The Nature of the Insensitivity of Gram-Negative Bacteria towards Penicillins

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SUMMARY

The insensitivity of Gram-negative bacteria towards different penicillins has been correlated with the inactivation of these compounds by the penicillinases produced by these bacteria. Penicillins such as phenethicillin, propicillin, methicillin, and cloxacillin were relatively inactive against Gram-negative bacteria, but resistance was not accompanied by inactivation of these compounds. With ampicillin and benzylpenicillin, however, bacterial resistance was associated with destruction of the penicillins. Ampicillin was more stable to the penicillinases produced by certain Gram-negative bacteria than was benzylpenicillin and was correspondingly more active against these organisms. Gram-negative bacteria which produced little or no penicillinase were two to four times more sensitive to ampicillin than to benzylpenicillin, but ampicillin was at least ten times more active than benzylpenicillin against several penicillinase-producing coliform organisms. All strains of bacteria which were resistant to both ampicillin and benzylpenicillin were capable of inactivating both compounds, but the insensitivity of these bacteria was not necessarily due solely to penicillinase production. Strains of *Pseudomonas aeruginosa* and certain strains of *Escherichia coli* and *Proteus mirabilis* were able to grow in high concentrations of these antibiotics, whereas with the strains of *Aerobacter aerogenes*, *Proteus morganii* and *Proteus vulgaris* examined, the resistance appeared to be due solely to inactivation of the penicillins.

INTRODUCTION

The destruction of benzylpenicillin by certain Gram-negative bacteria was reported first by Abraham & Chain (1940) and, later, by Bondi & Dietz (1944), but both groups of workers also observed that the resistance of bacteria to penicillins was not invariably associated with penicillinase formation. Similar findings with ampicillin were reported by Rolinson & Stevens (1961) who showed that certain Gram-negative bacteria were capable of destroying the antibiotic, but that others were capable of growing in high concentrations of it; these results have been confirmed by other workers (Stewart, Coles, Nixon & Holt, 1961; Trafford *et al.* 1962; Ayliffe, 1963). Thus, with ampicillin, as with benzylpenicillin, penicillinase appeared to occupy an indeterminate role in the resistance of Gram-negative bacteria towards penicillins. More recently, the characteristics of the penicillinases elaborated by certain Gram-negative bacteria have been investigated by various workers who have reported striking differences in the activities of these enzymes against various penicillins. For example, Auhagen *et al.* (1962) and Smith (1963) described strains of *Escherichia coli* which destroyed benzylpenicillin but which

had little or no effect on ampicillin, propicillin, methicillin or oxacillin. Ayliffe (1963) showed that ampicillin was more stable than benzylpenicillin to the penicillinase produced by a strain of *E. coli*, but, in addition, reported that ampicillin was inactivated as rapidly, or more rapidly, by penicillinases produced by strains of *Proteus* and *Klebsiella*. In these laboratories, Mr F. R. Batchelor (personal communication) has measured marked variation in the reaction of several penicillins to the penicillinases produced by a number of Gram-negative bacteria. It seemed possible that in some cases the differences in the sensitivities of some strains of Gram-negative bacteria towards various penicillins might be correlated with the relative stabilities of these compounds to the bacterial penicillinases. The results reported here were derived from experiments designed to investigate the relationship between the penicillinase activity of Gram-negative bacteria and the sensitivity of these organisms to penicillins, with a view to assessing the significance of these enzymes as factors in the resistance of bacteria to these antibiotics. The experiments comprised tests for antibacterial activity, measurement of the destruction of penicillins by bacteria, and observation of the growth of bacteria in the presence of penicillins. In addition, certain tests were also carried out to distinguish between penicillin inactivation by β -lactamases (penicillinases), which yield penicilloic acids, and deacylases (penicillin amidases) which yield 6-amino-penicillanic acid (6-APA).

METHODS

Measurement of the antibacterial activities of penicillins. Minimal inhibitory concentrations of various penicillins against bacteria were determined by twofold serial dilution in nutrient broth (Oxoid No. 2). The antibiotics were incorporated in graded concentrations in 5 ml. volumes of nutrient broth which were inoculated with 1 drop (0.03 ml.) of an overnight culture of the test organism. Inhibitory concentrations were measured after overnight incubation at 37°. In other experiments, the effect on the antibacterial activity of the penicillins of altering the inoculum size was measured.

Measurement of penicillin destruction. The destruction of penicillins incubated overnight in the antibacterial tests described above was estimated by microbiological assay of residual penicillin activity. Samples were taken from the cultures and were plated in punch-holes in large rectangular plates containing nutrient agar seeded with *Bacillus subtilis* (ATCC 6633). In these assays, standard lines were prepared from standard solutions of penicillins which had been incubated overnight at 37° in nutrient broth to compensate for thermal inactivation of penicillin. The plates containing test and standard samples were incubated overnight at 29° when the diameters of zones of inhibition were measured in the usual way. Tests were also carried out to estimate the extent of penicillin inactivation due to the experimental procedure adopted, and which represented destruction additional to that occurring in the antibacterial tests. Likely factors appeared to be change in pH value of cultures, and the penicillinase activities of test cultures during the microbiological assay procedures.

Measurement of the effect of penicillins on the growth of bacteria. Optically matched tubes containing selected concentrations of penicillins in 10 ml. volumes of nutrient broth were incubated with 0.5 ml. or 1.0 ml. of an overnight culture of test organism.

The solutions were incubated at 37° in a water bath, and growth was measured turbidimetrically over a period of 24 hr by means of an EEL nephelometer. In addition, a replicate set of cultures was prepared in an identical fashion and samples were taken from these at regular intervals and assayed for penicillin content as described above.

Quantitative estimation of penicillins by chromatography. Samples were taken from broth cultures of *Pseudomonas aeruginosa* which had been incubated overnight at 37° with known concentrations of ampicillin or benzylpenicillin and these were assayed as follows. One drop (about 6 μ l.) was placed on the origin of filter-paper tapes (1 cm. wide, Whatman No. 1) and developed by descending chromatography in a butanol+pyridine+water (1+1+1, by vol.) system. Tapes spotted with nutrient broths containing from 2.5 to 100 μ g. ampicillin or benzylpenicillin/ml. were treated in a like manner. After 16 hr, the tapes were placed on the surface of large rectangular assay plates containing nutrient agar seeded with *Bacillus subtilis* (ATCC 6638) and incubated for 24 hr at 29°. The penicillin concentrations of the test samples were estimated from standard lines derived from standard samples.

Chromatographic estimation of 6-aminopenicillanic acid. Samples were taken from the antibacterial tests and treated as above for quantitative estimation of penicillin by chromatography. The tapes were sprayed with 5% sodium bicarbonate solution and 5% phenylacetyl chloride in acetone before being plated for microbiological assay (Batchelor, Doyle, Nayler & Rolinson, 1959).

Chromatographic examination for penicilloic acid. Samples from antibacterial tests with ampicillin and benzylpenicillin were spotted on large sheets (Whatman paper No. 1) and developed in the butanol+pyridine+water system as described above. The chromatograms were sprayed with the starch-iodine reagent of Thomas (1961), and the zones which developed compared with those produced by the penicilloic acids which had been prepared by treatment of standard solutions of ampicillin and benzylpenicillin with penicillinase.

RESULTS

Preliminary

Destruction of penicillin due to change in pH values of bacterial cultures was considered to be negligible as a result of experiments which showed that there was little inactivation of standard solutions of benzylpenicillin incubated overnight in nutrient broth buffered at pH 5.0. This was the lowest pH value recorded for a number of overnight cultures of Enterobacteriaceae.

The amount of destruction of ampicillin or benzylpenicillin by bacterial penicillinase during the microbiological assay procedures was estimated by finding the effect of adding, at measured intervals, a culture of a penicillinase-producing strain of *Staphylococcus aureus* to standard solutions of these penicillins contained in punch-holes in an assay plate. There was significant inactivation only when the enzyme system was added within 30 min. of placing the penicillin solution on the assay plate, owing to the very rapid diffusion of the penicillins into the surrounding medium. It seemed improbable therefore that there would be any marked destruction of penicillins during the microbiological assay as a result of the penicillinase produced by the Gram-negative bacteria being examined.

Examination for production of 6-aminopenicillanic acid (6-APA)

Chromatographic examination of broth cultures in which there was marked destruction of penicillin as judged by microbiological assay, showed the presence of large amounts of the cognate penicilloic acid, and little or no 6-APA. The organisms tested included strains of *Escherichia coli*, *Proteus*, *Pseudomonas aeruginosa* and *Aerobacter aerogenes*. With one strain of *Proteus rettgeri*, however, an appreciable quantity of 6-APA was detected; the penicilloic acids of benzylpenicillin and 6-APA were also observed in cultures of this strain.

The activities of seven penicillins against Gram-negative bacteria

The relative activities of ampicillin, benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin, methicillin, and cloxacillin, against a variety of Gram-negative bacteria are shown in Table 1. From this it can be seen that of these compounds, ampicillin and benzylpenicillin alone showed appreciable activity. In general, ampicillin was two to ten times more active than benzylpenicillin, but certain strains were insensitive to both penicillins.

Table 1. *The relative activities of seven penicillins against certain Gram-negative bacteria*

Organism	Antibiotic						
	Ampicillin	Benzyl- penicillin	Phenoxy- methyl- penicillin	Phene- thicillin	Propi- cillin	Methi- cillin	Cloxa- cillin
			Minimal inhibitory concentration (μ g./ml.)				
<i>Escherichia coli</i> 2	2.5	12.5	125	> 500	500	> 500	> 500
<i>E. coli</i> 6	5.0	25	250	500	250	> 500	500
<i>E. coli</i> 87	25	500	> 500	500	250	> 500	500
<i>E. coli</i> 88	500	> 500	> 500	> 500	500	> 500	> 500
<i>Aerobacter aerogenes</i> A	250	125	500	> 500	500	500	500
<i>Klebsiella pneu- moniae</i> E	1.25	5.0	125	500	250	.	.
<i>Proteus mirabilis</i> 977	2.5	2.5	50	250	125	125	500
<i>P. mirabilis</i> 889	> 500	> 500	.	.	.	.	.
<i>P. morganii</i> A	125	> 500	> 500	500	500	.	.
<i>Pseudomonas aerugi- nosa</i> A	> 500	> 500	> 500	> 500	> 500	> 500	> 500
<i>Salmonella typhi</i>	1.25	2.5	50	250	125	> 500	500
<i>S. paratyphi</i> A	1.25	5.0	250	500	250	> 500	> 500
<i>Shigella sonnei</i>	5.0	25	125	500	250	> 500	> 500
<i>S. flexneri</i>	2.5	12.5	125	250	125	500	500

The relative stabilities of seven penicillins to the penicillinases of Gram-negative bacteria

The results of experiments designed to correlate the antibacterial activities of seven penicillins against two strains of *Escherichia coli* and the stabilities of these compounds to the penicillinases produced by the test bacteria are shown in Table 2. In these tests the minimal inhibitory concentrations of the antibiotics were measured in the usual fashion in nutrient broth using an ampicillin-sensitive strain and an

ampicillin-resistant strain of *E. coli*; in addition, the amount of destruction of penicillin which had occurred after overnight incubation was measured by microbiological assay. It will be seen that phenethicillin, propicillin, methicillin and cloxacillin were almost completely unaffected by the penicillinases produced by

Table 2. *The relative stabilities of ampicillin, benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin, methicillin, and cloxacillin to the penicillinases produced by two strains of Escherichia coli*

Minimal inhibitory concentrations of the penicillins against two strains of *Escherichia coli* were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Penicillin	Organism	Growth and penicillin concentration ($\mu\text{g./ml.}$) after incubation at 37° for 18 hr.								
bacteria-free control*		500	250	125	50	25	12.5	5.0	2.5	1.25
benzyl- penicillin	<i>E. coli</i> 2	—	—	—	—	—	+	+	+	+
		470	245	96	45	17.2	2.2	2.2	0.9	0.4
	<i>E. coli</i> 83	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
ampicillin	<i>E. coli</i> 2	—	—	—	—	—	—	—	+	+
		560	245	120	53	24.0	13.4	5.3	2.4	1.3
	<i>E. coli</i> 83	±	+	+	+	+	+	+	+	+
		400	82	24	1.3	0.7	0	0	0	0
phenoxymethyl- penicillin	<i>E. coli</i> 2	±	±	+	+	+	+	+	+	+
		520	240	115	34	13.0	5.8	3.0	1.3	0.6
	<i>E. coli</i> 83	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
phenethi- cillin	<i>E. coli</i> 2	+	+	+	+	+	+	+	+	+
		530	250	132	53	27.0	13.0	4.7	2.6	1.25
	<i>E. coli</i> 83	+	+	+	+	+	+	+	+	+
		550	195	93	41	16.5	8.4	2.6	0.6	0
propicillin	<i>E. coli</i> 2	±	+	+	+	+	+	+	+	+
		490	250	120	45	26.0	12.0	4.4	2.1	0.9
	<i>E. coli</i> 83	±	+	+	+	+	+	+	+	+
		510	250	127	40	23.0	11.0	3.0	1.3	0.4
methicillin	<i>E. coli</i> 2	.	+	+	+	+	+	+	+	+
		.	250	128	54	25.0	13.0	5.1	2.6	1.2
	<i>E. coli</i> 83	.	+	+	+	+	+	+	+	+
		.	250	130	49	25.0	12.5	4.6	1.8	0.9
cloxacillin	<i>E. coli</i> 2	.	+	+	+	+	+	+	+	+
		.	235	128	51	24.0	13.0	4.5	3.1	.
	<i>E. coli</i> 83	.	+	+	+	+	+	+	+	+
		.	245	130	51	25.0	13.0	4.0	0	.

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.

+ = Growth.

— = No growth.

± = Partial growth.

either strain of *E. coli*, but exerted little or no inhibitory effect on these cultures. In contrast, there was complete destruction of benzylpenicillin and marked destruction of ampicillin by the resistant strain of *E. coli* strain 83, and there was some

obvious destruction of benzylpenicillin by the sensitive strain of *E. coli* 2 at concentrations below the inhibitory value. Ampicillin was, however, unaffected by this strain. Phenoxymethylpenicillin appeared to occupy a mid-way position between these two groups of penicillins, inasmuch as this compound was almost inactive against one strain and was completely inactive against the other; but, nevertheless, there was obvious destruction of this penicillin by the ampicillin-sensitive organism and complete destruction by the second strain.

Table 3. *The activities of ampicillin and benzylpenicillin against Gram-negative bacteria causing minimal destruction of penicillins*

Minimal inhibitory concentrations of the penicillins against five strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration ($\mu\text{g./ml.}$) after incubation at 37° for 18 hr.								
		50	25	12.5	5.0	2.5	1.25	0.5	0.25	0.1
Bacteria-free control*										
<i>Salmonella typhi</i>	Benzyl-penicillin	.	.	—	—	±	+	+	+	+
		.	.	.	5.0	2.3	1.4	0.7	0.28	0.16
	Ampicillin	.	.	—	—	—	—	±	+	+
		.	.	.	5.0	2.7	1.4	0.53	0.28	0.1
<i>S. paratyphi</i> A	Benzyl-penicillin	.	.	.	—	—	±	+	+	+
		.	.	.	5.0	1.9	1.4	0.7	0.28	0.16
	Ampicillin	.	.	.	.	—	—	±	+	+
		.	.	.	.	2.5	1.8	0.44	0.23	0.12
<i>Proteus mirabilis</i> 4	Benzyl-penicillin	.	.	—	—	—	+	+	+	+
		.	.	12.5	5.0	2.0	1.0	0.52	0.29	0.16
	Ampicillin	.	.	—	—	—	—	—	+	+
		.	.	.	5.0	2.6	1.4	0.53	0.22	0.16
<i>P. mirabilis</i> c977	Benzyl-penicillin	.	.	—	—	+	+	+	+	+
		.	.	10.0	4.2	1.9	1.4	0.44	0.29	0.16
	Ampicillin	.	.	.	—	—	—	+	+	+
		.	.	.	5.0	2.6	1.4	0.53	0.21	0.1
<i>Klebsiella pneumoniae</i> E	Benzyl-penicillin	.	.	—	—	—	±	+	+	+
		.	.	10.5	5.0	2.4	1.4	0.45	0.28	< 0.1
	Ampicillin	.	.	.	—	—	—	—	+	+
		.	.	.	5.6	2.9	1.3	0.6	0.23	< 0.1

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.

+ = Growth.

— = No growth.

± = Partial growth.

The antibacterial activities and penicillinase stabilities of ampicillin and benzylpenicillin towards Gram-negative bacteria

As a result of numerous experiments to compare the relative antibacterial activities of ampicillin and benzylpenicillin, the Gram-negative bacteria were grouped as follows:

Group 1. *Gram-negative bacteria sensitive to both ampicillin and benzylpenicillin.* The results in Table 3 show the relative activities of ampicillin and benzylpenicillin against two *Salmonella* species, two strains of *Proteus mirabilis* and one strain of

Klebsiella pneumoniae. These organisms were inhibited by concentrations of 0.5–5.0 µg. penicillins/ml. but, in general, ampicillin was two to four times more active than was benzylpenicillin. Microbiological assay of these samples showed that there had been little or no destruction of either penicillin after incubation with the test organisms at 37° for 16–20 hr.

Group 2. Gram-negative bacteria sensitive to ampicillin but relatively resistant to benzylpenicillin. From Table 4 it can be seen that ampicillin inhibited the growth of three strains of *Escherichia coli* and one strain of each of *Shigella flexneri* and

Table 4. *Penicillinase activities of certain Gram-negative bacteria sensitive to ampicillin but moderately insensitive to benzylpenicillin*

Minimal inhibitory concentrations of the penicillins against five strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration ($\mu\text{g./ml.}$) after incubation at 37° for 18 hr.								
bacteria-free control*		50	25	12.5	5.0	2.5	1.25	0.5	0.25	0.1
<i>Shigella flexneri</i>	Benzyl penicillin	.	—	$\pm$	+	+	+	+	+	+
		.	13.5	13.0	0.44	0.2	0.16	0.12	< 0.1	< 0.1
	Ampicillin	.	.	.	—	—	—	+	+	+
		.	.	.	5.0	2.6	1.7	0.44	0.2	< 0.1
<i>S. sonnei</i>	Benzyl-penicillin	—	$\pm$	$\pm$	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
	Ampicillin	.	.	.	—	—	—	+	+	+
		.	.	.	5.0	2.1	1.05	0.12	< 0.1	< 0.1
<i>Escherichia coli</i> 2	Benzyl-penicillin	.	—	+	+	+	+	+	+	+
		.	17.0	1.9	0.64	0.46	1.8	0.12	< 0.1	< 0.1
	Ampicillin	.	.	.	—	—	$\pm$	+	+	+
		.	.	.	5.2	2.6	1.4	0.53	0.14	< 0.1
<i>E. coli</i> 61	Benzyl-penicillin	.	—	—	+	+	+	+	+	+
		.	20.0	6.8	0.84	0	0	0.12	0	0
	Ampicillin	.	.	.	—	—	—	+	+	+
		.	.	.	5.0	2.6	1.4	0.41	0.22	< 0.1
<i>E. coli</i> T 841	Benzyl-penicillin	—	—	+	+	+	+	+	+	+
		.	27.0	1.5	0	0	0	0	0	0
	Ampicillin	.	.	—	—	$\pm$	+	+	+	+
		.	.	15.0	4.4	2.65	0.63	0.24	0	0

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.

+ = Growth.

— = No growth.

± = Partial growth.

S. sonnei at concentrations of 1.25–2.5 µg./ml., whereas benzylpenicillin was inhibitory only at 12.5–25 µg./ml., thus showing a tenfold difference in the activities of these penicillins. Moreover, all five strains of bacteria destroyed most or all of the benzylpenicillin at concentrations below the minimal inhibitory concentrations, but there was little or no destruction of ampicillin by these organisms. These results showed the greater stability of ampicillin to the penicillinases produced by these bacteria, and suggested the possibility that the sensitivity of each strain to these

penicillins was related to the stabilities of the compounds to the penicillinase elaborated by each particular strain. This is supported by the results of an experiment shown in Fig. 1, illustrating the growth of one of these strains of bacteria, *E. coli* r341, in the presence of different concentrations of benzylpenicillin, and in Table 5 which shows the rate of destruction of the penicillin by this strain. It can be seen that at the lowest benzylpenicillin concentration (5 $\mu\text{g./ml.}$) growth was as rapid as in the control tube containing no antibiotic, while the concentration of antibiotic diminished steadily until none could be detected after 7 hr. At the next higher concentration (12.5 $\mu\text{g./ml.}$), there was little increase in growth for the first

Table 5. *Destruction of benzylpenicillin by cultures of Escherichia coli* r341

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Escherichia coli* r341 and incubated at 37° for 24 hr.; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration ($\mu\text{g./ml.}$)		
	25	12.5	5.0
0	25	12.5	5.0
1	24	11.5	5.4
2	20	9.2	5.0
3	16	6.3	4.5
4	15	3.0	2.5
5	12	1.3	1.3
6	11	1.3	0.9
7	10	0	0
24	0	0	0

3–4 hr., while the penicillin concentration fell gradually to 3.0 $\mu\text{g./ml.}$; during the next 3 hr the turbidity of the culture increased steadily and the penicillin concentration fell to zero. At the highest concentration (25 $\mu\text{g./ml.}$), there was no increase in turbidity over a period of 7½ hr, during which time the penicillin concentration decreased from 25 to 10 $\mu\text{g./ml.}$ After incubation overnight at 37° the turbidities of all these cultures were approximately equivalent to that of the control culture containing no penicillin, and no penicillin was detected for any concentration.

Group 3. Gram-negative bacteria resistant to both ampicillin and benzylpenicillin. Examples of bacteria resistant to ampicillin and to benzylpenicillin are shown in Table 6; it can be seen that all strains brought about marked destruction of both penicillins. In some cases, no penicillin could be detected in any of the culture tubes in which there was growth, e.g. *Escherichia coli* 70, *Proteus mirabilis* 889, *Klebsiella pneumoniae* c, *Aerobacter aerogenes* A and G. In other cases there was complete destruction of benzylpenicillin, but only partial destruction of ampicillin, for example, with strains of *P. rettgeri* A and *Ps. aeruginosa* A and c9. Yet again, with some strains there was only partial destruction of either penicillin, e.g. *E. coli* 37 and Paracolon WM29.

Quantitative estimation of penicillinase activity by *Pseudomonas aeruginosa* was made by bioautographic techniques, as it was noted that antibiotic-free cultures of this species displayed marked antibacterial activity against the assay organism, *Bacillus subtilis* ATCC 6633, with the result that microbiological assays showed little

Table 6. *Penicillinase activities of Gram-negative bacteria resistant to ampicillin and benzylpenicillin*

Minimal inhibitory concentrations of the penicillins against ten strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration ($\mu\text{g./ml.}$) after incubation at 37° for 18 hr.								
Bacteria-free control*		500	250	125	50	25	12.5	5.0	2.5	1.25
<i>Escherichia coli</i> 70	Benzyl-penicillin	.	+	+	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
	Ampicillin	.	+	+	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
<i>Proteus mirabilis</i> 889	Benzyl-penicillin	.	+	+	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
	Ampicillin	.	+	+	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
<i>Klebsiella pneumoniae</i> c	Benzyl-penicillin	.	+	+	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
	Ampicillin	.	+	+	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
<i>Aerobacter aerogenes</i> A	Benzyl-penicillin	.	—	—	+	+	+	+	+	+
		.	170	8	0	0	0	0	0	0
	Ampicillin	.	—	+	+	+	+	+	+	+
		.	90	0	0	0	0	0	0	0
<i>A. aerogenes</i> G	Benzyl-penicillin	.	—	+	+	+	+	+	+	+
		.	55	0	0	0	0	0	0	0
	Ampicillin	.	+	+	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
<i>Pseudomonas aeruginosa</i> A	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	.	.	.	.	.	.	.
	Ampicillin	+	+	+	+	+	+	+	+	+
		530	135	.	.	.	.	.	.	.
<i>Ps. aeruginosa</i> c9	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	.	.	.	.	.	.	.
	Ampicillin	+	+	+	+	+	+	+	+	+
		375	150	.	.	.	.	.	.	.
<i>Proteus rettgeri</i> A	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	—	—	—	±	+	+	+	+	+
		520	260	120	40	9.2	0.88	0.2	0	0
<i>E. coli</i> 37	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		8.5	18.5	12.5	3.2	0.3	< 0.1	0.5	0.4	< 0.1
	Ampicillin	—	—	+	+	+	+	+	+	+
		505	220	105	31	13.0	6.5	1.7	0.6	0.2
Paracolon WM 29	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		160	72	15	15	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		330	135	82	29	9.3	7.1	1.5	0.35	0.1

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
+ = Growth. — = No growth. ± = Partial growth.

or no destruction of either penicillin by this species. In fact, most strains of *P. aeruginosa* were capable of causing some destruction of both penicillins, ampicillin being more resistant than benzylpenicillin.

It is of interest that of some 30 strains of Gram-negative bacteria tested which were resistant to ampicillin and benzylpenicillin, all were capable of causing some degree of destruction of penicillin. In this series, no organisms which did not produce penicillinase and were inherently penicillin insensitive were isolated. In general, ampicillin was more stable than benzylpenicillin to the penicillinases produced by these Gram-negative bacteria, save that strains of *Aerobacter aerogenes* appeared to destroy ampicillin to a rather greater extent than benzylpenicillin.

Further experiments were designed to attempt to determine to what extent the resistance of these bacteria was due to penicillinase production. A simple type of experiment appeared to be estimation of the effect of decrease in inoculum size on

Table 7. *The effect of alteration of inoculum size on the sensitivity of Gram-negative bacteria to ampicillin and benzylpenicillin*

Organism	Inoculum*	Minimal inhibitory concentrations ($\mu\text{g./ml.}$)	
		Ampicillin	Benzylpenicillin
<i>Escherichia coli</i> 70	Neat	> 500	500
	10^{-3}	12.5	25
	10^{-6}	12.5	25
<i>Proteus vulgaris</i> D	Neat	> 500	> 500
	10^{-3}	5.0	12.5
	10^{-6}	1.25	2.5
<i>P. morganii</i> A	Neat	125	> 500
	10^{-3}	25	125
	10^{-6}	2.5	12.5
<i>Aerobacter aerogenes</i> A	Neat	500	125
	10^{-3}	12.5	12.5
	10^{-6}	2.5	5.0
<i>A. aerogenes</i> G	Neat	500	125
	10^{-3}	12.5	12.5
	10^{-6}	2.5	5.0
<i>Pseudomonas aeruginosa</i> A	Neat	> 500	> 500
	10^{-3}	500	> 500
	10^{-6}	500	> 500
<i>E. coli</i> 83	Neat	> 500	> 500
	10^{-3}	> 500	> 500
	10^{-6}	500	> 500
<i>P. mirabilis</i> 889	Neat	500	500
	10^{-3}	500	250
	10^{-6}	250	250

* 1 drop of an overnight culture diluted as specified.

the sensitivity of the organism to penicillin, the rationale being that intrinsically sensitive, penicillinase-producing bacteria would show a marked increase in sensitivity to penicillins with smaller inocula, whereas very small inocula of inherently insensitive organisms would still be capable of proliferating in high

concentrations of penicillin. It can be seen from Table 7 that *Escherichia coli* 70, *Proteus vulgaris* D and *P. morganii* A, and *Aerobacter aerogenes* A and G were very much more sensitive to ampicillin and benzylpenicillin when small inocula were employed, whereas with *Pseudomonas aeruginosa* A, *E. coli* 83, and *P. mirabilis* 889 there was little or no change in sensitivity to ampicillin or benzylpenicillin, even when the inoculum was diminished by a factor of one in a million. From these results it would seem that the former five strains might be examples of intrinsically sensitive, penicillinase-producing bacteria, and that the latter three strains might serve as examples of intrinsically insensitive organisms. These results prompted further experiments designed to confirm these findings by correlating growth with concomitant penicillin destruction.

Table 8. *Destruction of benzylpenicillin by cultures of Aerobacter aerogenes A*

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Aerobacter aerogenes* A and incubated at 37° for 24 hr; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration ($\mu\text{g./ml.}$)		
0	100	25	5.0
1	100	25	4.3
2½	90	20	2.6
4	37	2.8	1.6
5½	4.7	2.2	0.7
24	0	0	0

An example of an intrinsically sensitive, but penicillinase-producing organism is illustrated in Fig. 2, which shows the growth of *Aerobacter aerogenes* A in the presence of different concentrations of benzylpenicillin; the rate of destruction of the penicillin by this culture is shown in Table 8. The minimal inhibitory concentration of this culture after overnight incubation was 500 $\mu\text{g.}$ benzylpenicillin/ml., but during the first 6 hr a concentration of 100 $\mu\text{g./ml.}$ completely inhibited growth; during this period the penicillin concentration fell from 100 to 5 $\mu\text{g./ml.}$ Thereafter growth took place so that after 24 hr the turbidity of this culture equalled that of a penicillin-free control culture. These results appear to confirm that the growth of this culture in the presence of benzylpenicillin was related to the rate of destruction of the antibiotic by the organism.

In contrast, an intrinsically insensitive strain of *Escherichia coli* 83 multiplied in the presence of concentrations of up to 500 $\mu\text{g.}$ benzylpenicillin/ml. as rapidly as did an antibiotic-free culture (Fig. 3), although the rate of destruction of penicillin was relatively slow (Table 9), showing that the organism was able to proliferate readily in the presence of high concentrations of penicillin.

Thus, the inferences drawn from the inoculum-dilution experiments regarding the nature of the insensitivity of various Gram-negative bacteria to penicillins appeared to be supported by the growth experiments illustrated above, and would seem to confirm that whereas some penicillinase-producing strains of Gram-negative bacteria are inherently insensitive to penicillins, with other strains resistance is due mainly to destruction of the antibiotic.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Growth of *Escherichia coli* (T341) in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 5 µg./ml.; ○, culture containing penicillin, 12.5 µg./ml.; △, culture containing penicillin, 25 µg./ml.

Fig. 2. Growth of *Aerobacter aerogenes* A in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 5 µg./ml.; ○, culture containing penicillin, 25 µg./ml.; △, culture containing penicillin, 100 µg./ml.

Fig. 3. Growth of *Escherichia coli* (83) in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 25 µg./ml.; ○, culture containing penicillin, 125 µg./ml.; △, culture containing penicillin, 500 µg./ml.

Table 9. *Destruction of benzylpenicillin by cultures of Escherichia coli* 83

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Escherichia coli* 83 and incubated at 37° for 24 hr; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration (µg./ml.)		
	500	125	25
0	500	125	25
1	490	124	20
3½	465	115	16
5½	425	90	8
24	0	0	0

DISCUSSION

On the basis of the results reported here, the penicillins which display a relatively low order of activity against any of the Gram-negative bacteria examined may be readily distinguished from those penicillins which show a significant effect against certain strains. Compounds in the former group include phenethicillin, propicillin, methicillin and cloxacillin, and it is plain that the insensitivity of the Gram-negative bacteria to these penicillins is in no way associated with penicillinase. In contrast, the sensitivities of Gram-negative bacteria to ampicillin and benzylpenicillin were very varied, and in many cases the reaction of the organism to these drugs appeared to depend upon the ability of the organism to produce a penicillinase capable of destroying the specific penicillin. From the experiments described above, the bacteria tested could be divided into three groups, according to the effect of the specific penicillinase upon ampicillin or benzylpenicillin, e.g.:

(a) Strains which produced little or no penicillinase and were sensitive to

ampicillin and to benzylpenicillin, e.g. one *Salmonella* species, and some strains of *Proteus mirabilis* and *Klebsiella*.

(b) Strains which caused little or no destruction of ampicillin but more marked destruction of benzylpenicillin, and which were, accordingly, sensitive to ampicillin but less sensitive to benzylpenicillin, e.g. strains of *Escherichia coli*, *Shigella* species.

(c) Strains which were capable of destroying both compounds. This group, comprising penicillin-resistant organisms, could be further divided into: (i) intrinsically sensitive, penicillinase-producing, bacteria, e.g. strains of *Aerobacter aerogenes*, *P. vulgaris*, *P. morganii*; (ii) intrinsically insensitive, penicillinase-producing, bacteria, e.g. *Pseudomonas aeruginosa*, strains of *E. coli* and *P. mirabilis*.

It follows from these findings that the main difference in the activities of ampicillin and benzylpenicillin is associated with the increased stability of ampicillin towards the penicillinases produced by certain Gram-negative bacteria. Consequently, while ampicillin is from two to four times more active than benzylpenicillin against Gram-negative bacteria which produce little or no penicillinase, it is at least ten times more active against many strains which do produce penicillinase. The latter would apparently include the large number of strains of *Escherichia coli* which are sensitive to relatively low concentrations of ampicillin, but which require considerably higher concentrations of benzylpenicillin for inhibition. It is worth noting, however, that with these organisms growth is dependent upon destruction of the penicillin in the culture medium, so that benzylpenicillin might seem to be considerably more effective if antibacterial activity were measured over a shorter period of time than is conventional. Strains resistant to both penicillins are usually capable of destroying both compounds, although, again, ampicillin is generally more stable to the action of the penicillinases produced by these organisms. An exception to this finding is that benzylpenicillin appears to be more stable to *Aerobacter aerogenes* than is ampicillin but there is usually extensive destruction of both compounds. These findings are in good agreement with those of various other workers who have reported that ampicillin displays increased stability to the penicillinases produced by Gram-negative bacteria (Auhagen *et al.* 1962; Ayliffe, 1963; Smith, 1963). The last two workers also noted the superior stability of benzylpenicillin to the penicillinases produced by *Aerobacter* species.

In this series of experiments, all the resistant strains of bacteria produced penicillinase, whereas Trafford *et al.* (1962) reported that a relatively high proportion of bacteria which did not produce penicillinase were resistant to ampicillin. On the other hand, Barber (1962) reported that the majority of coliform bacteria are penicillinase-producing organisms capable of destroying penicillins. It is possible that in some cases at least, divergences in findings may be due to differences in techniques; this is being investigated. Likewise, although Knox & Smith (1962) have described strains of Gram-negative bacteria which are sensitive to ampicillin but with inherent resistance to benzylpenicillin, such strains have not been isolated in the experiments described above.

It thus seems that benzylpenicillin is active against strains of Gram-negative bacteria which produce little or no penicillinase, but is ineffective against penicillinase-producing bacteria. In contrast, ampicillin is relatively stable to the penicillinases produced by certain of the Gram-negative bacteria, and is, as a consequence, substantially more active than benzylpenicillin against many of these

bacteria. With resistant strains of bacteria, however, the situation is much more complex; here insensitivity may be due to penicillinase activity alone, or may be due to inherent resistance, although in the results reported here this inherent resistance was always accompanied by some degree of penicillin destruction.

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