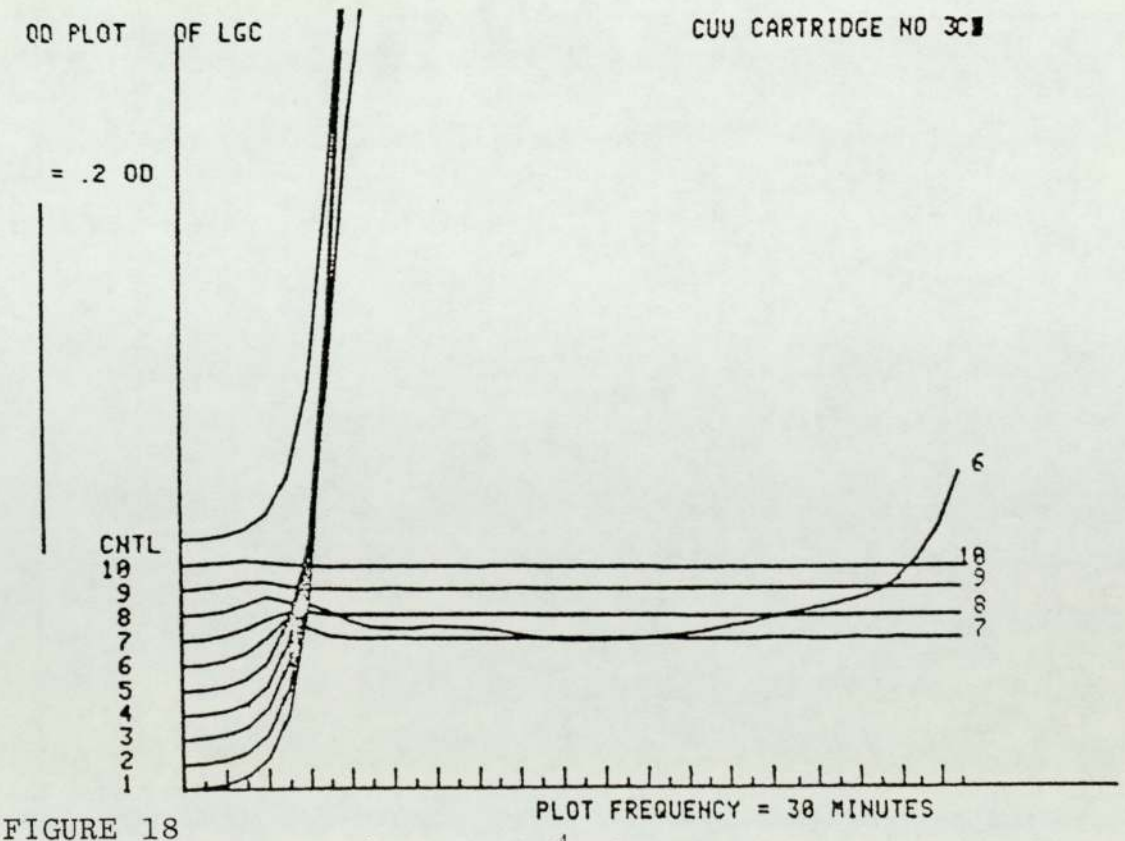


FIGURE 18

ESCHERICHIA COLI (B-LASE - TEM 1) 10^4orgs/ML V CLAVULANIC ACID 256 - 0.5 mg/L

3.1.6.2. Studies with ticarcillin.

Figure 19 shows the growth curves produced by the lac -ve E.coli in the presence of assay broth containing doubling dilutions of ticarcillin (256-0.5 mg/L) alone. It can be seen that growth of E.coli eventually occurred at all concentrations of the drug resulting in a turbidimetric M.I.C. of >256 mg/L. However, in all but the lowest two concentrations of antibiotic, this breakthrough did not occur until 12 hours or more had elapsed. This is reflected in the M.A.C. of 2 mg/L.

In contrast, when clavulanic acid was also present (at 8 mg/L) in the tubes - Figure 20 - this breakthrough was stopped in all but the lowest concentrations giving a turbidimetric M.I.C. of 2 mg/L and a M.A.C. of <0.5 mg/L.

When the lac +ve strain of E.coli was used the growth curves shown in Figures 21 and 22 were produced. Ticarcillin alone proved to be susceptible to beta-lactamase attack resulting in a turbidimetric M.I.C. of >256 mg/L and a M.A.C. of >256 mg/L. However, when clavulanic acid was also present these values were reduced in both cases to <0.5 mg/L.

Discussion.

Ticarcillin has greatest affinity for PBPs 1a and 3 (Curtis et al. 1979). Initially, this results in filaments being produced by the organism, followed by spheroplast formation and eventual lysis. This sequence of events occurs when there is no beta-lactamase present. The results shown in Figure 19 show that enough of the organisms survive

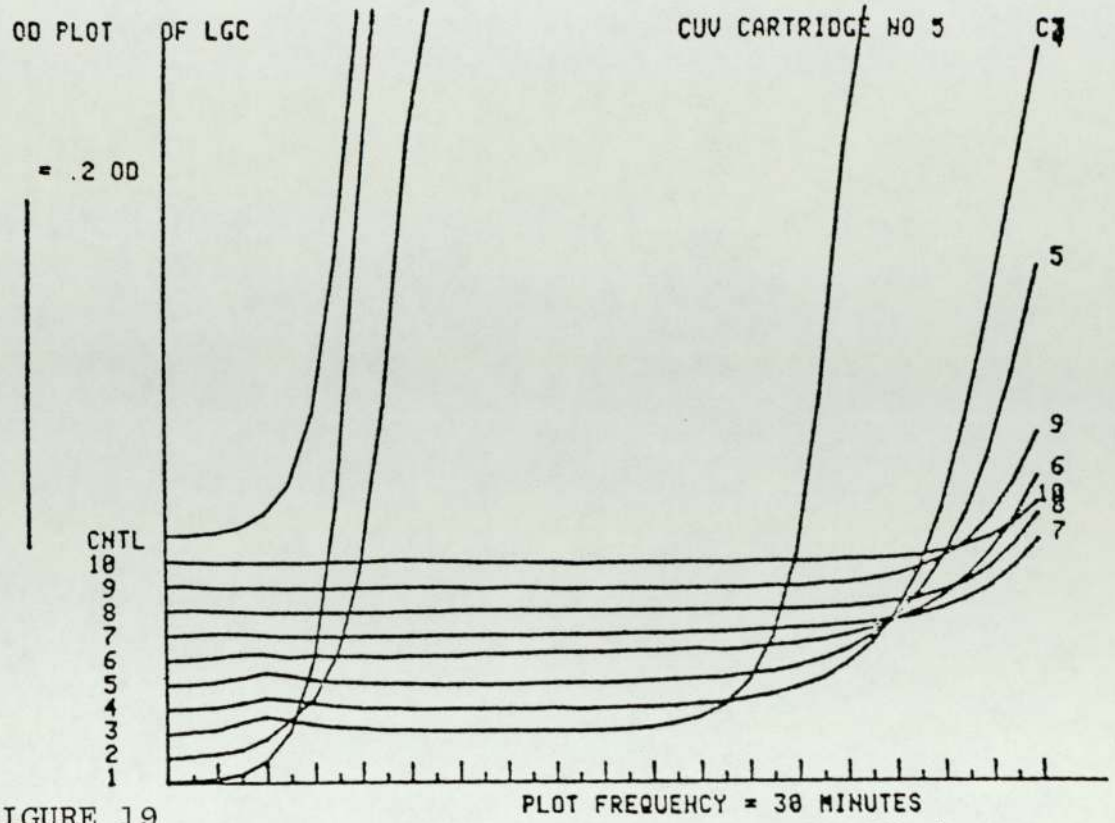


FIGURE 19

ESCHERICHIA COLI (BLASE - NEG) V TICARCILLIN 256 - 0.5 mg/L

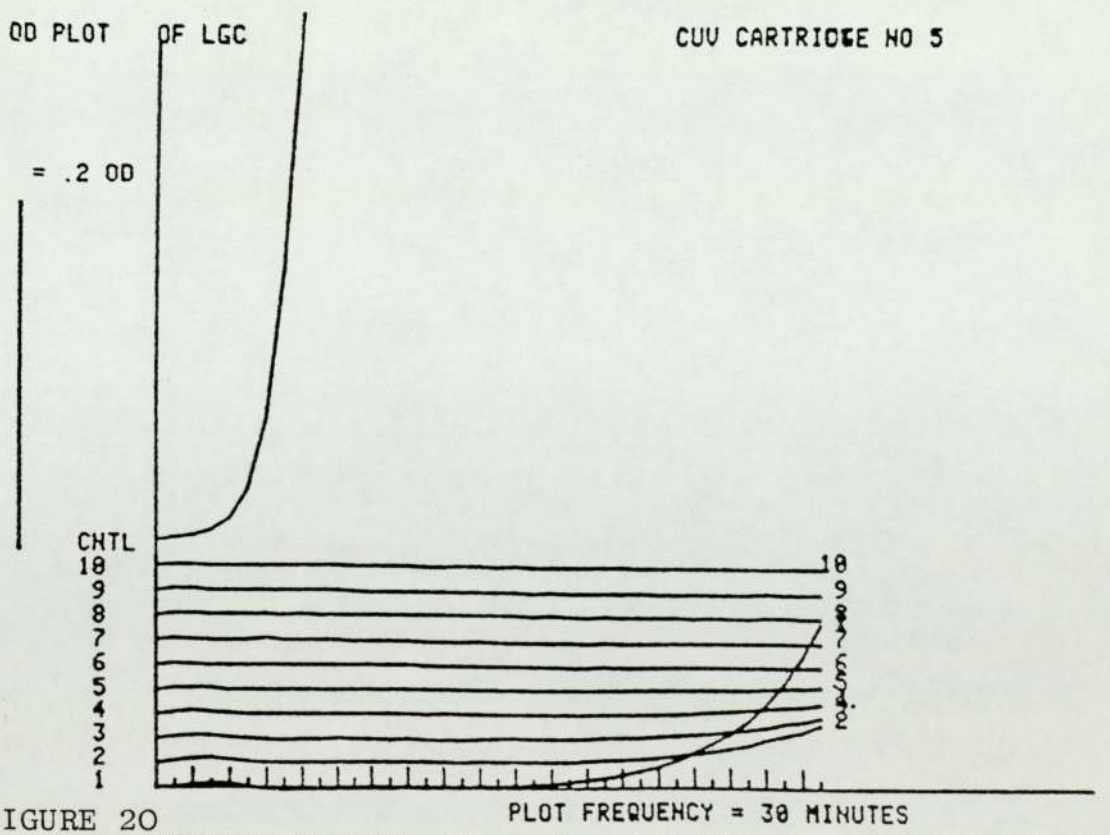


FIGURE 20

ESCHERICHIA COLI (BLASE - NEG) V TICARCILLIN 256 - 0.5 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

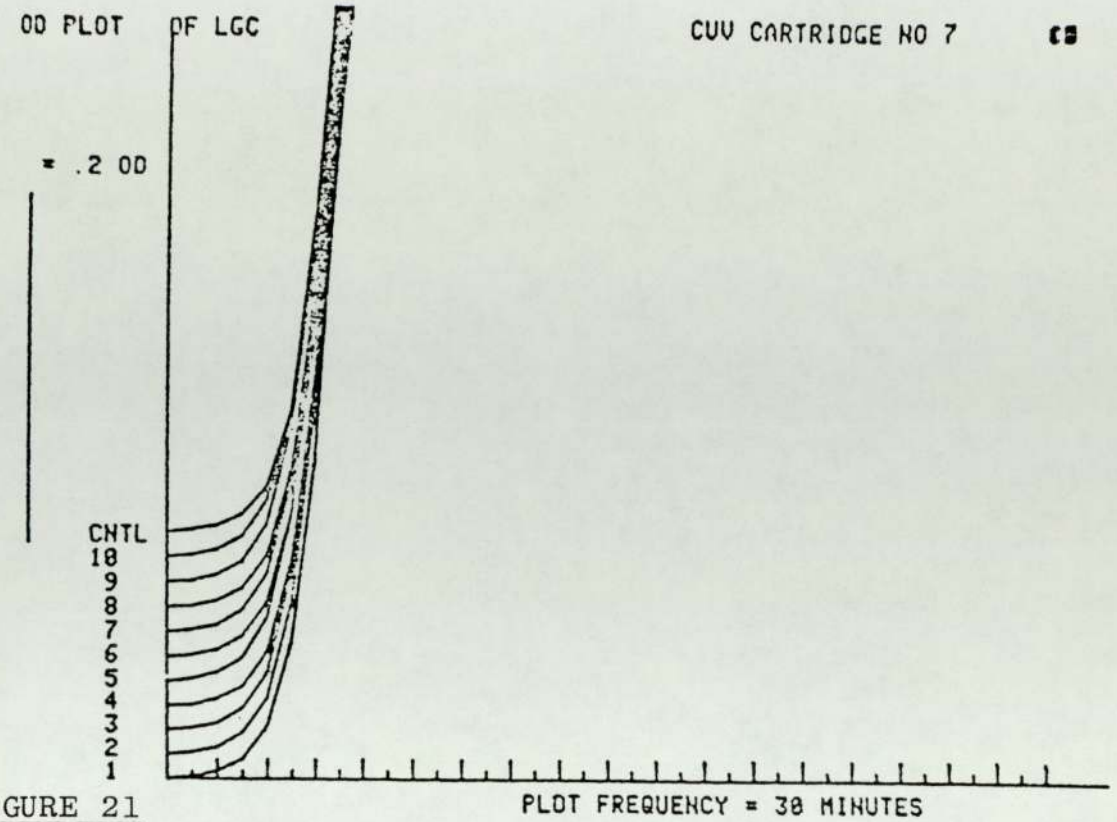


FIGURE 21

ESCHERICHIA COLI (BLASE - TEM 1) V TICARCILLIN 256 - 0.5 mg/L

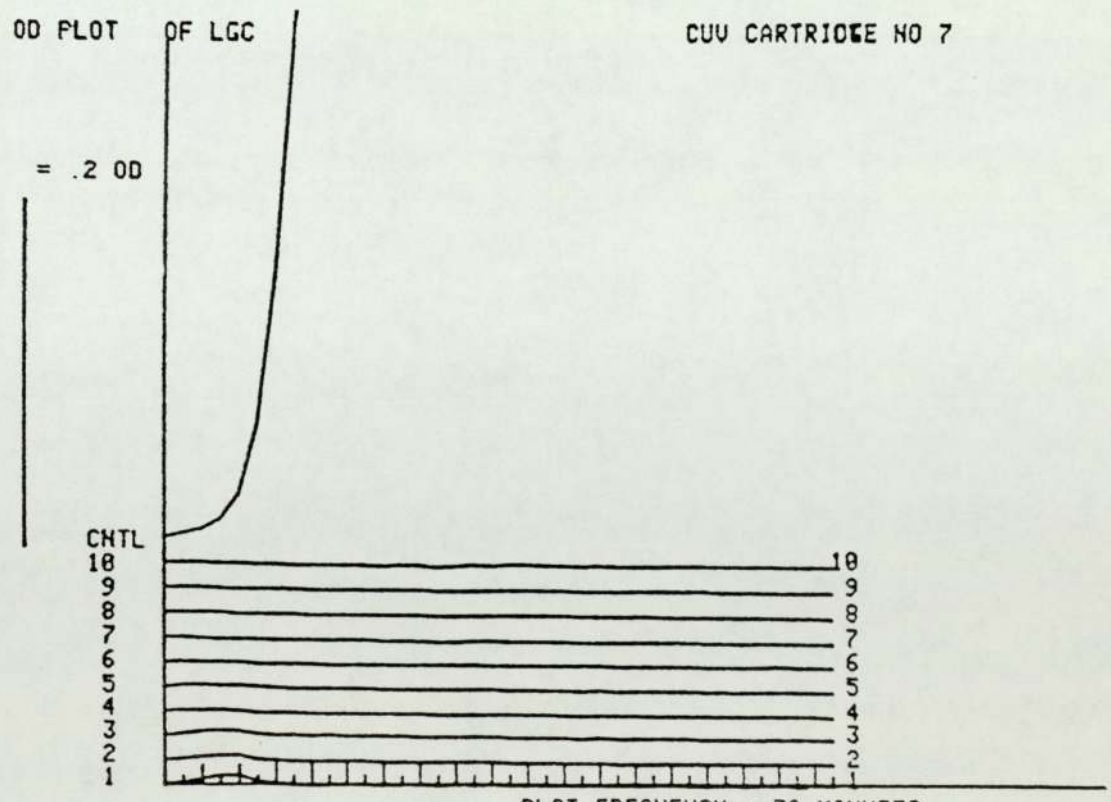


FIGURE 22

ESCHERICHIA COLI (BLASE - TEM 1) V TICARCILLIN 256 - 0.5 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

this attack to eventually break through at all concentrations. This effect is not seen with conventional agar incorporation techniques for M.I.C. determination. It is probably due to progressive antibiotic inactivation with increasing incubation time. Sherris and his co-workers (1967) compared broth and agar dilution methods in relationship to duration of incubation. They found the M.I.C.s increased twofold or more with the broth dilutions more than twice as often with prolonged incubation as compared with the changes between 12 and 24 hours with agar dilutions. If the growth curves shown in Figure 19 had been read after 14 hours incubation a turbidimetric M.I.C. of 4 mg/L would have been recorded agreeing to within one dilution to the agar incorporation M.I.C. of 2 mg/L (experimental details not shown).

Addition of clavulanic acid to the system caused a dramatic drop in the turbidimetric M.I.C. and M.A.C. suggesting again that a synergy was taking place between the two compounds due to the fact that clavulanic acid was binding to PBP2 in addition to the ticarcillin binding to PBPs 1A and 3. This two-pronged attack results in far fewer persisters being in a position to take advantage of any antimicrobial inactivation due to length of incubation. Figure 20 shows that any breakthrough is confined to only the lowest dilutions after 18 hrs incubation.

In the case of the lac +ve strain, ticarcillin on its own is ineffective due to destruction of the antibiotic by the beta-lactamase. Addition of clavulanic acid resulted in a dramatic drop in the turbidimetric M.I.C. reflecting not only the inactivation of the beta-lactamase by clavulanic

acid but also, perhaps, the PBP synergy between the two compounds.

3.1.6.3. Studies with Cefsulodin.

Curtis et al. (1979) showed that cefsulodin has very high affinity for PBPs 1a and 1b and little affinity for the other PBPs. It causes spheroplasting and rapid lysis of the bacterial cell.

Figure 23 shows the growth curves produced by the lac -ve E.coli when it was exposed to doubling dilutions of cefsulodin (256-0.5 mg/L). A turbidimetric M.I.C. of 32 mg/L was obtained and an M.A.C. of 16 mg/L. Figure 24 shows the growth curves produced when 8 mg/L of clavulanic acid was added to the cefsulodin concentration. A turbidimetric M.I.C. of 16 mg/L was obtained with an M.A.C. of <0.5 mg/L.

When the lac +ve strain of E.coli was exposed to cefsulodin alone the growth curves shown in Figure 25 were produced. These illustrate a turbidimetric M.I.C. of 128 mg/L with an M.A.C. of 32 mg/L. Addition of clavulanic acid again produced a dramatic effect (Fig. 26) resulting in a turbidimetric M.I.C. of 8 mg/L and an M.A.C. of < 0.5 mg/L.

Discussion.

As would be expected, addition of clavulanic acid to the cefsulodin when a lac +ve strain was undergoing test resulted in a considerable drop in the tubidimetric M.I.C. In fact the M.A.C. results show that the combination was affecting the organism even when cefsulodin was present at only low concentration.

With the lac -ve strain only a one dilution drop in turbidimetric M.I.C. was seen when clavulanic acid was added. This is usually accepted as being within

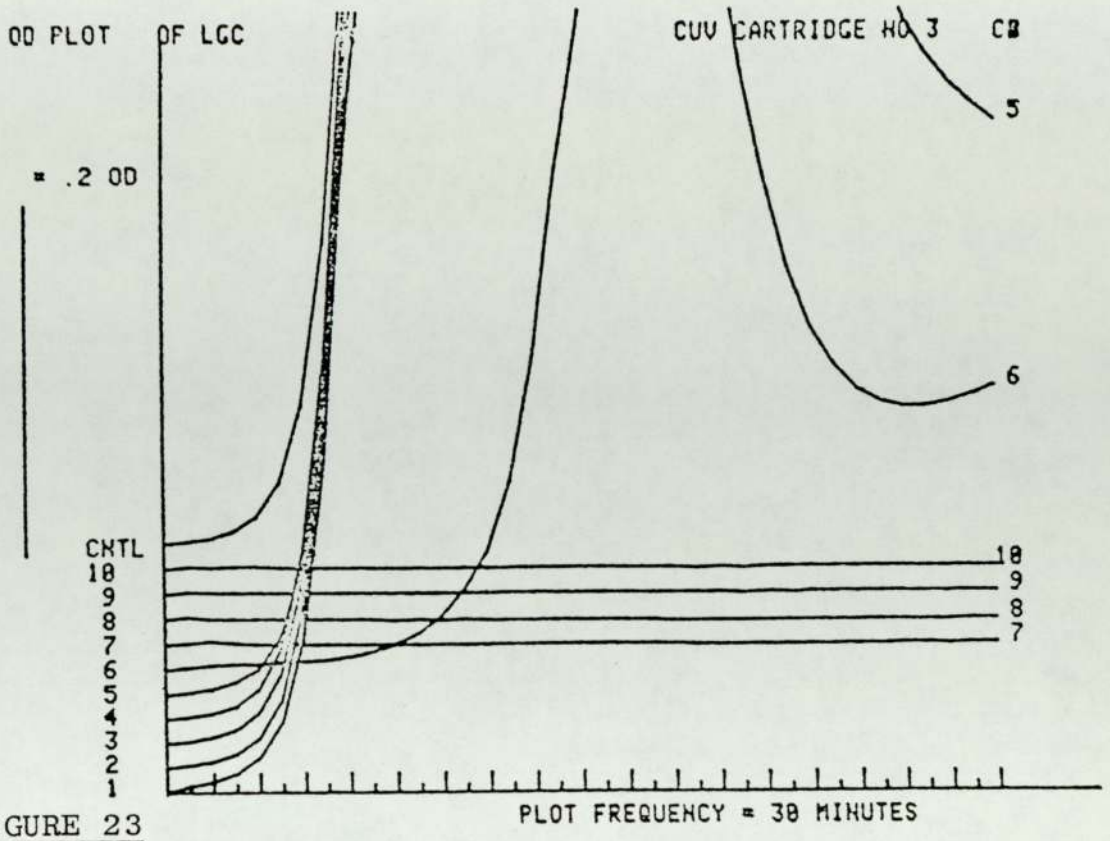


FIGURE 23

ESCHERICHIA COLI (B-LASE - NEG) V CEFSULODIN 256 - 0.5 mg/l

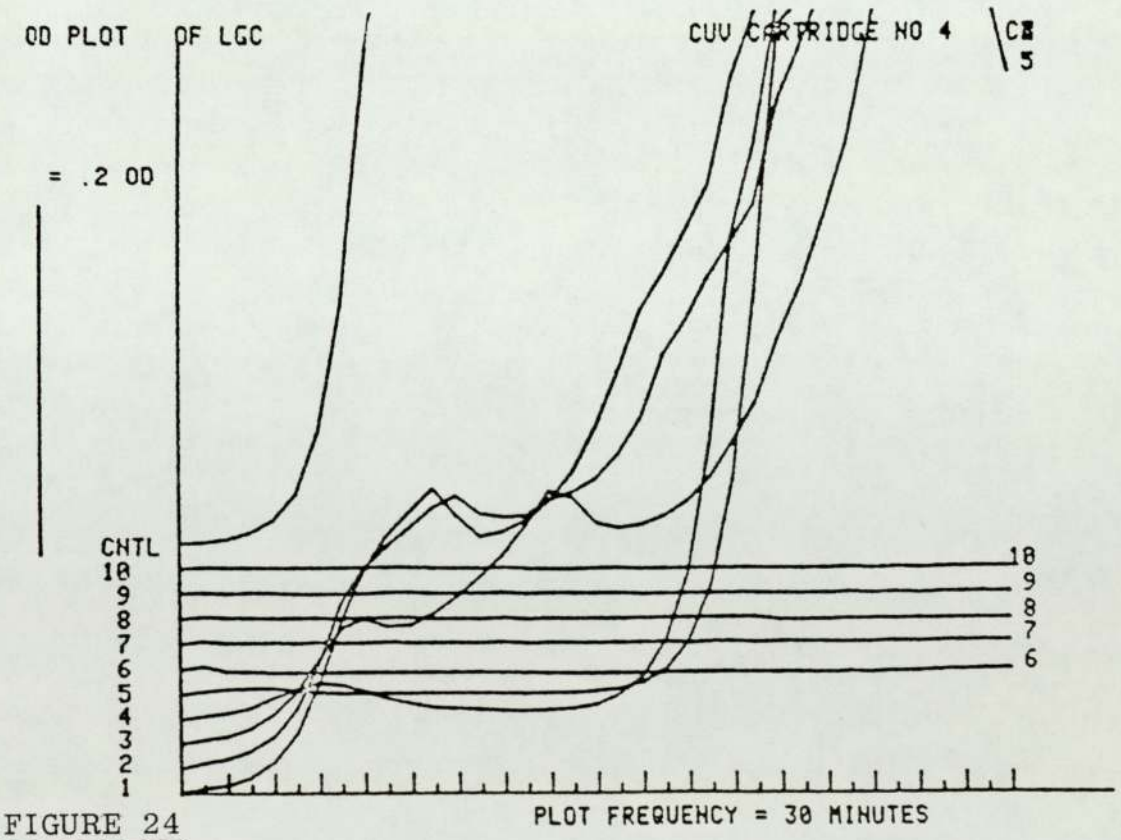


FIGURE 24

ESCHERICHIA COLI (B-LASE - NEG) V CEFSULODIN 256 - 0.5 mg/l. IN THE PRESENCE OF 8 mg/l. CLAVULANIC ACID

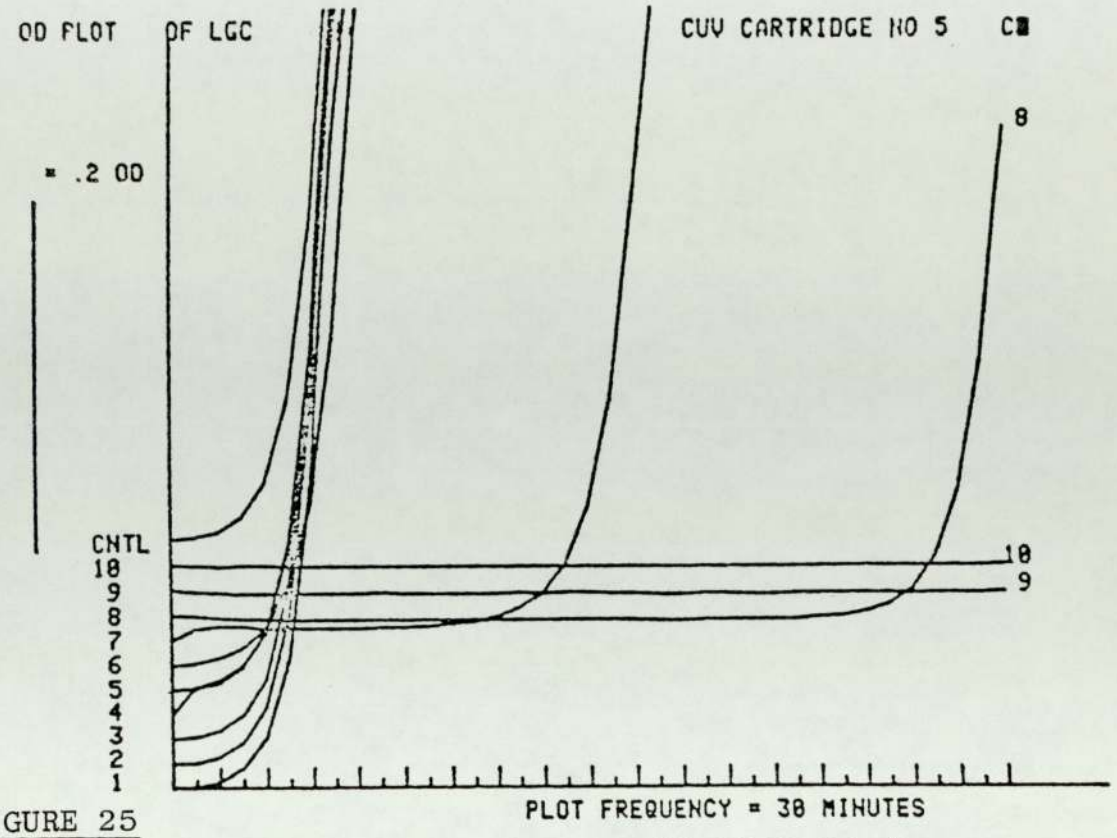


FIGURE 25

ESCHERICHIA COLI (β-LASE - TEM 1) V CEFSULODIN 256 - 0.5 mg/l.

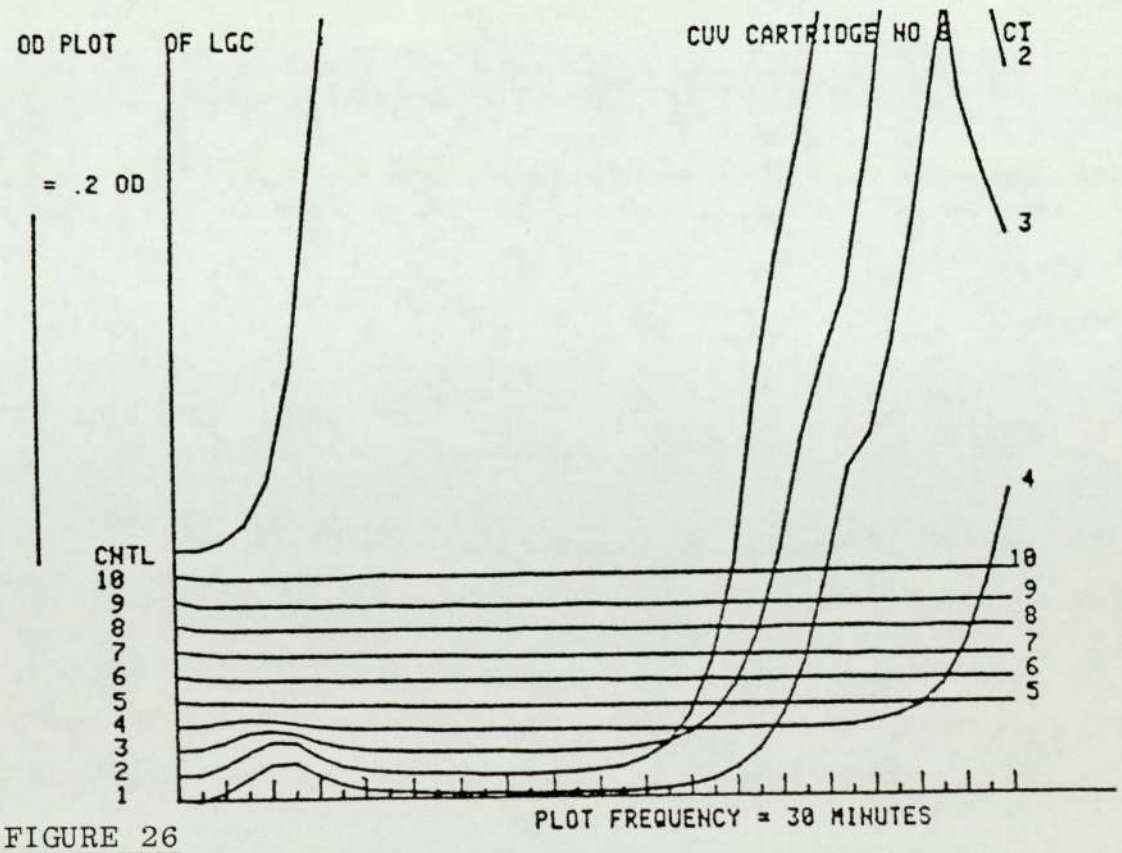


FIGURE 26

ESCHERICHIA COLI (β-LASE - TEM 1) V CEFSULODIN 256 - 0.5 mg/l. IN THE PRESENCE OF 8 mg/l. CLAVULANIC ACID

experimental error, however, if the M.A.C.s are compared a dramatic difference is seen indicating a synergy between the two compounds once again. The fact that the organism was able to recover from this attack up to 16 mg/L could either be due to the cefsulodin becoming unstable at lower concentrations or possibly the fact that in this case only PBPs 1 and 2 will have been bound compared to PBPs 1, 2 and 3 having been bound when clavulanic acid was added to amoxycillin or ticarcillin.

3.1.6.4. Studies with Aztreonam.

Aztreonam binds specifically to PBP 3 (Curtis - personal communication) causing filamentation and eventual lysis.

Figure 27 shows the growth curves produced when the lac -ve strain of E.coli was exposed to aztreonam alone; the turbidimetric M.I.C. and the M.A.C. were both <0.5 mg/L. When clavulanic acid was added to the system (Fig.28) the turbidimetric M.I.C. increased to 64 mg/L whilst the M.A.C. remained at <0.5 mg/L.

Figures 29 and 30 show the growth curves produced by the lac +ve strain of E.coli when it was exposed, in the first case, to aztreonam alone and, in the second, aztreonam and clavulanic acid. In both cases a turbidimetric M.I.C. of 128mg/L was obtained with an M.A.C. of <0.5 mg/L.

Discussion.

When the lac -ve strain was exposed to aztreonam alone it proved to be very susceptible, even at the lowest concentration there was no sign of growth after 18 hours incubation. Addition of clavulanic acid, however, caused growth to eventually occur in all but the two highest concentrations of aztreonam. This would suggest that an antagonism, rather than a synergy between clavulanic acid and aztreonam was occurring. The breakthrough in growth is not due to loss of activity on the part of aztreonam or a similar set of growth curves would have been seen when it was present alone. A possible explanation could be that although clavulanic acid primarily binds to PBP 2 it does

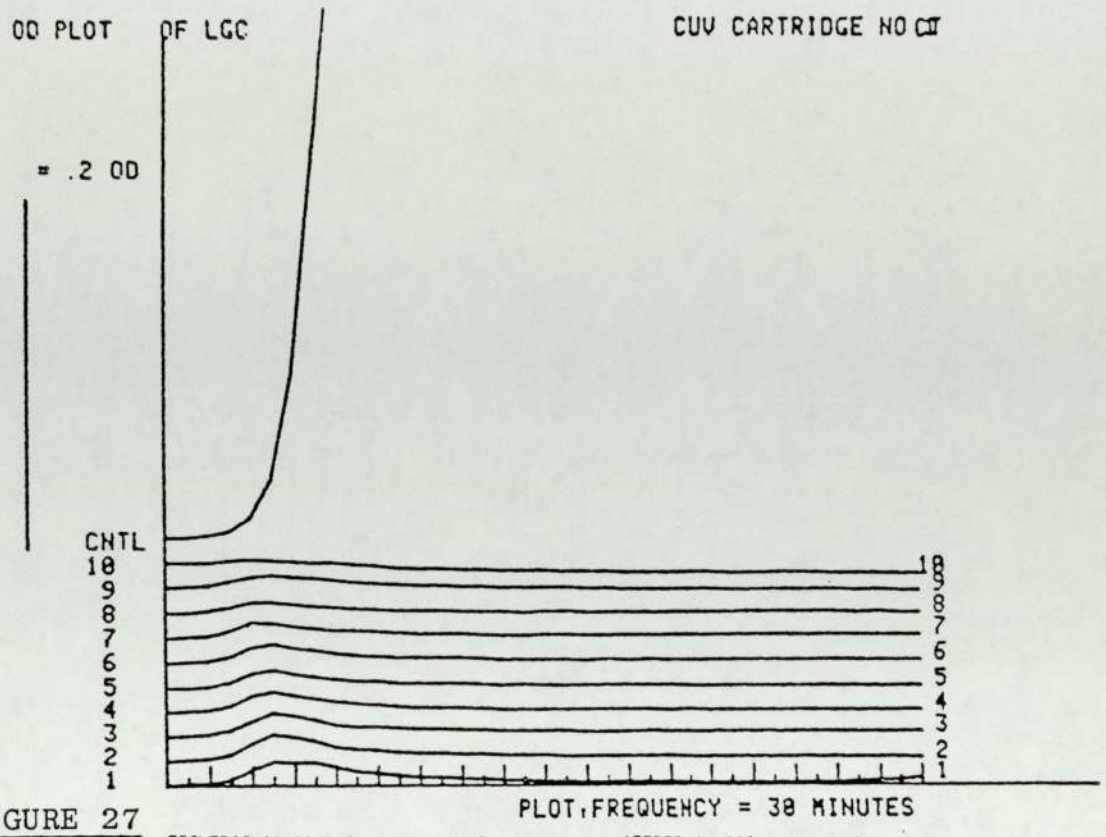


FIGURE 27

ESCHERICHIA COLI (B-LASE - NEG) V AZTREONAM 256 - 0.5 mg/L

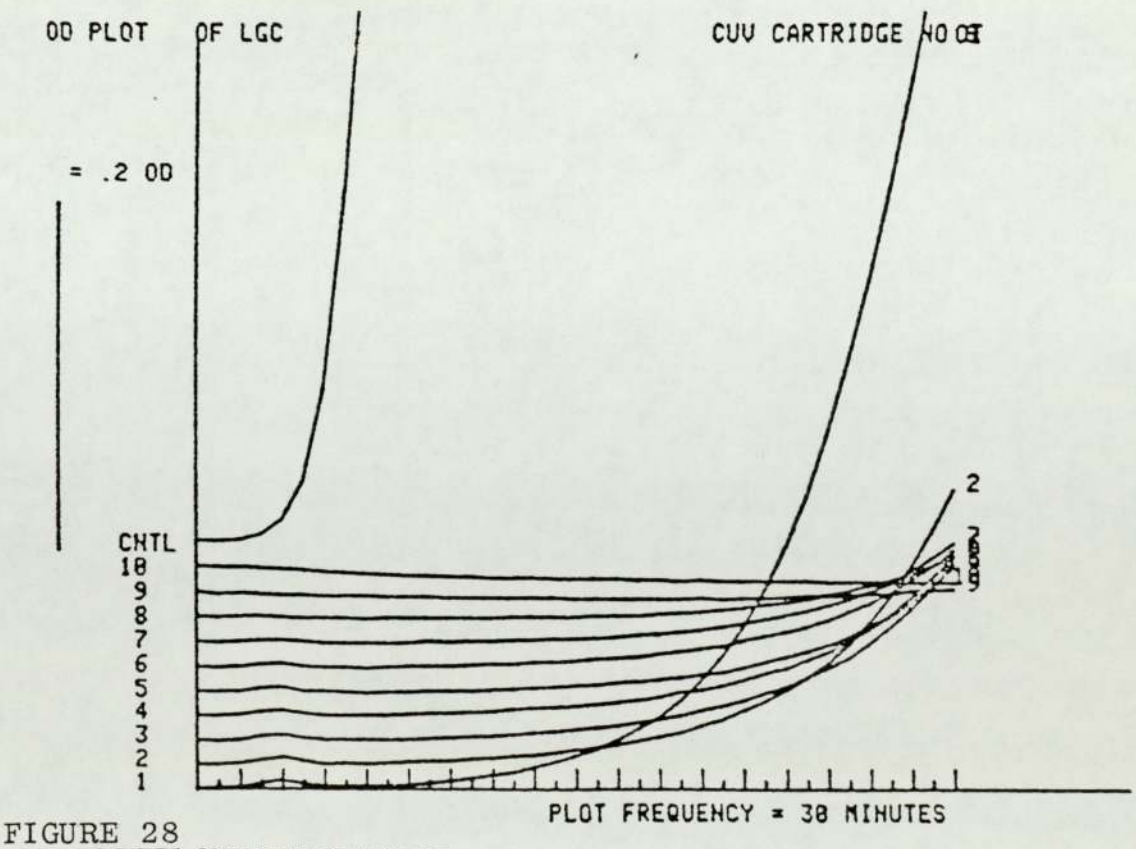


FIGURE 28

ESCHERICHIA COLI (B-LASE - NEG) V AZTREONAM 256 - 0.5 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

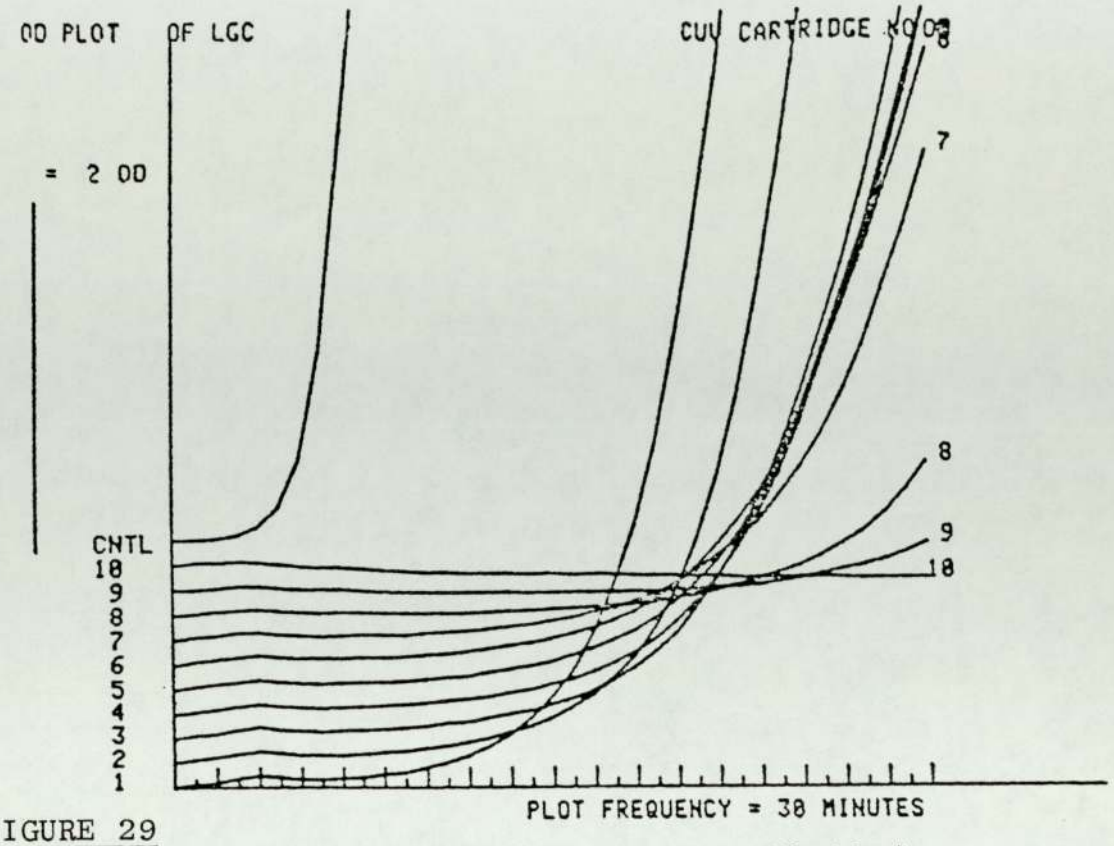


FIGURE 29

ESCHERICHIA COLI (B-LASE - TEM 1) V AZTREONAM 256 - 0.5 mg/L

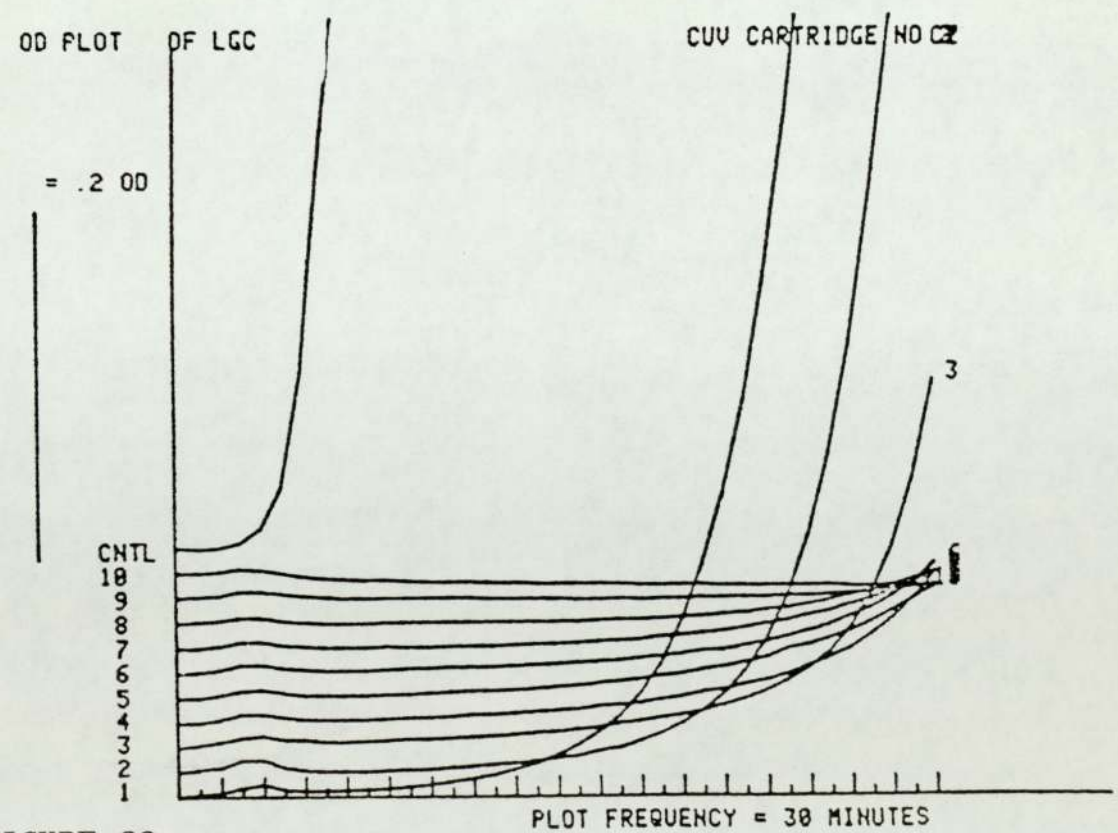


FIGURE 30

ESCHERICHIA COLI (B-LASE - TEM 1) V AZTREONAM 256 - 0.5 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

have an affinity for the other PBPs (Spratt 1977d) although PBP 3 is the one for which it has lowest affinity. Some of the bacterial cells would have had their PBP 3 bound by clavulanic acid, however, rather than by aztreonam. Eventually because there was insufficient clavulanic acid to lyse the cell these particular organisms would be able to grow again. The fact that the M.A.C. of the aztreonam was <0.5 mg/L whether clavulanic acid was present or not shows that for eight hours growth of organism was prevented.

The lac +ve strain on exposure to aztreonam alone initially responds well but after eight hours breakthrough of growth occurs presumably this is due to presence of the beta-lactamase. Perhaps it is initially inhibited only in a transient manner and eventually the enzyme is restored in an active state together with degradation products of aztreonam.

When clavulanic acid is also present the lac +ve strain again shows breakthrough after eight hours presumably due to similar reasons as those suggested for the lac -ve strain.

3.1.6.5. Studies with mecillinam.

Experiments with mecillinam were performed as described in section 2.2.2., with the exception that when clavulanic acid was present chamber 10 of the cuvette contained 8 mg/L of clavulanic acid only, in order to show that this concentration of clavulanic acid had no effect on the growth curve.

Figure 31 shows the growth curves produced by the lac -ve strain of E.coli when it was exposed to doubling dilutions of mecillinam in assay broth. A turbidimetric M.I.C. of >256 mg/L was produced with an M.A.C. of <0.5 mg/L. Breakthrough occurred first at the highest concentration.

Figure 32 shows the growth curves produced when the experiment was repeated with 8 mg/L of clavulanic acid present in the mecillinam dilutions. The curve produced by clavulanic acid alone (No.10) was identical to the control curve where no antibiotic was present. The turbidimetric M.I.C. was again >256 mg/L and the M.A.C. <1.0 mg/L; breakthrough in the lowest concentrations was delayed compared to the curves produced when mecillinam alone was present.

Figure 33 shows the growth curves produced by the lac +ve strain of E.coli when it was exposed to mecillinam alone. A turbidimetric M.I.C. of >256 mg/L once again occurred with an M.A.C. of 1 mg/L. Breakthrough in this case initially occurred at the lower concentrations, indeed the curve produced from the chamber containing 0.5 mg/L of mecillinam was identical to that given by the control. After

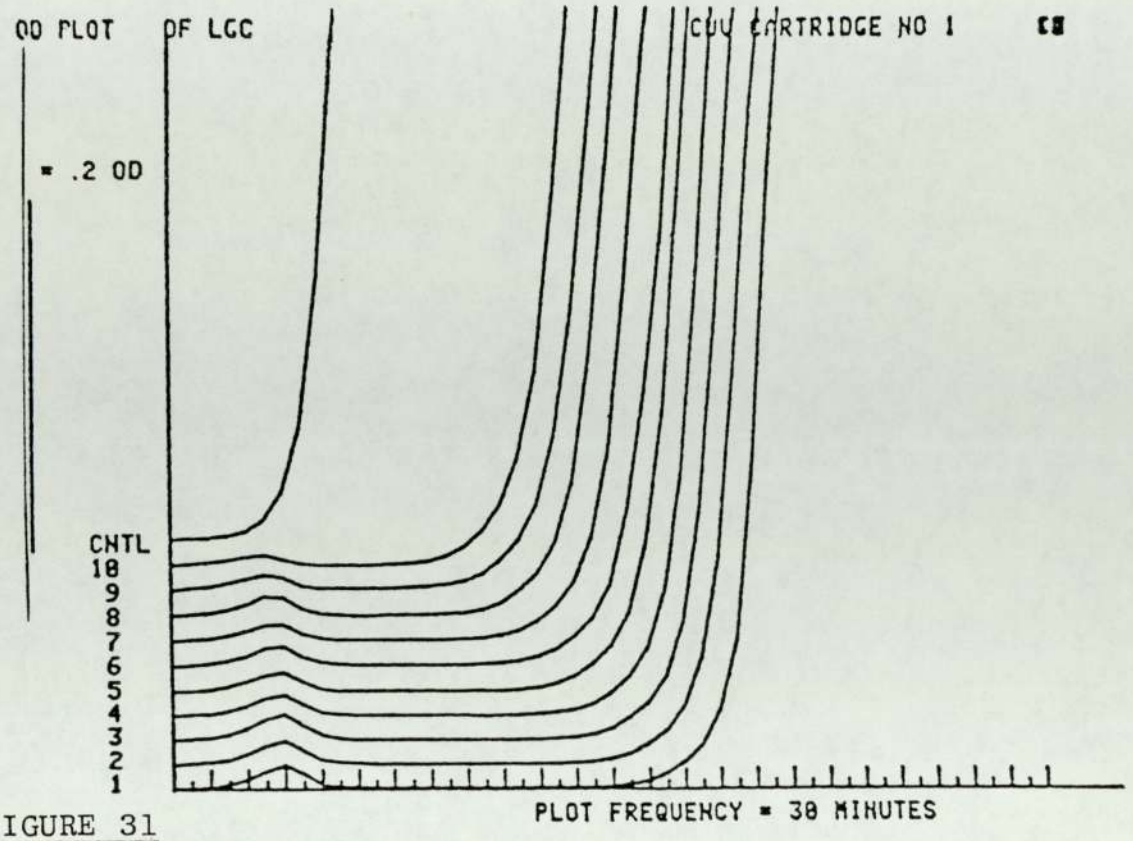


FIGURE 31

ESCHERICHIA COLI (B-LASE - NEG) V MECILLINAM 0.5 - 256 mg/L

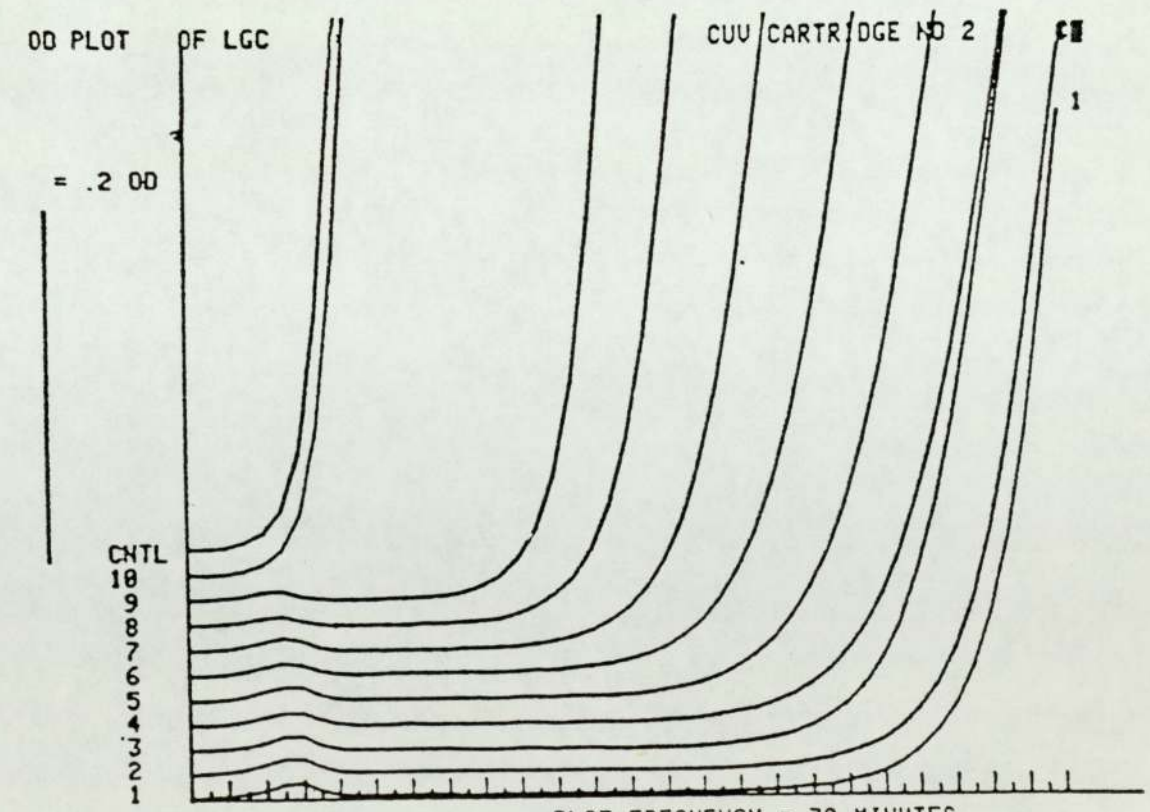


FIGURE 32

ESCHERICHIA COLI (B-LASE - NEG) V MECILLINAM 1.0 - 256 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

(CHAMBER 10 CONTAINED 8 mg/L CLAVULANIC ACID ONLY)

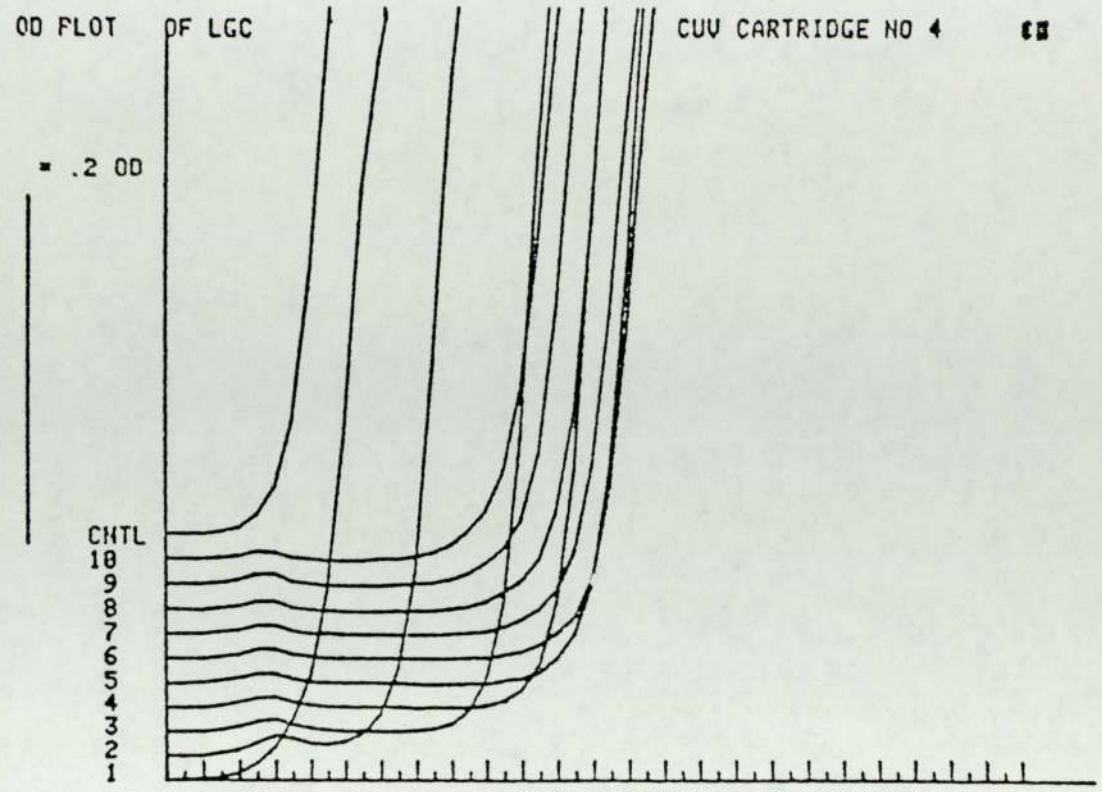


FIGURE 33

ESCHERICHIA COLI (β-LASE TEM 1) V MECILLINAM 0.5 - 256 mg/L

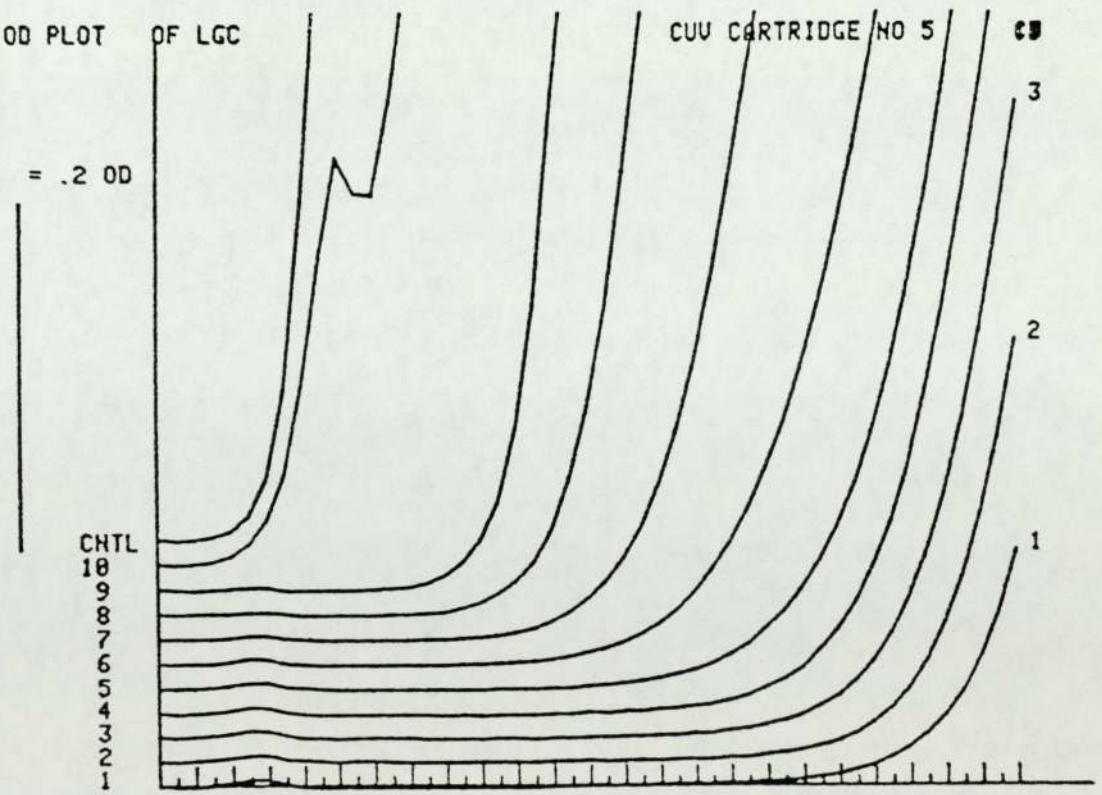


FIGURE 34

ESCHERICHIA COLI (β-LASE - TEM 1) V MECILLINAM 0.5 - 256 mg/L IN THE PRESENCE OF 8 mg/L CLAVULANIC ACID

(CHAMBER 10 CONTAINED 8 mg/L CLAVULANIC ACID ONLY)

nine hours breakthrough began occurring from the highest concentration down in a similar manner to the pattern given by the lac -ve strain.

Figure 34 shows the growth curves produced by the lac +ve strain when it was exposed to doubling dilutions of mecillinam and a standard amount of clavulanic acid in assay broth. Clavulanic acid alone (curve No. 10) again had no effect on the curve compared to the control.

A turbidimetric M.I.C. of >256 again occurred with an M.A.C. of <1.0 mg/L. Breakthrough in this case again was from the highest concentrations downwards.

Discussion.

Mecillinam is unique amongst the beta-lactams in that it binds specifically to PBP 2 and no others (Spratt 1977; Curtis et al. 1979). This means it was in direct competition with clavulanic acid for binding sites and if the theory of 'PBP synergy' is correct this should have been reflected in the growth curves.

When mecillinam was present alone the lac -ve strain of E.coli exhibited a type of 'Eagle effect', i.e. increasing the concentration of mecillinam present caused growth to occur rather than destroying it. This may be due to the fact that at higher concentrations of mecillinam PBP 2 will be bound more rapidly, yet the large spherical cells produced by this continue to grow and divide (Greenwood 1982).

When clavulanic acid was also present there was no change in the growth curves produced in the presence of high concentrations of mecillinam but at lower concentrations,

the time to breakthrough was delayed. This could be due to many more of the cells having their PBP 2 bound by clavulanic acid as there was more of this substance than mecillinam present, thus delaying the formation of sufficient numbers of the large spherical cells to cause the turbidity to increase.

With the lac +ve strain the beta-lactamase being produced was able to hydrolyse the mecillinam when it was present at low concentrations (Richmond 1977) and thus allow breakthrough to occur earlier; as the concentration of mecillinam increased, the effect of the beta-lactamase was less marked and growth curves similar to those produced by the lac -ve strain occurred.

Addition of clavulanic acid caused the beta-lactamase present to be inhibited and a set of growth curves identical to those seen with the lac -ve strain in the presence of clavulanic acid were produced.

Tybring and Melchior (1975) suggested that the production of large spherical forms of organism by mecillinam would cause the optical density to increase, when in fact, the number of colony-forming units was decreasing. As a consequence, morphology studies were performed with the lac -ve strain of E.coli in the presence of mecillinam alone and mecillinam plus clavulanic acid. As a comparison, and also to obtain more information, morphology studies were also carried out in the presence of amoxycillin and amoxycillin plus clavulanic acid.

3.1.7. Morphology Studies.

Changes in morphology were determined as described in section 2.2.8.

Figure 35 shows the morphological response of the E.coli to various concentrations of mecillinam in the absence and presence of a fixed amount of clavulanic acid.

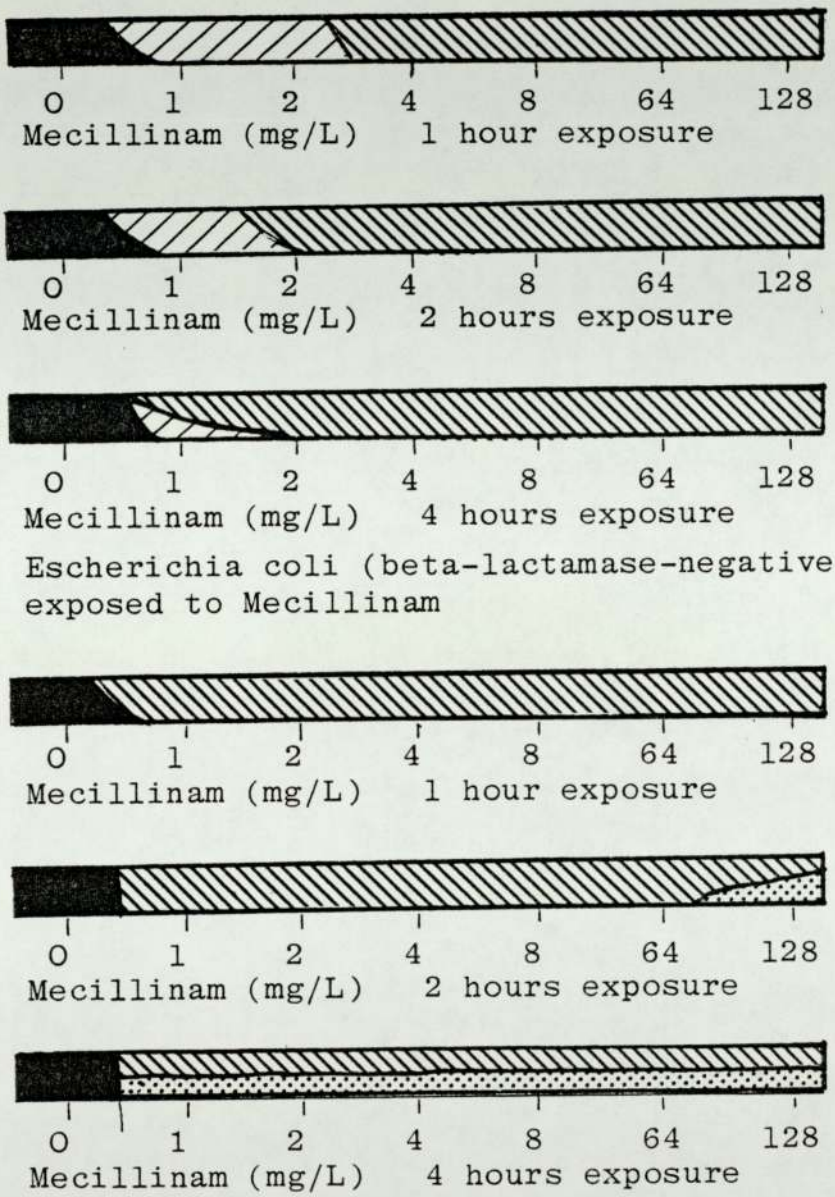
Mecillinam alone caused rounding of the cells after one hour exposure; at low concentrations this took the form of ovoid cells whilst higher concentrations formed large round cells - this was typical of what has been reported by other workers (Lund and Tybring 1972; Matsuhashu et al. 1974). Exposure for longer periods of time, up to four hours, resulted in more of these large round cells being produced.

When clavulanic acid was also present the large round cells were again formed, the only difference being that after four hours exposure fifty percent of the cells had lysed to cell debris.

Discussion.

Comparison of Figure 35 with Figures 31 and 32 shows that when mecillinam is present alone, the large round cells that are produced do not cause an increase in turbidity in the first four hours. Mecillinam presumably causes death of bacteria by osmotic rupture due to the round cells growing larger and larger. James and his co-workers (1975) have shown with electron micrographs that abortive attempts at septation occur in these large cells. If there are cells present that have a low internal osmolality then presumably these attempts at division could be successful so that

FIGURE 35. MORPHOLOGICAL RESPONSE PROFILES



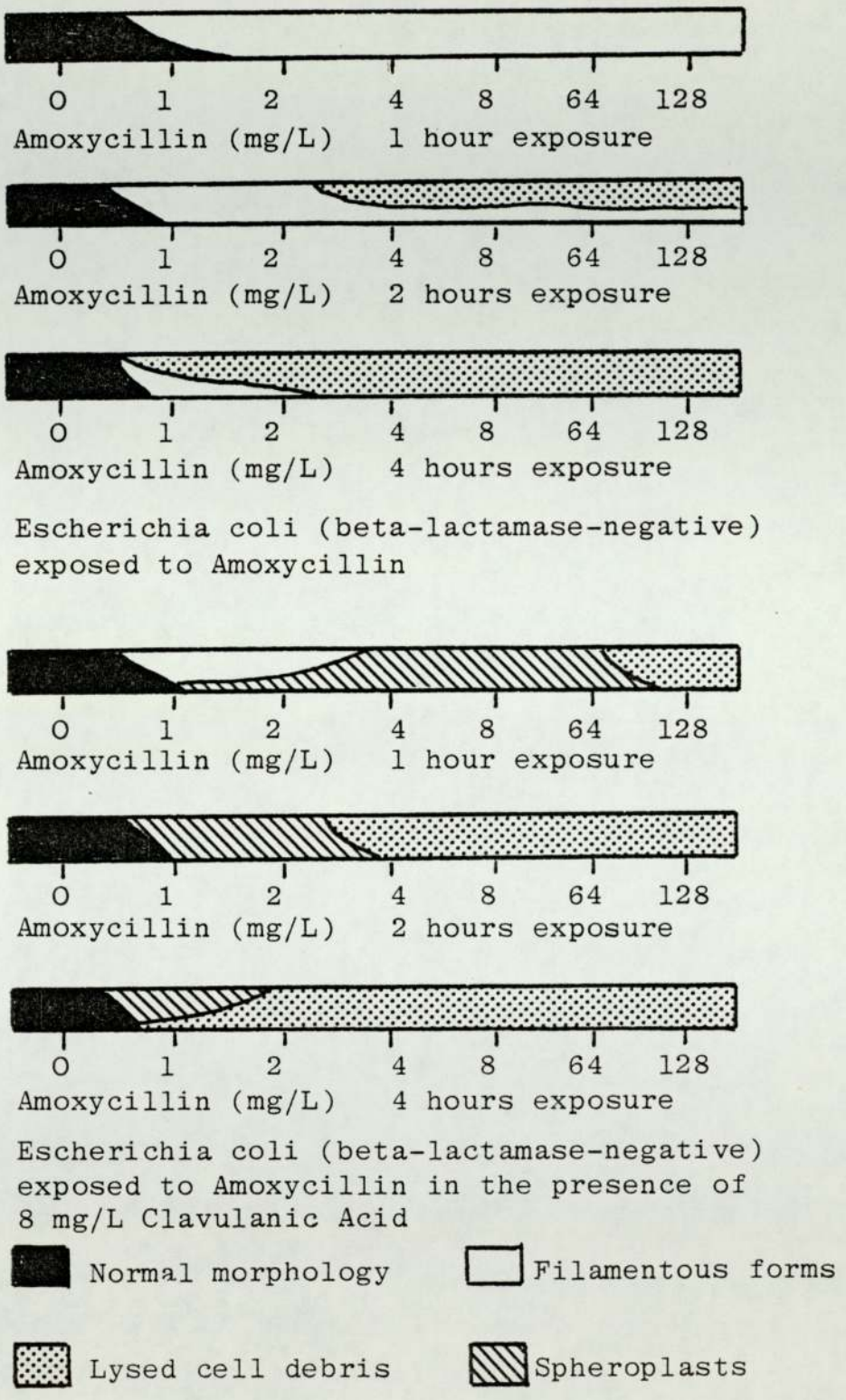
they combine to grow and divide as round cells, the delayed increase in turbidity seen with the growth curves could be a reflection of these lower internal osmolality cells beginning to increase their number.

When clavulanic acid is also present there is still no change in the growth curves over the first four hours, the lysis occurring at this time with the combination could be due to the fact that although clavulanic acid binds primarily to PBP2, it does have an affinity for the other PBPs (Spratt 1977d) unlike mecillinam (Spratt 1977c). Clavulanic acid after PBP2 shows affinity for PBPs 1, 4, 5 and 6 and least affinity for PBP 3. Possibly cells already damaged by mecillinam are more susceptible to lysis when clavulanic acid binds to the other PBPs even though the affinity for these PBPs is not great. Clavulanic acid on its own at this concentration of 8 mg/L does not affect the cell morphology (not shown in figure).

Obviously a great deal more work needs to be done to explain these events. If the growth curves shown in Figures 31 and 32 are representing resistant mutants then these should be slow growers (Matsuhashu et al. 1974; Tybring 1975) and it would be interesting to see if that was correct as well as looking at electron micrographs of the cells at the moment that turbidity begins to occur.

It is interesting, as a comparison, to look at the morphological changes produced in the lac -ve E.coli by amoxycillin in the absence and presence of clavulanic acid. Figure 36 illustrates the type of morphological response seen. Amoxycillin alone characteristically produced filamentous forms of the bacteria after one hour's exposure

FIGURE 36 MORPHOLOGICAL RESPONSE PROFILES



and these lysed over the next three hours, the ones exposed to higher concentrations being the first to lyse.

When clavulanic acid was also present, although a few filamentous forms were seen at low concentrations of amoxycillin in the first hour, the vast majority of concentrations showed spheroplasts as the response. Over the next three hours these spheroplasts lysed resulting in cell debris being the only thing visible on microscopical examination.

Spratt (1975) showed that the binding of PBP 3 resulted in the inhibition of cells division and the growth of E.coli as long filamentous cells. Binding of PBPs 1a and 1b resulted in rapid lysis of E.coli as these proteins appeared to be involved in maintaining the structural integrity of the bacterial cell.

Amoxycillin's primary affinity is directed towards PBPs 1A and 1B. However, if there is > 1 µg/ml present then binding to PBP 3 takes place also and thus the filamentous forms are seen (Curtis et al. 1979).

Clavulanic acid binding to PBP 2 in addition to the amoxycillin binding to PBPs 1 and 3 allows the development of spheroplasts rather than filamentous forms. Clavulanic acid alone at 8 mg/L did not cause any effect on the cells (not shown in figure).

3.1.8. Summary.

This section attempted to study the effect of clavulanic acid on the activity of amoxycillin against various selected strains of enterobacteria. It established the inhibitory effect of clavulanic acid on plasmid mediated beta-lactamases and a chromosomally mediated beta-lactamase belonging to Class IV. It confirmed that clavulanic acid has no effect on the class 1 chromosomally mediated beta-lactamases as seen in Ent. cloacae. These findings were illustrated by chessboard titration and turbidimetric studies. They also suggested that clavulanic acid may interact with other beta-lactams in a second manner due to it having a different binding site. The investigations into this possibility can be summarised as follows:

Antibiotic	PBP Bound	Expected result when combined with clavulanic acid	Observed result when combined with clavulanic acid
Amoxycillin	1 and 3	Synergy	Synergy
Ticarcillin	1 and 3	Synergy	Synergy
Cefsulodin	1	Synergy or indifference	Synergy
Aztreonam	3	Synergy or indifference	Antagonism
Mecillinam	2	Indifference or antagonism	Indifference/ synergy

From these results it can be seen that binding to PBP 1 needs to take place to ensure synergy. This is not surprising. Many workers (Reynolds and Chase 1981; Waxman and Strominger 1983) have shown the importance in binding to PBP 1 to produce a lethal effect. PBP 1 actually consists of two proteins (see Table 3) having distinct affinities for various beta-lactam antibiotics, termed PBP 1A and PBP 1B.

Spratt et al. (1977) suggested that because of the good correlation between cell lysis and binding to PBP 1B that this protein was the most important for cell elongation. However, Reynolds and Chase (1981) have shown that binding of PBP 1A is also important in producing the lethal effect.

Curtis et al. (1979) showed that amoxycillin's primary affinity was directed against PBPs 1A and 1B and 3 whilst ticarcillin's was directed against 1A and 3; both of these in combination with clavulanic acid showed enhanced activity against a lac -ve E.coli strain.

Cefsulodin has greatest affinity for PBPs 1A and 1B (Curtis et al. 1979) and once again combination with clavulanic acid led to enhanced activity against the lac -ve E.coli.

Mecillinam is specific in its binding to PBP 2 (Spratt 1977c) and on combination with clavulanic acid synergy was only seen when mecillinam was at low concentration and then only for a limited time.

Further work involving morphological studies and the use of PBP mutants, i.e. organisms which are deficient in one of the PBPs could elucidate whether PBPs 1A and 1B are vital to the lethal effect and if clavulanic acid's action on lac -ve strains was really due to PBP synergy.

3.2. An Investigation into the methods of determining the susceptibility of strains of bacteria to Amoxycillin in the presence of Clavulanic Acid.

3.2.1. Introduction

The previous section highlighted a problem in determining by disc diffusion techniques, the susceptibility of strains of bacteria to amoxycillin in the presence of clavulanic acid. In the case of lac +ve strains, although they were more susceptible to amoxycillin in the presence of clavulanic acid, an intermediate sensitivity, on this type of testing, was the common result. This section seeks to investigate using, for the most part 'wild strains', a number of different methods that are in laboratory use for determining susceptibility, with a view toward making recommendations as to which method should be used for testing sensitivity to amoxycillin in the presence of clavulanic acid.

3.2.2. Regression lines.

3.2.2.1. Methods and results.

The relationship between the M.I.C. of amoxycillin in the presence of clavulanic acid and the size of the zone of inhibition around a disc containing amoxycillin and clavulanic acid was investigated.

A pool of reference strains was selected which were known to have M.I.C.s in the upper, middle and lower ranges of clinically useful concentrations for most antibiotics (Ericsson and Sherris 1971). The strains are listed in Table 5.

Zone diameters and agar incorporation M.I.C.s were obtained as described in sections 2.2.1. and 2.2.2. Regression lines relating the result of the M.I.C. determination for each strain to the corresponding zone of inhibition were obtained according to the method of least squares (Colton 1974) and the corresponding correlation coefficients determined.

This technique was used with amoxycillin, augmentin (2 parts amoxycillin:1 part clavulanic acid) and, as a control of the technique, tetracycline. This latter antibiotic was chosen as Ericsson and Sherris (1971) had shown a close relation between zone size and M.I.C. with correlation coefficient $r = -0.90$ when testing it.

The regression lines produced for each of these antibiotics are shown in Figures 37, 38 and 39. The regression equations and correlation coefficients were as follows:-

FIGURE 37. REGRESSION LINE AND ERROR RATE BOUND ANALYSIS FOR THE 'ERICSSON ORGANISMS' AGAINST AMOXYCILLIN

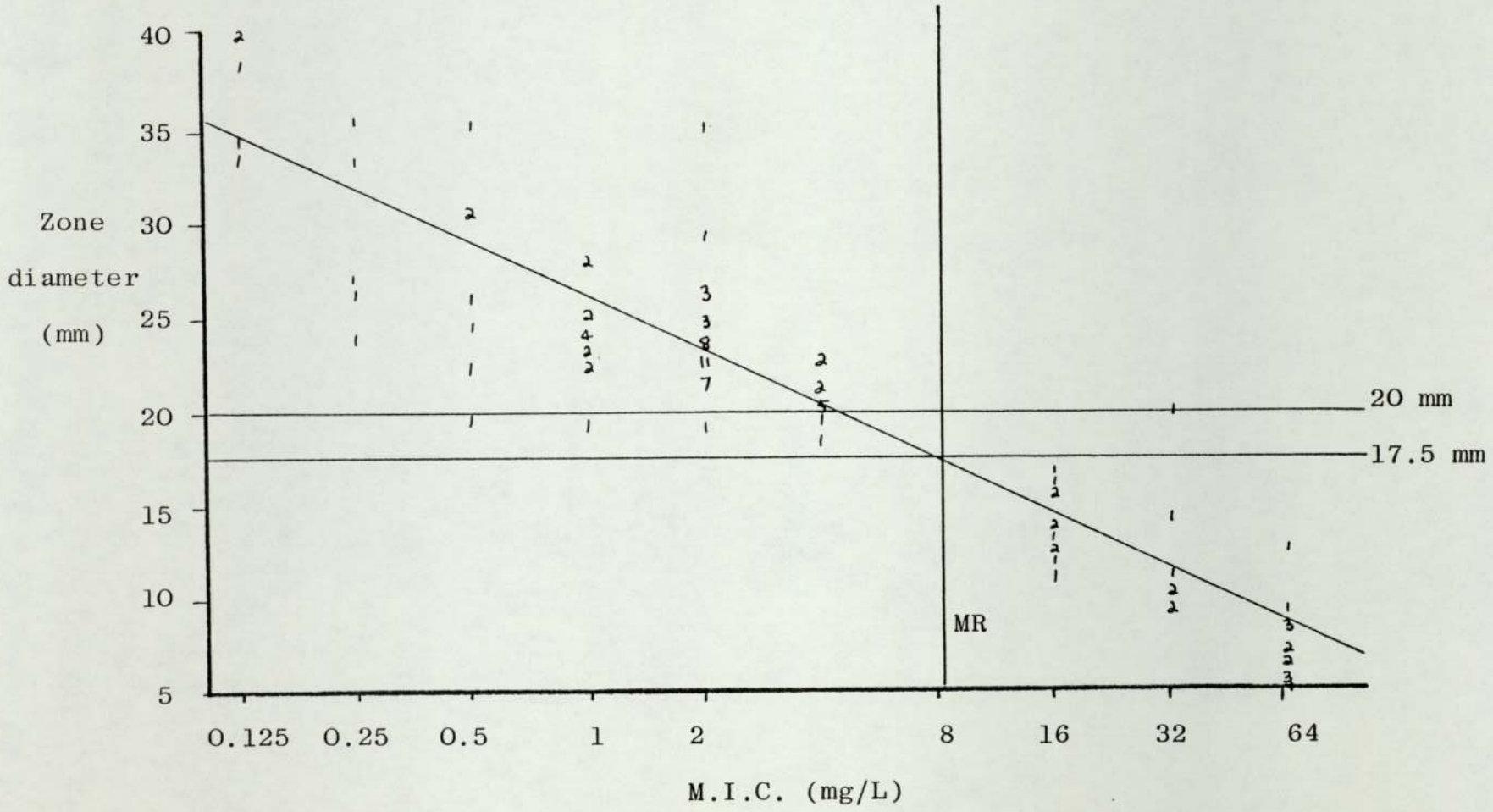


FIGURE 38 REGRESSION LINE AND ERROR RATE BOUND ANALYSIS FOR THE 'ERICSSON ORGANISMS' AGAINST TETRACYCLINE

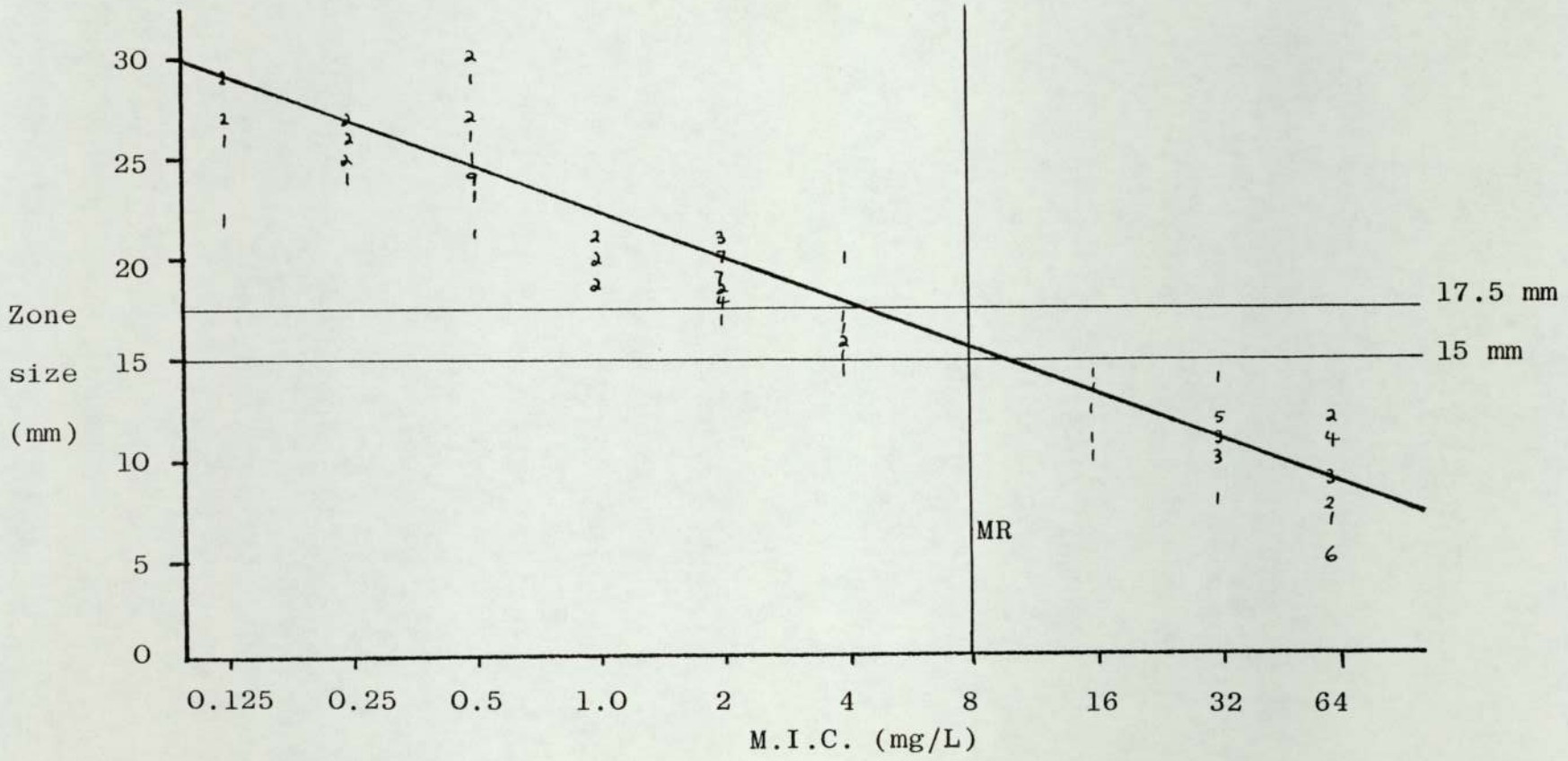
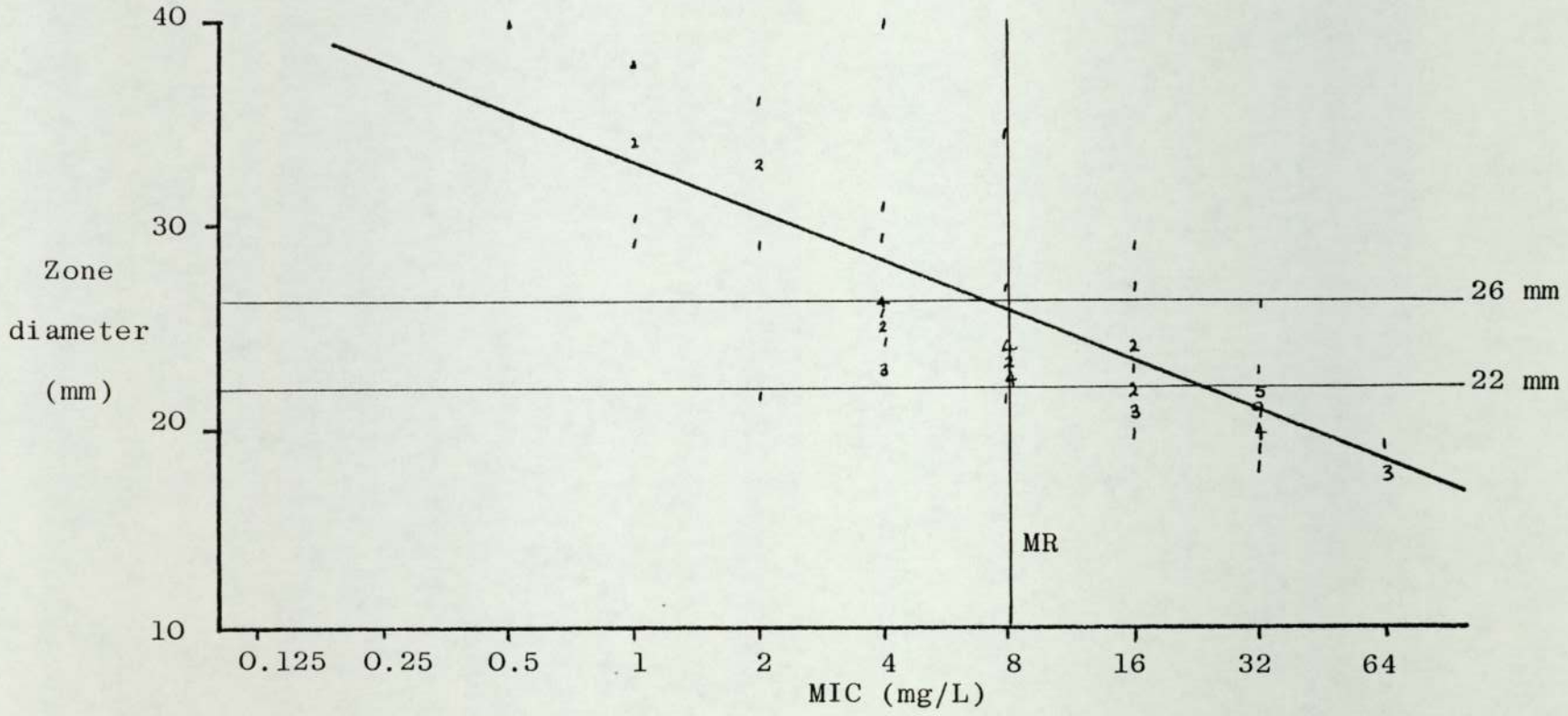


FIGURE 39. REGRESSION LINE AND ERROR RATE BOUND ANALYSIS FOR 'ERICSSON ORGANISMS' AGAINST AUGMENTIN (EXPRESSED AS CONCENTRATION OF AMOXYCILLIN COMPONENT)



Amoxycillin	y = -9.5x+25.7	r : -0.92
Tetracycline	y = -75.1x+223.6	r : -0.91
Augmentin	y = -8.2x+33.08	r : -0.80

The correlation coefficients of amoxycillin and tetracycline indicate a close relationship between the zone of inhibition around a disc and the M.I.C. of the drugs for the reference strains tested.

The correlation coefficient obtained for Augmentin, however, does not indicate a close relationship between zone size and M.I.C.

3.2.3. Error rate bounded analysis.

Metzler and DeHaan (1974) pointed out that regression line analysis is not without its faults -the effect of extreme values on the regression line e.g. very high M.I.C.s or very large zone sizes are excluded from calculations of the regression and their is no assessment of the rate of error or percentage of misclassification that will occur.

They proposed that an M.I.C. value (the MR) be chosen based on the pharmacokinetics and the indicated clinical usages of the antibiotic, that will divide strains of bacteria into a resistant group and a susceptible group, i.e. strains with M.I.C.s > MR are resistant and strains with M.I.C.s. < MR are susceptible.

Because false classification of a bacterial isolate as susceptible when it is in fact resistant is a major error they suggest a maximal tolerable rate of error of 0.01, thus no more than 1% of strains would be classified as susceptible by their zone diameter reading when in fact they were really resistant.

False classification as resistant when the strain is in fact susceptible is not as serious, so a maximal tolerable rate of error of 0.05 was suggested giving a maximum 5% of strains being classified as resistant by their zone diameter reading when in fact they were susceptible.

Having set these maximal tolerable rates of error the possible zone diameters will be divided into three intervals, susceptible, indeterminate and resistant with the indeterminate ideally as small as possible without exceeding the maximal rates of error.

Using these criteria an MR value of 8 mg/L was selected for amoxycillin, tetracycline, and Augmentin (expressed as the amoxycillin concentration). Figures 37, 38 and 39 show the zone diameter breakpoints calculated by error rate-bounded analysis. They were for the three antibiotics:

Tetracycline	$\leq$ 15 mm	and	$\geq$ 17.5 mm
Amoxycillin	$\leq$ 17.5 mm	and	$\geq$ 20.0 mm
Augmentin	$\leq$ 22.0 mm	and	$\geq$ 26.0 mm

Discussion

Brown and Blowers (1978) stated that if a regression line showed poor correlation it was indicative that disc tests by all methods with that agent - not just the ones that relied on regression lines - were of limited reliability. The correlation coefficient 'r' for Augmentin with a value of 0.80 falls into this category.

In a similar manner the percentage of strains classified as indeterminate by the error rate-bounded analysis is a measure of the strength of the relationship between M.I.C.s and zone diameters (Metzler and DeHaan 1974). If this value is small, then zone diameters are useful for classification of the susceptibility of bacteria to the antibiotic. If it is large, then the zone diameters are not useful. The percentage of isolates classified in this indeterminate category with the three antibiotics was:

Tetracycline : 7.7%

Amoxycillin : 8.1%

Augmentin : 29.4%

To see if the evidence presented here against disc

testing, by all methods, with a panel of reference strains was valid a modification of the Stokes comparative method was used to test a number of 'wild strains'.

3.2.4. Sensitivity Testing using the Comparative Method.

3.2.4.1. Escherichia coli strains.

The one hundred *E. coli* strains described in section 2.1.1. were used for these experiments. M.I.C.s were determined by agar incorporation for amoxycillin alone and in the presence of increasing amounts of clavulanic acid up to a concentration of 8 mg/L. The M.I.C.s of Augmentin (expressed as the amoxycillin concentration) were also determined. The method used was that described in section 2.2.2.

The results obtained are shown, in the form of histograms, in Figure 40 and 41.

The distribution of the M.I.C.s of amoxycillin exhibits a typical bimodal pattern, dividing the strains into sensitive and resistant populations with very few isolates falling into what could be termed an intermediate group.

As increasing concentration of clavulanic acid was added to the amoxycillin the bimodal pattern was lost until when 8 mg/L of clavulanic acid was present seventy-five of the strains were inhibited by 0.5 mg/L of amoxycillin and all of the strains were inhibited by < 8 mg/L of amoxycillin.

When Augmentin itself was tested, the M.I.C.s (expressed as the amoxycillin concentraton) showed a pattern akin to a normal distribution curve with many of the M.I.C.s falling into the 8 and 16 mg/L categories - values which would be regarded as of intermediate susceptibility.

Testing the one hundred strains for beta-lactamase production by the methods described in section 2.2.6., 52 of

FIGURE 40 100 STRAINS OF ESCHERICHIA COLI

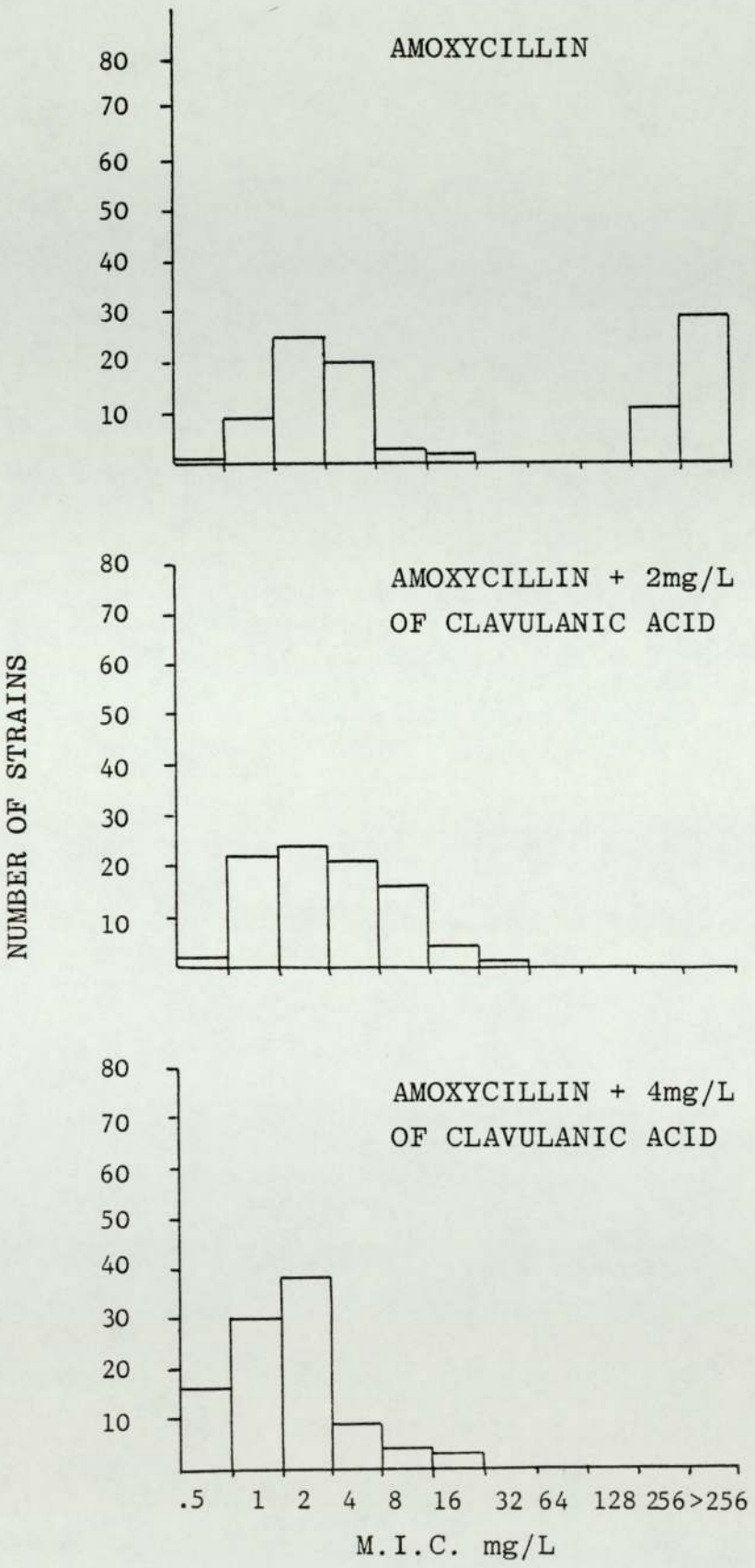
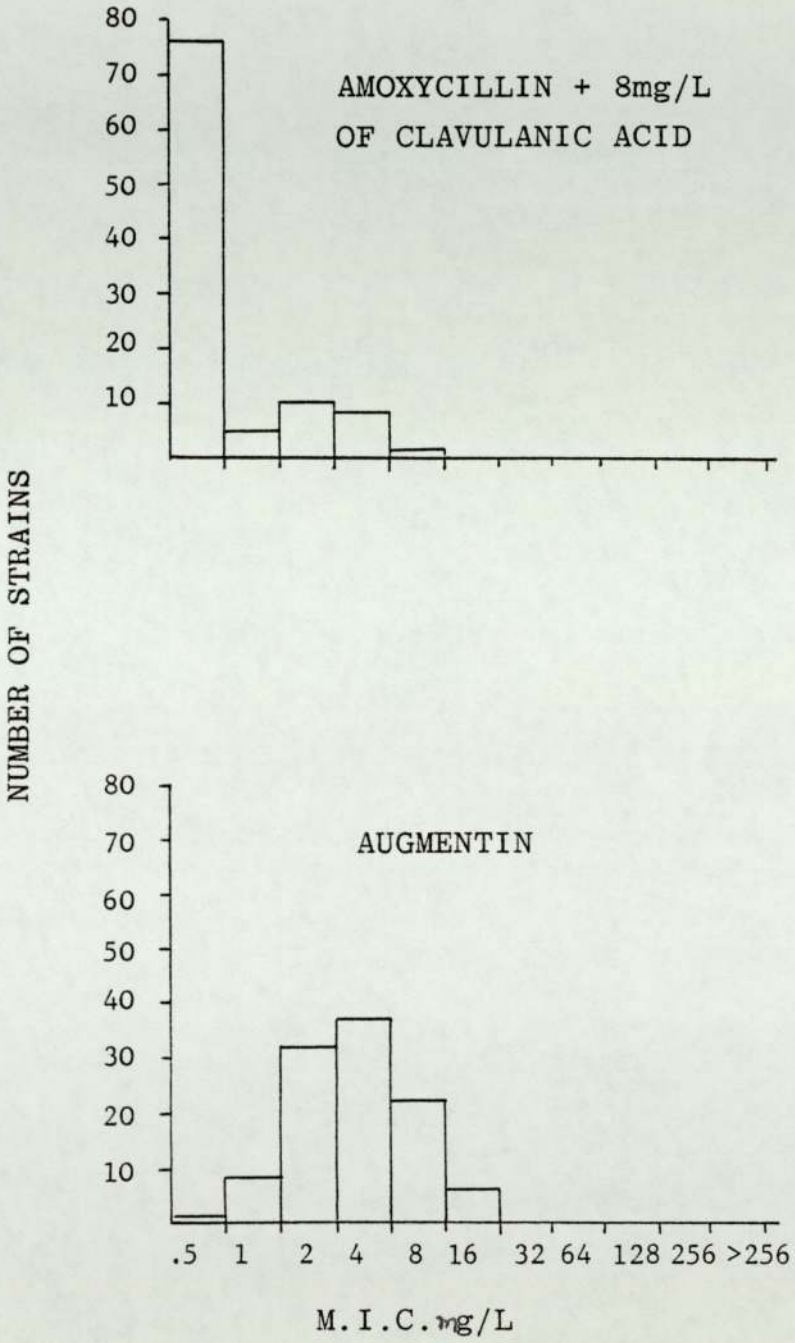


FIGURE 41. 100 STRAINS OF ESCHERICHIA COLI



the strains were positive by one or more of the methods. Thirty-nine of the forty strains which were inhibited by $\geq$ 256 mg/L of amoxycillin when tested alone produced beta-lactamase, the odd strain showed no beta-lactamase production, at least not with the three methods of testing used here. Augmentin had an M.I.C. of 8 mg/L to this strain suggesting the clavulanic acid was having some effect - perhaps due to PBP synergy.

The other thirteen strains which were positive for beta-lactamase production had amoxycillin M.I.C.s as follows:-

3 had an M.I.C. of amoxycillin of 8 mg/L

4 had an M.I.C. of amoxycillin of 4 mg/L

5 had an M.I.C. of amoxycillin of 2 mg/L

1 had an M.I.C. of amoxycillin of 1 mg/L

These thirteen strains were therefore either only producing a small amount of beta-lactamase or they were producing one of the beta-lactamases that did not hydrolyse amoxycillin at a fast rate (Matthew 1979). In fact in all thirteen cases the M.I.C. of Augmentin was identical to that obtained with amoxycillin.

The same one hundred E. coli strains also had their sensitivity determined to amoxycillin and Augmentin by disc diffusion techniques as described in section 2.2.1. The results obtained are shown in Tables 8 and 9.

In the case of amoxycillin alone it can be seen that using this comparative method of disc sensitivity testing the one hundred strains are divided into three groups:-

the resistant group (n = 40) all having an M.I.C. of amoxycillin $\geq$ 256 mg/L;

TABLE 8.

M.I.C. of AMOXYCILLIN OF 100 STRAINS OF ESCHERICHIA COLI FROM U.T.I. CATEGORIZED BY DISC SENSITIVITY

	M.I.C. (mg/L)											
Disc Result (No)	<0.5	0.5	1	2	4	8	16	32	64	128	256	>256
Sensitive (39)		1	9	19	10							
Intermediate (21)				6	10	3	2					
Resistant (40)											11	29

TABLE 9.

M.I.C. of AUGMENTIN (EXPRESSED AS AMOXYCILLIN CONCENTRATION IN mg/L) OF 100 STRAINS OF ESCHERICHIA COLI FROM U.T.I. CATEGEGORIZED BY DISC SENSITIVITY

	M.I.C. (mg/L)											
Disc Result (No)	<0.5	0.5	1	2	4	8	16	32	64	128	256	>256
Sensitive (69)		1	7	32	24	4						
Intermediate (32)					7	19	6					
Resistant (O)												

the sensitive group (n = 39) all having an M.I.C. of amoxycillin $\leq$ 4 mg/L;

and an intermediate group (n = 21) which had M.I.C.s of amoxycillin between 2 and 16 mg/L.

When Augmentin discs were used in the comparative method the one hundred strains divided into only two groups:-

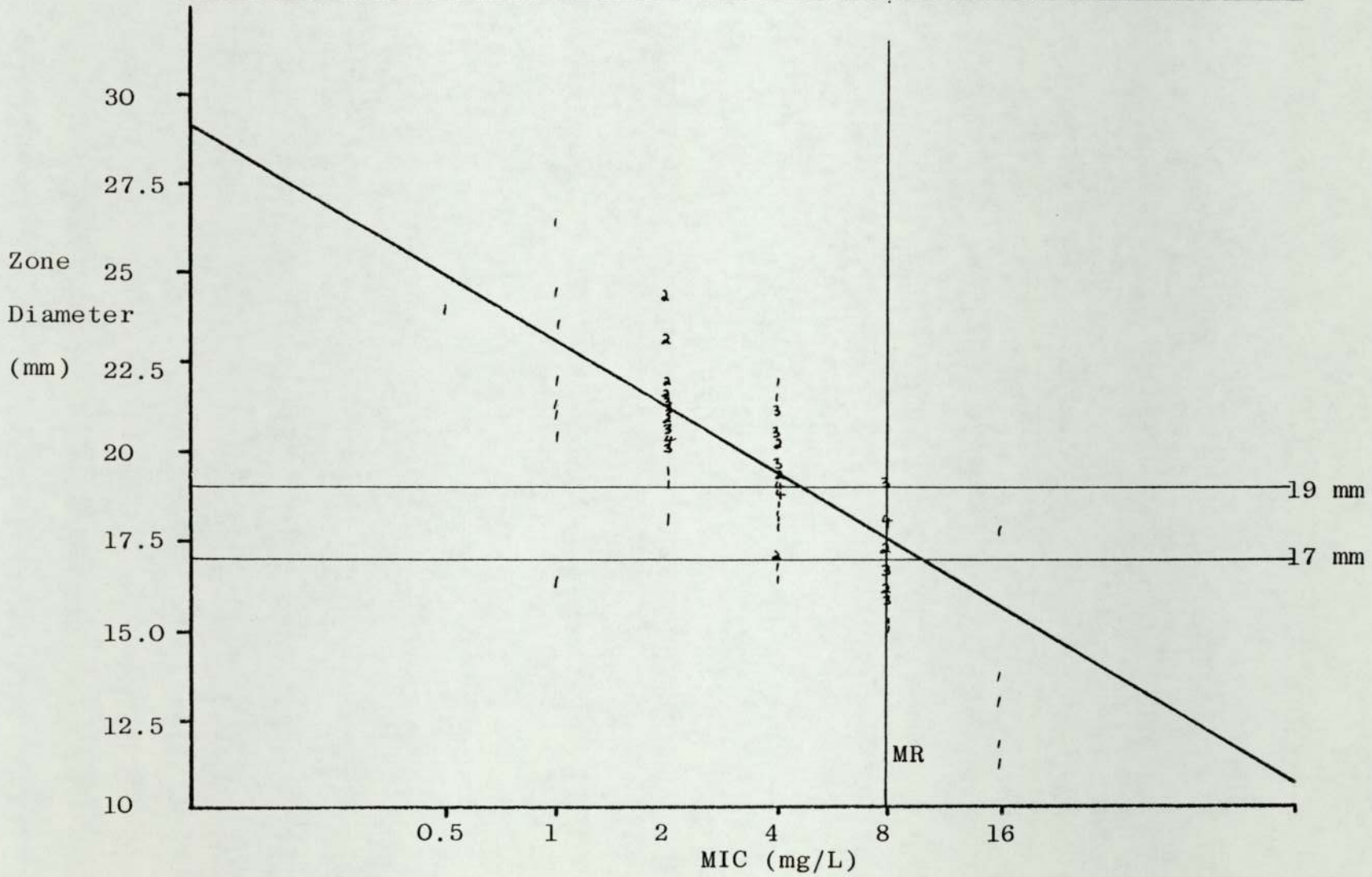
a sensitive group (n = 68) all having an M.I.C. to Augmentin (expressed as the amoxycillin content) of $\leq$ 8 mg/L;

an intermediate group (n = 32) having Augmentin M.I.C.s between 4 and 16 mg/L.

Of the 40 strains that were resistant to amoxycillin alone, 11 are classified as sensitive to Augmentin (3 with an M.I.C. of 8 mg/L, 7 with an M.I.C. of 4 mg/L and 1 with an M.I.C. of 2 mg/L). The remaining 29 strains are all classified as of intermediate sensitivity to Augmentin.

If 8 mg/L of Augmentin is taken as the MR (see section 3.2.3., then 26 strains which should be classified as sensitive are in fact placed in the intermediate group. This is illustrated in Figure 42 which shows the regression line and error rate-bounded analysis for the one hundred strains. The correlation coefficient (r) = -0.79 and twenty-two of the strains fall into an intermediate category on error rate-bounded analysis. The zone-diameter breakpoints for these one hundred strains of E.coli, $\leq$ 17 mm and $\geq$ 19 mm are much lower than the ones obtained from the error rate bounded analysis of the reference strains (section 3.2.3. Fig. 39) where the values were $\leq$ 22 mm and $\geq$ 26 mm; indeed if these latter criteria had been used as zone breakpoints

FIGURE 42 REGRESSION LINE AND ERROR RATE BOUND ANALYSIS FOR 100 ESCHERICHIA COLI STRAINS AGAINST AUGMENTIN (EXPRESSED AS CONCENTRATION OF AMOXYCILLIN COMPONENT)



for the E.coli strains only one organism would have been classified as sensitive, thirteen as intermediate and eighty-six as resistant!

3.2.4.2. Amoxycillin-resistant strains.

In the previous section the one hundred E.coli strains were consecutive isolates and no differentiation was made as to their sensitivity. In this next section fifty-seven amoxycillin resistant isolates were tested against Augmentin. The isolates are listed in section 2.1.1.

Sensitivity testing by the comparative method and M.I.C. determinations were carried out as described in sections 2.2.1. and 2.2.2.

The M.I.C.s of amoxycillin alone and in the presence of clavulanic acid were determined and the results are shown in Figure 43. All of the isolates, except one, had an amoxycillin M.I.C. of > 128 mg/L; the one exception having an amoxycillin M.I.C. of 16 mg/L.

Using the methods described in section 2.2.6. all but two of the fifty-seven isolates produced beta-lactamase. The two exceptions were a Prot. vulgaris and a Kleb. aerogenes. They had an amoxycillin M.I.C. of > 256 and 128 mg/L respectively so it could be that the particular methods used for detecting beta-lactamase production were not detecting it and not that it was absent (Rubin et al. 1981).

The amoxycillin M.I.C. in the presence of 8 mg/L of clavulanic acid was reduced in all but eight of the isolates. Thirty-nine of the isolates (68%) had an amoxycillin M.I.C. (in the presence of clavulanic acid) of < 8 mg/L.

The results of the comparative disc testing of the fifty-seven isolates against amoxycillin and Augmentin are shown in Tables 10 and 11. As would be expected all of the

FIGURE 39. 57 URINARY PATHOGENS

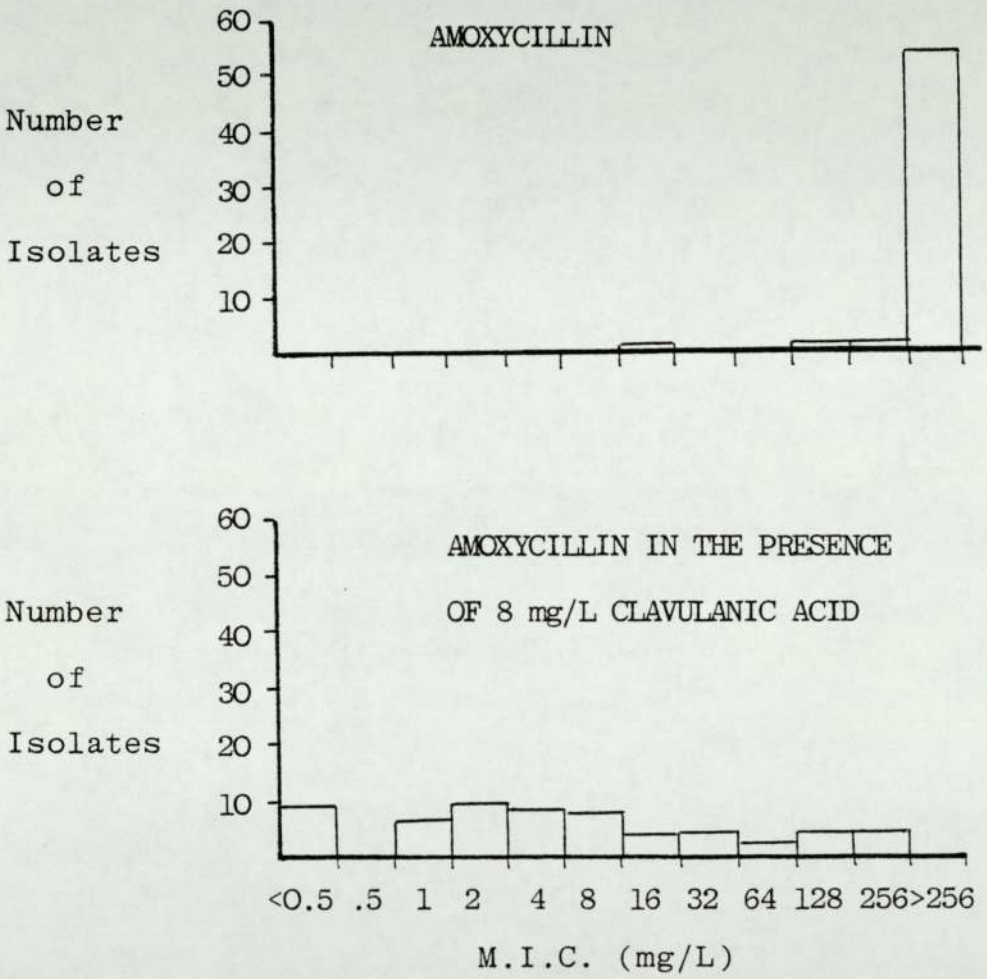


TABLE 10.

M.I.C.of AMOXYCILLIN (mg/L) OF 57 URINARY PATHOGENS CATEGORIZED BY DISC SENSITIVITY RESULT

	M.I.C. (mg/L)											
Disc Result (No)	<0.5	0.5	1	2	4	8	16	32	64	128	256	>256
Sensitive (0)												
Intermediate (1)							1					
Resistant (56)										1	1	54

TABLE 11.

M.I.C. of AUGMENTIN (EXPRESSED AT AMOXYCILLIN CONCENTRATION IN mg/L) OF 57 URINARY PATHOGENS CATEGORIZED BY DISC SENSITIVITY RESULT

	M.I.C. (mg/L)											
Disc Result (No)	<0.5	0.5	1	2	4	8	16	32	64	128	256	>256
Sensitive (20)	9		5	3	2	1						
Intermediate (23)			1	6	6	5	2	2		1		
Resistant (14)						1	2	2	2	3	4	

isolates when tested with an amoxycillin disc proved to be in the resistant group, except for the Kleb. aerogenes with an amoxycillin M.I.C. of 16 mg/L. This was classified in the intermediate group.

When Augmentin discs were used in the comparative method the 57 isolates could be divided into three groups:-

a sensitive group (n = 20) all with an M.I.C. $\leq$ 8 mg/L;

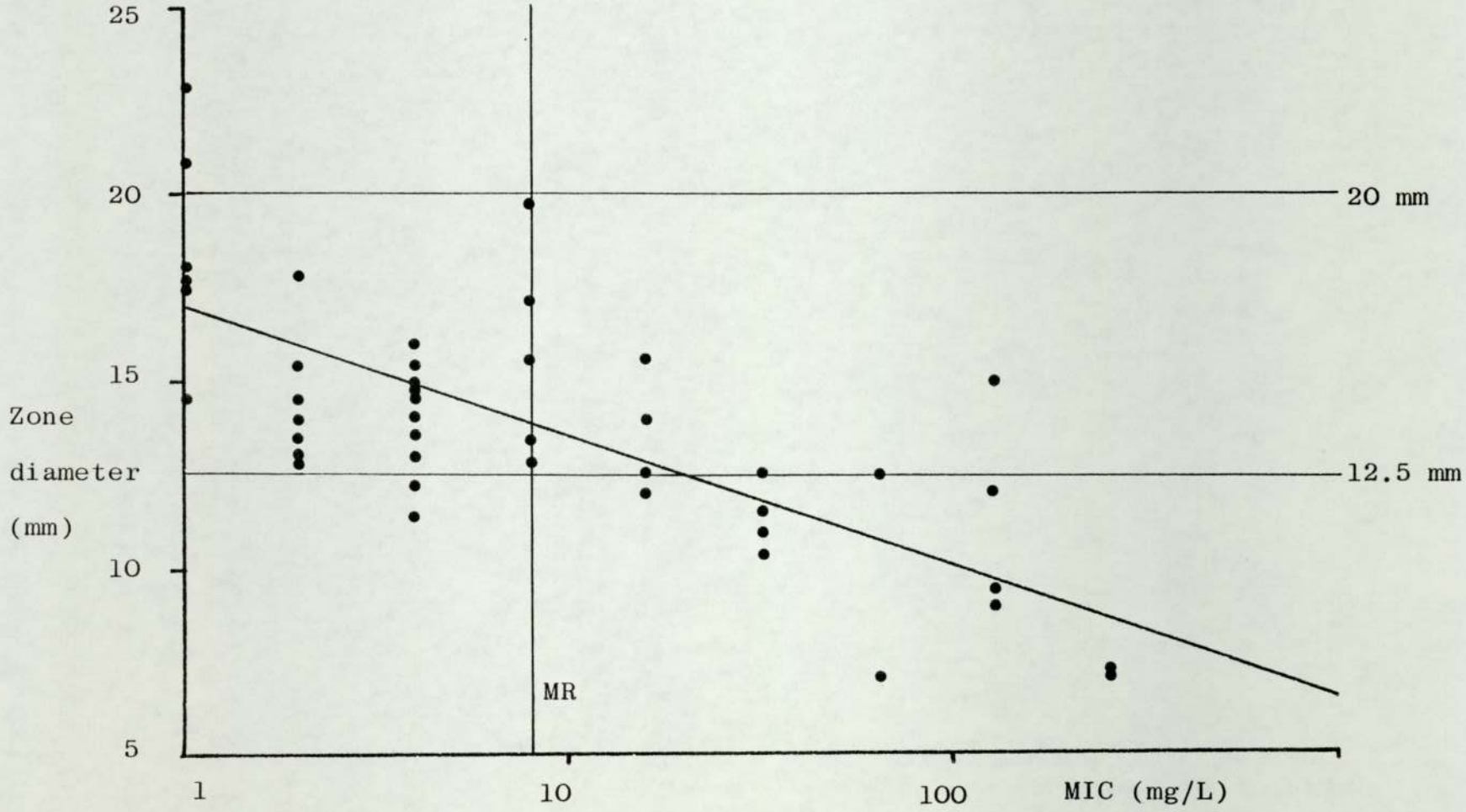
a resistant group (n = 14) all but one with an M.I.C. > 16 mg/L (the exception had an M.I.C. of 8 mg/L);

an intermediate group (n = 23) with M.I.C.s ranging from 1 mg/L to 128 mg/L.

If an MR of 8 mg/L is accepted, 19 strains (33.3%), which should be classified as sensitive, are placed in either the intermediate category (18 of the strains) or resistant category (one strain). Figure 44 shows the regression line and error rate-bounded analysis for these 57 isolates. In order to conform to Metzler and DeHaan's proposals (1974) breakpoint zone diameters of 12.5 mm and 20 mm are necessary and this results in 33 of the isolates (57.8%) being classified as intermediate. The correlation coefficient (r) was -0.75.

In this group of isolates, 96% were beta-lactamase producers. Therefore this is where one would expect Augmentin to do well and indeed 68% of the strains have their M.I.C.s to amoxycillin reduced to <8 mg/L in the presence of clavulanic acid, unfortunately the comparative disc test does not show this with the majority of the strains being classified as intermediate.

FIGURE 44 REGRESSION LINE AND ERROR RATE BOUND ANALYSIS FOR THE 57 URINARY ISOLATES AGAINST AUGMENTIN (EXPRESSED AS CONCENTRATION OF AMOXYCILLIN COMPONENT)



3.2.4.3. Staph. aureus isolates.

The two previous sections dealt with the comparative disc testing of Gram-negative isolates; with regard to the Gram-positive organisms the important clinical isolate in respect of Augmentin will be Staph. aureus. Reading and Cole (1977) have shown that the beta-lactamases synthesized by strains of Staph. aureus are efficiently inhibited by clavulanic acid.

The 52 strains of Staph. aureus described in section 2.1.1. had their benzylpenicillin M.I.C.s determined alone and in the presence of two concentrations of clavulanic acid:- 8 mg/L and 1 mg/L as described in section 2.2.2. Two different inocula were used; 10^3 and 10^6 organisms/ml. This was because Staph. aureus produces, in the main, extracellular beta-lactamase (Richmond 1981) - thus the more organisms present the more beta-lactamase that is produced to attack the benzylpenicillin.

Figure 45 shows the results of these experiments. The inoculum effect is clearly seen in that when 10^3 organisms/ml was the inoculum 94% of the strains would not grow in a benzylpenicillin concentration > 0.25 mg/L; when the inoculum was increased to 10^6 organisms/ml only 19% of the strains remained in this category and 80% would still grow at a concentration of 8 mg/L and above.

Clavulanic acid had a marked effect on M.I.C. values, even when present at only 1 mg/L, causing all the strains to be inhibited by a concentration of benzylpenicillin $\leq$ 0.5 mg/L. This applied whether the inoculum was 10^3 organisms/ml or 10^6 organisms/ml.

FIGURE 45. 52 STRAINS OF STAPHYLOCOCCUS AEREUS

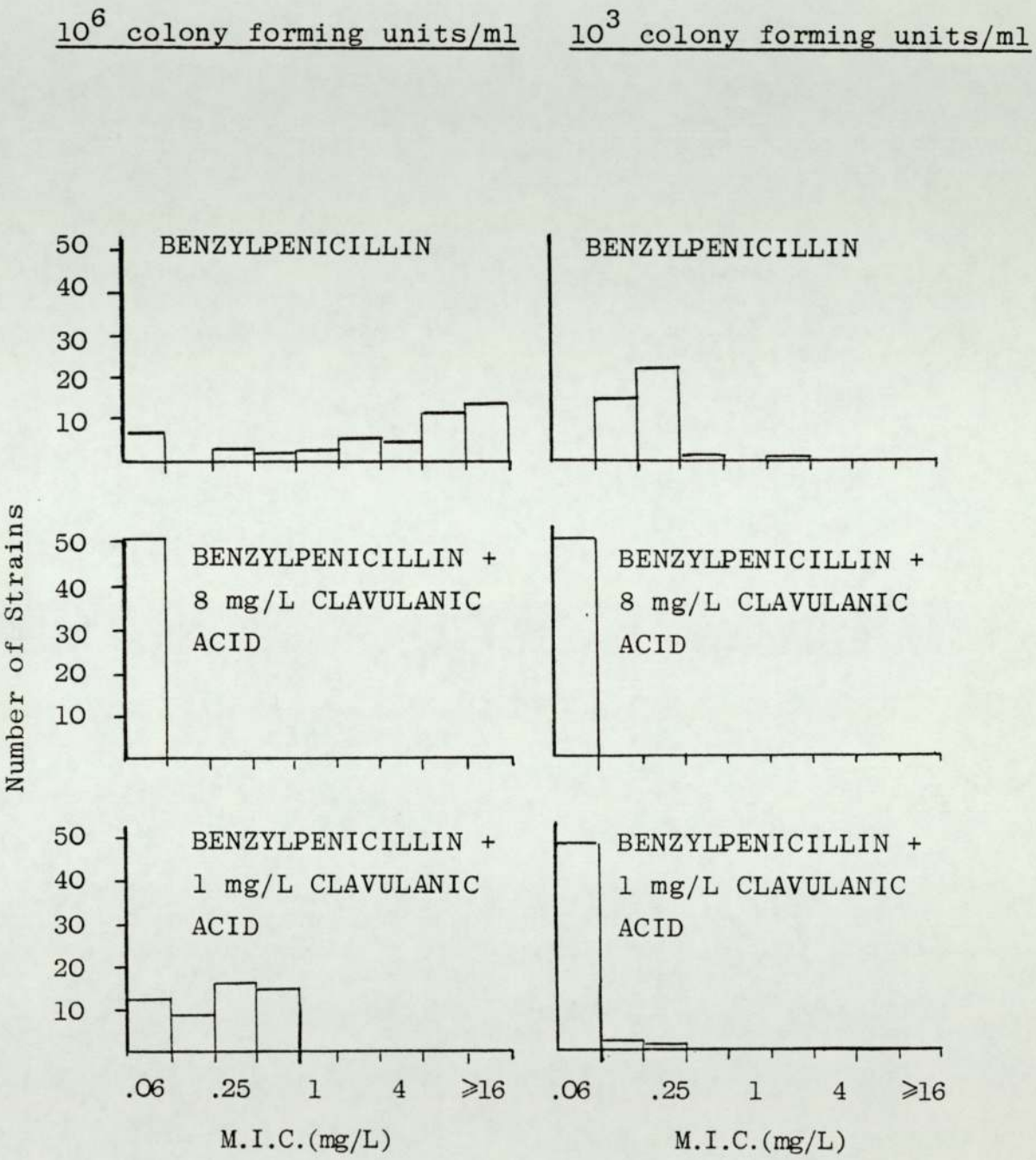


TABLE 12.

M.I.C. of PENICILLIN (mg/L) OF 52 STAPHYLOCOCCUS AUREUS STRAINS CATEGORIZED BY DISC SENSITIVITY RESULT

	M.I.C. (mg/L)										
Disc Result (No)	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32
Sensitive (7)	7										
Intermediate (5)			1	1	2			1			
Resistant (40)			2	1	1	6	5	11	4	5	5

TABLE 13.

M.I.C. of PENICILLIN (mg/L) IN PRESENCE OF 8 mg/L CLAVULANIC ACID OF 52 STAPHYLOCOCCUS AUREUS STRAINS CATEGORIZED BY DISC SENSITIVITY RESULT

	M.I.C. (mg/L)										
Disc Result (No.)	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32
Sensitive (17)	17										
Intermediate (35)	35										
Resistant (0)											

Comparative disc sensitivities, using Staph. aureus N.C.T.C. 6571 as the control, were carried out as described in section 2.2..1. Penicillin and Augmentin discs were used. The results obtained are shown in Tables 12 and 13.

Penicillin alone grouped the strains into three groups:-

a sensitive group (n = 7) with penicillin M.I.C.s. = 0.06 mg/L;

an intermediate group (n = 5) with penicillin M.I.C.s between 0.25 and 8 mg/L;

a resistant group (n = 40) with penicillin M.I.C.s between 0.25 and >32 mg/L.

It is of interest to note that when benzylpenicillin was first introduced in the 1940's, benzylpenicillin was effective against virtually all staphylococci but emergence of beta-lactamase proucing strains has resulted in the type of distribution shown here with 77% of the strains proving to be resistant (Sleigh 1982).

The addition of clavulanic acid to the penicillin results in two groups of staphylococci being classified on disc testing:-

(i) a sensitive group (n = 17) with M.I.C.s $\leq$ 0.06 mg/L;

(ii) an intermediate group (n = 35) with M.I.C.s $\leq$ 0.06 mg/L.

This means that 67% of the staphylococcal strains would be classified as of intermediate susceptibility to Augmentin even though their M.I.C.s to this compound are identical to the 33% classified as sensitive.

3.2.4.4. Discussion.

The principles involved in the formation of a zone of inhibition around an antibiotic containing disc were formulated by Cooper (1963). Antibiotic diffuses through the culture medium at a rate that depends on the molecular size, and chemical nature of the antibiotic and of the medium; hence the sizes of zones around discs containing similar amounts of different agents do not necessarily indicate the comparative in vitro activity of the agents.

A concentration gradient of antibiotic is formed with a high concentration close to the disc and a decreasing concentration away from it. A zone of inhibition is formed when a critical concentration of drug - that amount that is just capable of inhibiting microbial growth under test conditions - reaches, for the first time, a density of cells too large for it to inhibit. The size of the zone of inhibition is determined by the distance that the critical inhibitory concentration can diffuse into the agar medium before a particular density of cells is reached.

The size of the zone of inhibition can be affected by many variables; the most important being the size of the inoculum - more time is required to reach the critical density of cells if the inoculum is light, consequently the critical concentration of drug can diffuse further and a larger zone of inhibition results. Conversely if a high inoculum is used a smaller zone of inhibition will be produced. This effect is very important when testing oganisms such as Staph. aureus which produces extracellular beta-lactamases, this organism often appearing to be

susceptible to penicillin and cephalosporins when the inoculum is light, but with a denser inoculum enough beta-lactamase is produced and liberated into the surrounding medium to destroy the antibiotic and permit microbial growth. This effect is readily illustrated in Figure 45 when the M.I.C.s of benzylpenicillin for the 52 strains of Staph. aureus are compared for the two inoculum sizes; 10^3 organisms/ml and 10^6 organisms/ml.

The composition of the agar medium is also important. It affects the activity of different antibiotics by factors such as the presence of cations in the medium, the pH of the medium and the presence of various antagonists. The medium should have sufficient nutrient to minimize strain-to-strain variation in growth rates (Cooper 1963).

Ericsson and Sherris (1971) suggested many ways in which these variables, and others, could be standardised. Antibiotic disks should be controlled as to their concentration. In America the F.D.A. has stated limits of 67-150% of the labelled potency (Barry and Badal 1978). The antibiotic disks used throughout this study had their potency checked by Mast Laboratories and were found to be within these limits (Ashley - personal communication).

Zone diameter of individual antibiotics are classified in terms of susceptible, intermediate or resistant by reference to an interpretative chart (Acar 1980). The rationale of these charts assumes that the relationship between the diameter of the zone of inhibition around the antibiotic disc and the minimum inhibitory concentration is known, and that the limit for the categorisation of susceptibility takes into account the distribution of

strains as to susceptibility ranges and the levels of the antibiotic achievable in vivo. The outer limit of the zone of inhibition contains an antibiotic concentration which is similar to the minimum inhibitory concentration of that antibiotic to a specific organism. If the minimum inhibitory concentration is expressed in logarithmic form and plotted against the zone diameter produced by a given disc a line of best fit (regression line) can be plotted. From this line interpretative zone sizes can be selected by establishing minimum inhibitory concentration breakpoints. If there is good correlation then a bimodal distribution will occur with the bacterial strains tested being grouped at either end of the scale and being classified as sensitive or resistant; this type of distribution occurs with tetracycline, kanamycin and naladixic acid (Acar 1980).

Unfortunately this does not occur with Augmentin discs, the majority of the bacterial strains falling into the intermediate category; this category should perform three functions (Acar 1980):-

(i) it categorises strains that are neither clearly resistant nor fully susceptible, i.e. either an overlapping between susceptible and resistant populations or distinct populations exhibiting a multimodal distribution;

(ii) from a therapeutic point of view the intermediate category may refer to the relationship of minimum inhibitory concentration to the in vivo effectiveness of the level of the drug;

(iii) it has been considered to provide a buffer zone that minimizes the significance of minor technical

variables that cannot be completely controlled in routine susceptibility testing.

Taking these points in turn and applying them to the results obtained on testing Augmentin the following statements may be made:-

Point one does not apply to the Staph. aureus; it can be seen from the agar plate M.I.C. results (Table 13) that all of the isolates have a benzylpenicillin M.I.C. < 0.06 mg/L yet 35 of the 52 strains (67%) were classified in the intermediate category.

With regard to point two, all the Staph. aureus isolates were from soft-tissue sites or conditions where a high dose of the drug would not be given nor high levels obtained by concentration in the body. Even when urinary tract infections are considered, where the drug would be more concentrated, there are still a considerable number of strains (Tables 9 and 11) sharing the same M.I.C. to Augmentin yet some are classified as sensitive and others as resistant.

The third point covers experimental error, the ideal relationship between zone size and M.I.C. assumes a continuous M.I.C. gradient when in fact $\log_2$ dilution steps were used. Brown and Blowers (1978) have stated that errors of $\pm$ one dilution step, and of $\pm$ 2 mm in zone size measurements are not uncommon. However, this should still lead to only a few strains being placed in the intermediate category and not the large number that are.

The most likely explanation for the large number of isolates being placed in the intermediate category is that a combination of substances is being tested. Ericsson and

Sherris (1971) and Acar (1980) all stated that when two antimicrobials are being tested this should not be done by the use of a single disc. Augmentin, of course, is unique in that a combination is being tested, but for all general purposes the clavulanic acid moiety will not be exhibiting an antimicrobial action in the accepted sense - for this reason the manufacturers claim that a single disc may be used.

In British Laboratories, two control organisms are commonly used as reference organisms for antibiotic sensitivity testing; they are:-

For systemic isolates: Staph. aureus N.C.T.C. 6571

For urinary isolates: E.coli N.C.T.C. 10418.

These two organisms were used in this study as controls for the Stokes comparative method as described in section 2.2.1. Neither of these organisms produces beta-lactamases and both are highly sensitive to amoxycillin, and in the case of the Staph. aureus, penicillin. This fact results in a large zone size being produced around the disc by the control organism. The zone size obtained around the disc by a beta-lactamase producing test organism, however, will be smaller as its zone is determined not by the amoxycillin but by the concentration of inhibitor (clavulanic acid) in the agar. As there is already only half as much clavulanic acid as amoxycillin present in the Augmentin disc, this will obviously diffuse at a different rate on this fact alone, apart from differences in the size and shape of the two molecules and their overall ionic change - all factors which affect the rate of diffusion through a given medium (Barry 1980).

Because smaller zone sizes are produced by the beta-lactamase +ve organisms, when they are compared to the zone size produced by the control organism, the bacterial strains are placed in the intermediate sensitivity group. This is borne out by the fact that all 39 of the E.coli isolates that were sensitive to amoxycillin alone (Table 8) were also sensitive to Augmentin (Table 9) as were the seven Staph. aureus isolates which were sensitive to benzylpenicillin (Table 12) when they were tested against Augmentin (Table 13). This is because they are all beta-lactamase negative strains and therefore the size of the zone of inhibition is totally dependent on the amoxycillin part of the combination.

Pien (1983) reported similar findings with the Staph. aureus isolates in his study, the majority falling into an intermediate category on disc testing although clinically they responded to Augmentin in an identical manner to the ones he classified as sensitive.

Fuchs et al. (1983) question the validity of an intermediate category being recognised with staphylococci and suggest a zone size of $\geq$ 20 mm being selected as the susceptible breakpoint. If this criterion is applied to the staphylococcal isolates in this study, 16 of them (30.7%) would still fall outside this range even though their M.I.C.s to Augmentin as determined by agar incorporation techniques were $<$ 0.06 mg/L identical to the value for the 36 isolates which do have zone sizes of $\geq$ 20 mm. If this M.I.C. value of 0.06 mg/L is accepted as indicative of susceptibility then a breakpoint zone size of $\geq$ 17 mm would have to be used for the Staph. aureus strains included in

this study.

Fuchs and his co-workers (1983) also suggested, on the basis of error rate-bounded analysis, zone sizes of $\geq$ 18 mm susceptible and $\leq$ 13 mm resistant for Gram-negative isolates with a breakpoint concentration of 8 mg/L amoxycillin. Using the criteria that they used i.e. tolerating classification of no more than 1% of the strains as susceptible when they are really resistant and no more than 5% of the strains as resistant when they are really sensitive (Metzler and DeHaan 1974) then from the data obtained with the reference strains zone sizes of $\geq$ 26 mm susceptible and $\leq$ 22 mm resistant (see Fig. 39) would be suggested. This still leaves 29.4% of the isolates in the intermediate category, as the number of strains occurring in this category is a measure of the strength of the relationship between M.I.C. and zone diameter. It can be seen that this is not the ideal way to test for Augmentin susceptibility.

A possible way around this problem could be to use breakpoint sensitivity testing; as both components of the combination are dissolved in agar plates then the diffusion problems outlined above should not occur.

3.2.5.1. Breakpoint Sensitivity Testing.

M.I.C. breakpoints have to satisfy three concepts (Acar 1980):-

(i) the M.I.C. upper limit for susceptibility must be lower than the level of antibiotic obtainable in the blood or tissues with clinically accepted dosage and route of administration;

(ii) the M.I.C. breakpoint should fit within the limits of clusters of microbial organisms having comparable susceptibilities;

(iii) the population of strains defined within a susceptible or resistant category by M.I.C.-breakpoints should be documented as responding clinically and reasonably correlated to in vivo results.

Using these criteria and the recommendation of the NCCLS that 8 mg/L should be the breakpoint for amoxycillin testing (1982) plates were prepared as described in section 2.2.2. containing the drug concentrations shown in Table 14.

Only one plate was used for penicillin/clavulanic acid combination testing and growth on it caused the organism to be classified as resistant.

Three concentrations of amoxycillin with a standard amount of clavulanic acid were used for testing the urinary isolates. Growth on all three plates by the organism classified it as resistant, on the lower two intermediate and on the lowest only sensitive.

Table 15 shows a comparison of the results obtained by breakpoint testing to those obtained by the comparative

TABLE 14.

Antibiotics	Breakpoint Concentrations mg/L		
Amoxycillin + Clavulanic Acid	2	8	32
	8	8	8
Penicillin + Clavulanic Acid	1		
	1		

TABLE 15. BREAKPOINT CLASSIFICATION

		SENSITIVE	INTERMEDIATE	RESISTANT
	SENSITIVE	68	0	0
Disc Classification	INTERMEDIATE	32	0	0
	RESISTANT	0	0	0

COMPARISON OF DISC TESTING AND BREAKPOINT TESTING OF 100 ESCHERICHIA COLI STRAINS AGAINST AUGMENTIN

method of disc testing for the one hundred E.coli strains. With breakpoint testing all one hundred strains were classified as sensitive including the thirty-two strains that the comparative method had classified as intermediate. Eight-three of the strains did not grow on any of the three plates containing amoxycillin and clavulanic acid, the remaining seventeen strains grew only on the plate containing 2 mg/L amoxycillin and 8 mg/L clavulanic acid.

When the fifty-seven amoxycillin-resistant urinary isolates were tested for Augmentin susceptibility by this method the results given in Table 16 were obtained. Forty-one of the isolates were classified as sensitive (72%). The one isolate classified as resistant by the comparative method but sensitive on breakpoint testing was an E.coli with an M.I.C. of 8 mg/L to Augmentin. This isolate only grew on the plate containing 2 mg/L amoxycillin and 8 mg/L clavulanic acid.

Six isolates were classified as intermediate by breakpoint testing. These were two E.coli also classified as intermediate by the comparative method with M.I.C.s of 16 and 32 mg/L respectively to Augmentin, and three E.coli and a Kleb. aerogenes all classified as resistant by the comparative method with M.I.C.s of 16, 32, 128 and 32 mg/L respectively to Augmentin.

Ten isolates were classified as resistant by breakpoint testing. One of these isolates, a Cit. freundii with an M.I.C. to Augmentin of 128 mg/L, was classified as intermediate by the comparative method. The remaining nine isolates were classified as resistant by both methods, eight of them having Augmentin M.I.C.s > 64 mg/L and one, a Ent.

TABLE 16. BREAKPOINT CLASSIFICATION

		SENSITIVE	INTERMEDIATE	RESISTANT
	SENSITIVE	20	0	0
Disc Classification	INTERMEDIATE	20	2	1
	RESISTANT	1	4	9

COMPARISON OF DISC TESTING AND BREAKPOINT TESTING OF

57 URINARY PATHOGENS AGAINST AUGMENTIN

cloacae having an Augmentin M.I.C. of 16 mg/L.

Table 17 shows the comparison of the breakpoint results with the comparative disc results for the fifty-two Staph.aureus strains. All of the isolates were classified as sensitive on breakpoint testing.

TABLE 17 BREAKPOINT CLASSIFICATION

		SENSITIVE	INTERMEDIATE	RESISTANT
	SENSITIVE	17	0	0
Disc Classification	INTERMEDIATE	35	0	0
	RESISTANT	0	0	0

COMPARISON OF DISC AND BREAKPOINT TESTING OF 52 STAPHYLOCOCCUS AUREUS STRAINS AGAINST AUGMENTIN

3.2.5.2. Discussion.

As was postulated in the previous dicussion (section 3.2.4.4.) the fact that the two parts of the Augmentin combination are uniformly dispersed throughout the medium rather than having to diffuse from a central reservoir overcomes the categorisation of many strains into that of intermediate sensitivity. If the breakpoint method is used then all of the Staph. aureus isolates are classified as sensitive and all but 14 of the 157 Gram-negative isolates (91%). These results agree much more with the M.I.C. determinations and the results of many clinical trials with Augmentin (Leigh 1982, Millard 1982).

Added advantages to the breakpoint method of sensitivity testing are that large numbers of strains can be tested at one time and that no measurement of endpoint has to take place, merely a recording of the presence or absence of growth.

The stability of the compounds in agar is obviously important especially if the commercial firms are going to supply them but careful examination of results should soon indicate if a compound has lost its activity.

Inoculum size does not seem to be the problem with breakpoint testing that it can be with disc sensitivity testing. Waterworth (1981) found that varying the inoculum 100 fold had very little effect on results and when there were discrepancies these occurred more often with a light inoculum rather than a heavy one.

3.2.6.1. Turbidimetric Studies.

These studies were carried out as outlined in section 2.2.5. with the exception that chamber 10 of each cuvette contained clavulanic acid (8 mg/L) only. Chambers 9 to 1 had doubling dilutions of amoxycillin 256-1.0 mg/L in the presence of 8 mg/L clavulanic acid. The experiments were run for six hours (this is the time that the Abbott MS.2 takes for other antibiotic sensitivities) and the growth curves plotted. The 57 amoxycillin-resistant urinary isolates (see section 2.1.1.) were used for these experiments.

The M.A.C. (minimum antibacterial concentration) was defined as the lowest concentration of Augmentin to produce a significant deviation from the normal growth curve produced by the organism in the absence of antibiotic.

Table 18 shows the relationship between the results obtained by this method compared to the M.I.C. results obtained from agar dilution experiments.

Clavulanic acid alone had no effect on any of the isolates; the growth curve produced being identical to that of the control.

Forty of the isolates (72%) had an M.A.C. determined from the six hour growth curves that was within one dilution of the M.I.C. obtained from agar incorporation techniques.

Twelve organisms had an M.A.C. that was fourfold or less than the M.I.C. and five organisms had an M.A.C. that was fourfold or greater than the M.I.C.

TABLE 18.

COMPARISON OF THE M.A.C. AFTER 6 HOURS TURBIDIMETRIC MONITORING WITH THE M.I.C. TO AUGMENTIN OBTAINED BY AGAR INCORPORATION FOR 57 GRAM-NEGATIVE ISOLATES

	MINIMUM ANTIBACTERIAL CONCENTRATION									
	MIC 16	MIC 8	MIC 4	MIC 2	MIC	MICx2	MICx4	MICx8	MICx16	MICx32
Number of Organisms	1	4	7	9	18	13	4	0	0	1

3.2.6.2. Discussion.

Clinical microbiology today is the most labour and skill intensive discipline of laboratory medicine (Isenberg 1982). It attempts to identify microorganisms and to provide an antibiotic profile as quickly as possible. With few exceptions, this information takes 24-36 hours to be available; automation, therefore, has attempted to abbreviate this time period. The Abbott MS-2 (see section 2.1.6.) uses multichambered cuvettes, shaking incubation and light-transmission nephelometry to monitor the growth of an organism in the absence and presence of antibiotic. Results of optical density measurements made every five minutes are stored in a computer unit and growth rate constants are calculated compared to the control chamber. These kinetic analyses are automatically translated into major susceptibility categories analogous to the standard disc diffusion guides (Bossi et al. 1982). For the majority of antibiotics an answer as to whether an isolate is sensitive or not will be obtained within six hours.

With the 57 strains tested here good agreement with the M.I.C. as determined by agar incorporation occurred in 72%. This is a similar result to that obtained by Johnston and Griffiths (1982) when they compared a four hour incubation in the Abbott MS-2 to an 18 hour broth dilution test. They found, as this study does, that the majority of disagreement was due to the MS-2 result indicating greater susceptibility. They also found that if inactivation in the MS-2 was prolonged to 18 hours correlation was improved. This was not attempted here as the whole point of the MS-2

system, and similar systems, is that an earlier result is obtained.

4. CONCLUSIONS

Clavulanic acid extends the spectrum of amoxycillin remarkably. However, with conventional disc sensitivity testing, due to the reasons outlined, a large proportion of organisms will be wrongly placed in an intermediate category of moderate sensitivity.

The manufacturers of Augmentin have suggested two alternatives to overcome this problem (Arnstein - personal communication):-

(i) Readjustment of the Stokes comparative criteria so that on testing with a 30 µg Augmentin disc the following are adopted.

Sensitive: zone radius equal, wider, or:-

In the case of Gram-negative bacilli not more than 5 mm smaller than the controls strain, N.C.T.C. E.coli 10418 or:-

In the case of staphylococci not more than 10 mm smaller than the control strain of Staph. aureus N.C.T.C. 6571.

Intermediate: zone of 3 mm radius or greater but insufficiently large to fall into the above category.

Resistant: zone of 2 mm radius or less.

The consequence of these proposals would be that many more zone sizes would have to be measured with calipers than is the case at present. The direct eye comparison between the control organism and the test organism that is one of the advantages of the Stokes method would be lost.

The second alternative suggested is that two new control organisms which are beta-lactamase producers are used instead of the two beta-lactamase negative organisms employed at the moment.

The two proposed organisms are E.coli BRL 1077 and Staph. aureus BRL 1555. Although this would overcome the problem of Augmentin disc sensitivity testing it would create problems as regards the testing of other antibiotics. This is because all the information on disc sensitivity testing of antibiotics is based (when the comparative method is used) on the controls being E.coli N.C.T.C. 10418 or Staph. aureus N.C.T.C. 6571. Therefore, Augmentin could not be incorporated into a multidisk ring and a separate sensitivity plate would need to be set up in order to determine its result.

The breakpoint method of sensitivity testing would seem to offer a realistic alternative. The problem of wrongly classifying an organism's sensitivity is overcome due to the amoxycillin and clavulanic acid being uniformly dispersed throughout the sensitivity medium. Large numbers of isolates may be tested and the cost is not expensive.

The automated turbidimetric method of sensitivity testing is expensive and is still undergoing development, having said that it is rapid and if the growth curves are actually produced a great deal of information can be studied. With conventional disc sensitivity testing no indication of what is happening at sub M.I.C. levels is obtained. However, the turbidimetric system will show if there is a delay in growth and if it is linked to morphological studies a great deal of information can be obtained. In this way not only the beta-lactamase inhibition effect of clavulanic acid can be studied but also its complementation effect. This effect is due to the fact that clavulanic acid has greatest affinity for penicillin -binding-protein 2 whereas the majority of beta-lactam

antibiotics have greatest affinity for PBPs 1 and 3. From the studies carried out in this thesis it is apparent that to obtain maximum complementation, clavulanic acid needs to be combined with a beta-lactam that binds to PBP 1; fortunately amoxycillin does this.

Another antibiotic that has affinity for PBP 1 is ticarcillin and there is a combination of clavulanic acid and ticarcillin under development to be known as Timentin. This should have an extremely broad spectrum (Clarke and Zemcov 1984) due not only to its beta-lactamase inhibitory properties but also to its anti-pseudomonal ones. From studies conducted in this thesis, Timentin will also show a complementation effect on beta-lactamase negative strains due to the different PBPs attacked by the components. However, similar problems could well occur in disc sensitivity testing and breakpoint testing should again be encouraged.

Penicillanic acid sulphone is another beta-lactamase inhibitor derived by chemical manipulation of 6-aminopenicillanic acid (English et al. 1978). Like clavulanic acid it has little antibacterial activity in its own right and it binds to PBP 2. It is being partnered by ampicillin and once again could well exhibit a complementation effect due to PBP synergy. The disc sensitivity test will again probably have many problems due to similar reasons as given here for Augmentin.

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