The Nature of Carbenicillin Resistance in *Pseudomonas aeruginosa*

By A. H. THOMAS AND R. A. BROADBRIDGE

Division of Biological Standards, National Institute for Medical Research,
Mill Hill, London, N.W.7

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SUMMARY

Carbenicillin resistance in two strains of *Pseudomonas aeruginosa* has been investigated. *Pseudomonas aeruginosa* NCTC 10490, a carbenicillin sensitive strain, produced a β -lactamase when grown in the presence of carbenicillin. This enzyme although active against benzylpenicillin did not hydrolyse carbenicillin. Cell walls of *P. aeruginosa* NCTC 10490 habituated to the presence of carbenicillin showed a higher lipid content compared with the walls of the parent strain. Hospital-isolated, carbenicillin-resistant *P. aeruginosa* 69/4992 synthesized a β -lactamase which hydrolysed carbenicillin as well as benzylpenicillin. The two types of enzyme were characterized by their substrate profile, sensitivity to enzyme inhibitors and resolution by isoelectric focusing. Enzymes active against carbenicillin were differentiated by isoelectric focusing from those unable to hydrolyse carbenicillin; both types of enzyme were shown to be complex mixtures by this technique.

INTRODUCTION

Carbenicillin (disodium salt of 6 (α -carboxyphenyl acetamido) penicillanic acid) is a semisynthetic penicillin active against *Pseudomonas aeruginosa* *in vitro* (Acred *et al.* 1967; Knudsen, Rolinson & Sutherland, 1967). Because the sensitivities of many strains of *P. aeruginosa* to carbenicillin are within a reasonably narrow range of concentrations which can be safely obtained *in vivo*, carbenicillin has been used successfully in the treatment of pseudomonal infections (Brumfitt, Percival & Leigh, 1967; Jones & Lowbury, 1967; Richardson, Spittle, James & Robinson, 1968; Stratford, 1968). The original reports of carbenicillin-resistant strains of *P. aeruginosa* described small colony variants some of which produced β -lactamase, although none of these strains inactivated carbenicillin (Brumfitt *et al.* 1967; Jones & Lowbury, 1967; Smith & Finland, 1968). Recently an increasing number of carbenicillin-resistant strains of *P. aeruginosa* have been isolated (Stratford, 1968; Bell & Smith, 1969; Darrell & Waterworth, 1969; Stephenson, 1969; Lowbury *et al.* 1969; News on, 1969) and the last two reports described strains of *P. aeruginosa* able to inactivate carbenicillin.

In an attempt to understand the mechanism of carbenicillin resistance, both cell wall preparations and β -lactamase enzyme preparations from carbenicillin-sensitive and carbenicillin-resistant *Pseudomonas aeruginosa* have been compared.

METHODS

Materials. The following antibiotics were used: Sodium Benzylpenicillin, Cephaloridine (Glaxo Laboratories Ltd, Greenford, Middlesex), Ampicillin Sodium and Carbenicillin Disodium (Beecham Research Laboratories, Betchworth, Surrey); all were of British Pharmacopoeial quality. The penicillin concentrations were based on an assumed 100%

purity of the salts. The carbenicillin available at present may contain as much as 5% (w/w) benzylpenicillin (British Pharmacopoeia 1968); the material we used contained 4% (w/w) of benzylpenicillin.

The following chromatographic solvent systems were used to identify the penicillin degradation products and the cell wall constituents:

1. The upper phase of butan-1-ol + acetic acid + water (12 + 3 + 5, v/v);
2. Butan-1-ol + pyridine + water (1 + 1 + 1, v/v) (Cole & Sutherland, 1966);
3. Chloroform + methanol + water (65 + 25 + 4, v/v);
4. Propan-2-ol + butanone + 1 N-hydrochloric acid (60 + 15 + 25, v/v);
5. 2-Methylpropan-2-ol + butanone + propanone + methanol + water + (0.88) ammonia (40 + 20 + 20 + 1 + 14 + 5, v/v) (Haworth & Heathcote, 1969);
6. Ethyl acetate + pyridine + butan-1-ol + *n*-butyric acid + water (10 + 10 + 5 + 1 + 5, v/v) (Mukerjee & Ram, 1964);
7. Ethyl acetate + pyridine + water + acetic acid (5 + 5 + 3 + 1, v/v);
8. The upper phase of ethyl acetate + acetic acid + water (3 + 1 + 3, v/v);
9. The upper phase of butan-1-ol + ethanol + water + (0.88) ammonia (40 + 10 + 49 + 1, v/v);
10. Propan-1-ol + (0.88) ammonia + water (6 + 3 + 1, v/v) (Ellwood, Keleman & Baddiley, 1963).

The solvents were of 'Analar' grade except for 2-methyl propan-2-ol and *n*-butyric acid which were 'Laboratory Reagent' grade (British Drug Houses Chemicals Ltd, Godalming, Surrey).

The amino acids, amino sugars, carbohydrates and phospholipids were chromatographically homogenous (British Drug Houses Chemicals Ltd, and Koch-Light Laboratories, Colnbrook, Buckinghamshire).

Strains and growth conditions. The strains of *Pseudomonas aeruginosa* used were NCTC 8203, NCTC 10490, 69/3425 and 69/4992; the two latter carbenicillin-resistant strains were kindly supplied by Dr E. J. L. Lowbury of the Birmingham Accident Hospital. The minimum inhibitory concentrations of carbenicillin and of benzylpenicillin against these organisms were determined using twofold serial dilutions of the antibiotics in nutrient broth; one ml amounts of these were inoculated with one drop (10^5 organisms) of an overnight broth culture. After incubation overnight at 30° the tubes were inspected visually and the lowest antibiotic concentration to cause complete inhibition of growth was considered to be the minimum inhibitory concentration. Habituation of strain NCTC 10490 to the penicillins was made by serial transfer in nutrient broth containing increasing subinhibitory concentrations; final concentrations of benzylpenicillin (7.5 mg/ml; 2.107×10^{-2} M) and carbenicillin (0.1 mg/ml; 2.367×10^{-4} M) were tolerated by substrains which are here designated RB and RC respectively. Strain NCTC 8203 was grown in the presence of benzylpenicillin (5 mg/ml; 1.404×10^{-2} M) to increase the yield of β -lactamase. The nutrient broth had the following composition (% w/v): peptone 0.6; pancreatic digest of casein, 0.4; yeast extract, 0.3; beef extract, 0.15 and glucose, 0.1, adjusted to pH 7.0.

The organisms used for metabolic and chemical investigations were grown as batches of 1.2 l, in 4 l conical flasks, shaken at 30° for 18 h. Morphological examination of the suspensions, which usually contained 1.0 to 1.3×10^8 viable organisms per ml, was by phase-contrast microscopy. The organisms were collected by centrifuging at 2075 g for 2 h at 4° and were washed three times with distilled water. The culture supernatants were tested for β -lactamase activity; in addition the antibiotic containing supernatants were biologically assayed and examined for the degradation products of benzylpenicillin and of carbenicillin.

The concentrated bacterial suspensions after disruption at 175 (atm) with a Hughes press, were diluted with distilled water and centrifuged at 12,000 *g* for 30 min. The supernatant, which contained some particulate material, was freeze-dried and stored at -20° . The residue, which consisted of cell wall debris, was washed three times with distilled water and retained as a freeze-dried powder at -20° .

Examination of culture supernatants. All the culture supernatants were examined for β -lactamase activity by incubating with benzylpenicillin (2.806×10^{-3} M) or carbenicillin (2.367×10^{-3} M) for 60 min at 35° . Samples (1 μ l) of each reaction mixture and the appropriate control solutions were examined for the penicillin and its corresponding penicilloic acid by agarose-starch gel electrophoresis. The gel was prepared from agarose 1 % (w/v) and hydrolysed starch 1 % (w/v) in a phosphate buffer, pH 7.0, ionic strength 0.02. A potential of 2 V mm $^{-1}$ was applied across the gel for 30 min when the penicillins and penicilloic acids were detected with iodine vapour (Thomas & Broadbridge, 1970). The antibiotic containing supernatants were examined for penicillin degradation products by chromatography using Whatman paper no. 1 and solvent systems 1 and 2 as described by Cole & Sutherland (1966) and by electrophoresis as above. The residual antibiotic activity was estimated biologically. Since the assay organism used, *Bacillus subtilis* NCTC 8236, is some sixty times more sensitive to benzylpenicillin than to carbenicillin, trace amounts of benzylpenicillin had first to be separated. The assay of both antibiotics in the carbenicillin containing supernatants was performed after thin-layer electrophoresis (Lightbown & De Rossi, 1965).

Examination of bacterial supernatants. Freeze-dried material (200 mg) was dissolved in water, applied to a Sephadex G-25 column and eluted with 0.02 M-sodium acetate buffer, pH 7.6 (Sabath, Jago & Abraham, 1965). The fractions having β -lactamase activity were collected and freeze-dried. The potency of an enzyme preparation and its Michaelis constant for benzylpenicillin were determined by potentiometric titration (Wise & Twigg, 1950) using a pH-stat (Radiometer, Copenhagen, Denmark). The enzyme solution, adjusted to pH 7.0 with 0.01 M-KOH, was contained in a temperature-controlled polystyrene vessel maintained at 30° , and a steady stream of helium was passed over the reaction mixture. The substrate, benzylpenicillin (2.806×10^{-4} M), was added and the rate of hydrolysis monitored by measuring the alkali required to maintain the pH at 7.0. These precautions were necessary to maintain the blank at pH 7.0 for a period of an hour without the addition of even minute amounts of alkali; by this means a sharp end-point was obtained even with the low concentration of benzylpenicillin and carbenicillin used. The hydrolysis of carbenicillin and the effect of the β -lactamase inhibitors, cloxacillin and *p*-chloromeribenzoate (*p*-CMB) were evaluated potentiometrically; it was necessary to maintain the pH at 8.0 in the case of *p*-CMB. Substrate profiles for certain enzyme preparations were obtained by comparing the constant, steady-state rates of hydrolysis of ampicillin, carbenicillin and cephaloridine relative to benzylpenicillin. The rate of hydrolysis of ampicillin at pH 7.8 was determined by potentiometric titration whilst the formation of aminobenzylpenicilloic acid was confirmed by gel electrophoresis and by an increased absorption at 340 nm. Enzymatic cleavage of the β -lactam bond of cephaloridine was measured as the decrease in absorption at 255 nm at pH 7.0.

As the enzyme preparations could not be adequately resolved by agarose-starch gel electrophoresis further characterization was achieved by isoelectric focusing in thin layers of polyacrylamide gel. The following solutions were used to prepare the gel. Acrylamide solution: acrylamide, 6.86 g; *N,N*¹-methylenebisacrylamide, 0.18 g; and water to 100 ml. Catalyst solution: *N,N,N*¹,*N*¹-tetramethylethylenediamine, 0.06 ml; riboflavin, 1.06 mg and water to 100 ml. The chemicals were 'Laboratory Reagent' grade (British Drug Houses

Chemicals Ltd), the carrier ampholyte pH range 3 to 10 was supplied as a 40% solution (LKB Produkter A.B., Croydon, Surrey).

A polyacrylamide gel containing carrier ampholytes was prepared immediately before use by mixing 90 ml of acrylamide solution with 30 ml of catalyst solution and 12 ml of carrier ampholyte solution. This was sufficient for three 28×13 cm plates which were prepared by the method of Humphreys (1970). Solutions of the samples, $50 \mu\text{g}$ in $5 \mu\text{l}$, were applied to the gel over an area 2.0 cm long and 1.0 cm wide; the samples were spaced 2.0 cm from the edge of the plate and 1.5 cm apart. Electrical contact was made by inverting the plate so that the gel rested in a horizontal plane across two carbon rods (20×1.25 cm) 20 cm apart. Before use the cathode was moistened with 5% (v/v) ethylenediamine and the anode with 5% (v/v) phosphoric acid. Isoelectric focusing was performed in a humid chamber at 4° by applying 400 V (2 V mm^{-1}) from a constant voltage supply for 16 h. The initial current was 5 mA which steadily decreased to 0.6 mA as the pH gradient of the carrier ampholytes was established in the gel. The pH gradient was measured at 1 cm intervals along the gel with a glass surface electrode (type GM 23, Electronic Instruments Ltd, Richmond, Surrey).

The gel was stained for proteins with a solution of 0.2 g bromophenol blue, 50 ml ethanol, 5 ml glacial acetic acid and water to 100 ml for 1 h. The above solution without the bromophenol blue was used for decolorizing the gel.

The β -lactamase activity was located by biological development. The gel was overlaid with a slab of assay agar, medium A, pH 7.0 (British Pharmacopoeia, 1968), inoculated with a spore suspension of *Bacillus subtilis* NCTC 8236 and contained one of the following: ampicillin, 8.07×10^{-5} M; benzylpenicillin, 2.80×10^{-6} M; carbencillin, 7.10×10^{-5} M or cephaloridine, 2.40×10^{-6} M. The overlaid gel was incubated at 35° for 12 h, zones of exhibition indicated the destruction of the antibiotic.

Examination of cell wall material. The loosely bound lipids of the cell wall were extracted under nitrogen with chloroform + methanol (2 + 1, v/v), as described by Wilkinson (1968). The amount of lipid extracted was determined gravimetrically from both the dry weights of the lipid extract and the weight change of the residual cell wall. The lipid extract was separated into two fractions, free fatty acids and phospholipids, by acetone precipitation using the method described by Bobo & Eagon (1968), gravimetric determinations were made on both fractions. All materials were dried to constant weight in vacuum over phosphorus pentoxide.

The phospholipid fractions were compared by chromatography on silica gel G using solvent system 3. Iodine vapour, ninhydrin, a modified sulphuric acid spray (Ziminski & Borowski, 1966) and a molybdenum spray (Dittmer & Lester, 1964) were used to detect the spots.

The cell wall preparations were hydrolysed by heating at 100° with HCl both for 2 h with 1 N and for 20 h with 6 N and examined for amino acids, amino sugars and carbohydrates. Amino acids were identified by two-dimensional thin-layer chromatography on cellulose plates using solvent systems 4 followed by 5 (Haworth & Heathcote, 1969), the spots were detected with ninhydrin/cadmium acetate reagent. Descending paper chromatography on Whatman no. 4 previously washed with 2 N-acetic acid and rinsed with distilled water employing solvent systems 6 and 7 was used to identify the amino sugars; the spots were detected with alkaline silver nitrate. Carbohydrates were detected by descending paper chromatography as above using solvent systems 7 and 9 and by ascending paper chromatography with Whatman no. 4 using solvent systems 8 and 10. Spots were detected with alkaline silver nitrate, aniline hydrogen phthalate, ninhydrin or Schiff's periodate reagent.

The phosphorus content of the cell walls was determined using the modified method of Allen (1940). To estimate the cations, the cell walls (200 mg) were digested with 10 ml 60 % (w/v) perchloric acid at 150°. The solutions were evaporated to 2 ml and then made up to 10 ml with distilled water. The resulting solutions were analysed using an atomic absorption spectrophotometer (Perkin Elmer 303).

RESULTS

Growth of the organisms. *Pseudomonas aeruginosa* NCTC 10490 and its two antibiotic habituated substrains (RB and RC) were grown in antibiotic-free nutrient broth producing cultures designated (RB/O and RC/O) they were also grown in nutrient broth containing the corresponding penicillins producing cultures designated (RB/B and RC/C). The different cultures are indicated in Table 1 together with the final pH of each culture. Filamentous forms, characteristically produced by penicillin, were present in the substrains of *P. aeruginosa* NCTC 10490 which had been cultured in the presence of either benzylpenicillin or carbenicillin. Impairment of cell wall synthesis was not evident amongst cells of the carbenicillin resistant strain, 69/4992, grown in the presence of carbenicillin. The minimum inhibitory concentrations of benzylpenicillin and of carbenicillin against the strains of *P. aeruginosa* are shown in Table 2. The substrains of *P. aeruginosa* NCTC 10490 habituated to benzylpenicillin and to carbenicillin reverted to sensitivity to these antibiotics on repeated subculture in antibiotic free media.

Residual penicillin content and β -lactamase activity of the culture supernatants. Examination of the supernatants from cultures grown in the presence of antibiotics showed that

Table 1. *The growth conditions used for the strains of Pseudomonas aeruginosa and the presence (+) or absence (–) of β -lactamase activity in the resulting culture supernatants*

Strain of <i>P. aeruginosa</i>	Growth conditions		Laboratory designation of 18 h culture	β -lactamase activity against	
	Antibiotic concentration $\times 10^{-3}$ M	pH after incubation		benzylpenicillin	carbenicillin
NCTC 10490	None	7.18	S	–	–
NCTC 10490/RB	None	7.19	RB/O	–	–
NCTC 10490/RB	Benzylpenicillin 21.07	6.40	RB/B	+	–
NCTC 10490/RC	None	7.11	RC/O	–	–
NCTC 10490/RC	Carbenicillin 0.24	7.09	RC/C	+	–
69/4992	None	7.14	4992/O	+	+
69/4992	Carbenicillin 4.73	7.12	4992/C	+	+

RB indicates strain habituated to benzylpenicillin, RC to carbenicillin.

Table 2. *Minimum inhibitory concentrations of benzylpenicillin and carbenicillin against Pseudomonas aeruginosa determined using twofold serial dilution in nutrient broth*

Strain of <i>P. aeruginosa</i>	Benzylpenicillin (μ g/ml)	Carbenicillin (μ g/ml)
NCTC 10490	1,000	2
NCTC 10490/RB	> 16,000	800
NCTC 10490/RC	> 16,000	800
NCTC 8203	8,000	4
69/4992	> 16,000	8,020
69/3425	> 16,000	8,000

benzylpenicillin had been completely hydrolysed by *Pseudomonas aeruginosa* NCTC 10490, habituated to benzylpenicillin. Two acid degradation products were detected by thin-layer gel electrophoresis, corresponding to benzylpenicilloic acid and benzylpenicillin. Paper chromatography showed the second product corresponded with a prepared sample of benzylpenicilloic acid (Mozingo & Folkers, 1949), the decarboxylation product of benzylpenicilloic acid. In acid solution benzylpenicilloic acid will be decarboxylated (Schwartz, 1969) though the exact mechanism has not been studied. By monitoring the optical rotation of the enzymatic hydrolysis of benzylpenicillin this second reaction can be seen to proceed at a much slower rate than the hydrolytic reaction (unpublished observation). Only the benzylpenicillin present in the carbenicillin containing supernatant had been hydrolysed by the carbenicillin habituated strain of *P. aeruginosa* NCTC 10490, there was no significant decrease in the potency of the carbenicillin itself. By contrast *P. aeruginosa* 69/4992 degraded both penicillins to their respective penicilloic acids (Table 1).

β -Lactamase activity of the cell supernatants. The potencies of the different enzyme preparations, expressed as units of penicillinase as defined by Pollock & Torriani (1953), are shown in Table 3, together with their Michaelis constant for benzylpenicillin. The rates of hydrolysis, obtained under steady-state conditions, when the enzyme was saturated, for ampicillin, carbenicillin or cephaloridine are shown relative to benzylpenicillin. All the enzyme preparations obtained from the strains of *Pseudomonas aeruginosa* NCTC 10490 were completely inactivated by cloxacillin (2.0×10^{-4} M); in the presence of carbenicillin (5.0×10^{-4} M) activity of the enzymes was reduced by 50%; *p*-CMB (5.0×10^{-4} M) caused no inhibition. The hydrolysis of benzylpenicillin or of carbenicillin by the β -lactamase of 69/4992 was completely inhibited by cloxacillin (5.0×10^{-4} M), *p*-CMB (5.0×10^{-4} M) had no inhibitory effect.

Isoelectric focusing resolved the total soluble proteins of the enzyme preparations within the range of pH 4.0 to 6.8. Subsequent protein staining did not reveal any marked differences between any of the preparations but biological development differentiated two distinct groups of active material, each of which had an isoelectric focusing pattern which was complex (Fig. 1). Enzyme preparations from the same strain had identical isoelectric focussing patterns when developed on different substrates, even when the bacteria were grown in differing environments. Because the substrate profiles of the β -lactamase of strain NCTC 10490 and 69/4992 resembled respectively those described for *Pseudomonas aeruginosa* NCTC 8203 (Sabath *et al.* 1965) and *P. aeruginosa* 69/3425 (Sykes & Richmond, 1970),

Table 3. Comparison of the β -lactamase preparations from *Pseudomonas aeruginosa*, potency (penicillinase units/mg) and Michaelis constant (K_m) determined against benzylpenicillin 2.8058×10^{-4} M and the maximum rates of hydrolysis of ampicillin, carbenicillin and cephaloridine at stated concentration relative to benzylpenicillin (100)

Preparation	Penicillinase (units/mg)	Michaelis constant for benzylpenicillin (10^{-5} M)	Maximum rates of hydrolysis		
			Ampicillin 2.69×10^{-4} M	Carbenicillin 2.37×10^{-4} M	Cephaloridine 2.41×10^{-4} M
S	0	0	.	.	.
RB/O	100	3.005	.	.	.
RB/B	430	2.952	23	0	995
RC/O	370	2.952	.	.	.
RC/C	400	3.025	25	0	915
4992/O	1420	2.935	111	13	96
4992/C	1020	2.922	113	9	104

Isoelectric focusing, using pH 3 to 10 carrier ampholytes, of β -lactamase preparations synthesized by strains of *Pseudomonas aeruginosa* grown in nutrient broth containing different penicillins.

Fig. 1 to 4. Preparations top to bottom: NCTC 10490 (benzylpenicillin, 21.07×10^{-3} M); NCTC 10490 (carbenicillin, 0.24×10^{-3} M); 69/4992, (o) and 69/4992 (carbenicillin, 4.73×10^{-3} M). Development was on assay agar seeded with *Bacillus subtilis* NCTC 8236 containing ampicillin, 3 μ g/ml (Fig. 1); benzylpenicillin, 0.2 μ g/ml (Fig. 2); cephaloridine, 0.1 μ g/ml (Fig. 3) and carbenicillin, 3.0 μ g/ml (Fig. 4).

Fig. 5, 6. Preparations top to bottom: NCTC 10490 (benzylpenicillin, 21.07×10^{-3} M); 69/4992 (o); 69/3425 (o); NCTC 8203 (benzylpenicillin, 14.04×10^{-3} M). Development as above; benzylpenicillin, 0.2 μ g/ml (Fig. 5) and carbenicillin, 3.0 μ g/ml (Fig. 6).

enzyme preparations from these strains were compared by isoelectric focusing. The isoelectric focusing pattern of strains NCTC 10490 and NCTC 8203 were similar though the major component of the latter enzyme had an isoelectric point at pH 6.5 compared with the former at pH 6.6. The β -lactamases from the two carbenicillin-resistant strains 69/4992 and 69/3425 were very similar to each other except that the latter enzyme contained a greater proportion of material with an isoelectric point of pH 4.4.

Composition of the cell walls. The amount of readily extractable lipids from the different batches of *Pseudomonas aeruginosa* NCTC 10490 varied from 14.6% to 19.2% of the dry weight of the cell walls. The greater proportion of the lipids consisted of free fatty acids and neutral lipids, the residue was made up of phospholipids (Table 4).

The phospholipids were separated into four distinct components by thin-layer chromatography. The main component was tentatively identified as phosphatidyl ethanolamine, the remaining three spots corresponding to disphosphatidyl glycerol, phosphatidyl glycerol and phosphatidyl choline though the true identity of the latter is doubtful (Bobo & Eagon, 1968; Hancock & Meadow, 1969). There were no qualitative differences in the phospholipid fraction from any of the cell wall preparations.

Chromatographic examination of the different cell wall acid hydrolysates revealed no qualitative nor quantitative differences in composition. The principal compounds detected were *N*-acetyl glucosamine, muramic acid, alanine, glutamic acid, 2,4-diaminopimelic acid, asparagine and glutamine. Determination of the cell wall content of cations and phosphorus showed no significant differences (Table 4).

DISCUSSION

The very stringent precautions used in the β -lactamase assays enable the hydrolysis of very small quantities of penicillin to be monitored. It is significant that with such a sensitive method the hydrolysis of carbenicillin was not detected when examining the enzyme preparation from *Pseudomonas aeruginosa* NCTC 10490. In the presence of either benzylpenicillin or carbenicillin this strain of pseudomonas produces a β -lactamase though neither whole bacteria nor enzyme preparations inactivate carbenicillin. Both carbenicillin and cloxacillin inhibit the activity of this β -lactamase which suggests that carbenicillin has some affinity for the enzyme though it is resistant to hydrolysis.

The presence of filamentous cells amongst the cultures of *Pseudomonas aeruginosa* NCTC 10490 which had grown in contact with carbenicillin indicates that this penicillin impairs cell wall formation as does higher concentrations of benzylpenicillin. No gross differences were detected in the composition of the cell wall of the sensitive and penicillin habituated strains of NCTC 10490. The cell walls of the penicillin habituated strains contained slightly more readily extractable lipid, phosphorus, Ca^{2+} , Mg^{2+} and Na^+ than the

Table 4. *Comparison of the cell walls of Pseudomonas aeruginosa* NCTC 10490 grown in the presence of benzylpenicillin 21.07×10^{-3} M, RB/B and carbenicillin 0.24×10^{-3} M, RC/C

Expressed as percentage (w/w) of cell wall dry weight.									
Cell wall	REL	NL	PL	Ca^{2+}	Mg^{2+}	Zn^{2+}	Na^+	K^+	P^{5+}
S	14.5	9.46	4.06	0.23	0.38	0.004	0.029	0.0015	1.16
RB/B	15.9	11.00	4.28	0.33	0.58	0.004	0.043	0.0009	1.28
RC/C	19.2	12.56	5.13	0.30	0.44	0.005	0.040	0.0006	1.80

Readily extractable lipids (REL), free fatty acids and neutral lipids (NL) and phospholipids (PL).
S, Control no antibiotic.

sensitive strain. The overall cell wall composition of *P. aeruginosa* NCTC 10490 is similar to that reported for the cell walls of *P. aeruginosa* OSO-64 (Bobo & Eagon, 1968) and a polymyxin sensitive strain of *P. aeruginosa* NCTC 6750. An increase in the readily extractable lipid content of the cell walls of NCTC 6750 has been associated with polymyxin resistance (Brown & Watkins, 1970). No difference has been detected between the mucopeptide content of the cell walls of penicillin-sensitive and penicillin-resistant staphylococci (Rogers & Jeljaszewicz, 1961; Dyke, 1969) though there is a report that the cell walls of penicillin-resistant staphylococci contain a higher proportion of readily extractable lipids (Dunnick & O'Leary, 1970).

Garber & Friedman (1970) described a β -lactamase obtained from *Pseudomonas aeruginosa* 1978 whose substrate profile, especially the lack of activity against carbenicillin, was similar to that of *P. aeruginosa* NCTC 10490. They suggested that the intrinsic resistance of the pseudomonads was an important factor in penicillin resistance. The carbenicillin resistance of *P. aeruginosa* NCTC 10490 habituated to carbenicillin is not due to the β -lactamase but it may be attributed to an increase in the lipid content of the cell walls. This is in accord with reports of a carbenicillin-resistant *P. aeruginosa* which did not hydrolyse carbenicillin (Brumfitt *et al.* 1967; Smith & Finland, 1968; Watanakunakorn, Phair & Hamburger, 1970) even when the bacteria produced a diffusible β -lactamase (Jones & Lowbury, 1967). A permeability barrier which limits the access of certain penicillins into the cell is a feature of Gram-negative penicillinase producing bacteria and this barrier has been implicated in penicillin resistance (Smith, Hamilton-Miller & Knox, 1969). Our results suggest that the readily extractable lipids may be required for such a barrier.

The high degree of carbenicillin resistance exhibited by *Pseudomonas aeruginosa* 69/4992 can be attributed to the rapid destruction of the antibiotic by the constitutive β -lactamase of this organism, which was not inhibited by cloxacillin. Similarly, Neu & Swarz (1969) recorded that methicillin did not allow carbenicillin to exert its antibacterial effect against resistant strains of *Escherichia coli* and *P. aeruginosa*.

The enzymes obtained from *Pseudomonas aeruginosa* were differentiated into two categories by the method of isoelectric focusing. Enzymes that hydrolysed carbenicillin formed one category and those enzymes not active against carbenicillin were in the second category. Genetic transfer of β -lactamase activity has been shown to be mediated amongst pseudomonads by extrachromosomal R-factors (Fullbrook, Elson & Slocombe, 1970; Sykes & Richmond, 1970). Therefore it was anticipated that the β -lactamases able to hydrolyse carbenicillin would be similar, if not identical, as the strains 69/3425 and 69/4992 which produced these enzymes were isolated in the same hospital unit. The similarity of the substrate profiles of the enzymes from *P. aeruginosa* NCTC 10490 and NCTC 8203 indicated that they too were related to each other.

The isoelectric point reflects the total composition of the charge amino acids but the exact distribution of the charge residues will affect the isoelectric focusing position. The complex isoelectric focusing patterns exhibited by both categories of β -lactamase are an expression of this type of variation. This complex composition of the β -lactamase enzyme has not been reported and if confirmed it will complicate the study of β -lactamase kinetics. Using starch gel electrophoresis, Jack & Richmond (1970) noted the presence of two distinct β -lactamases in a strain of *Escherichia coli*; such heterogeneity may be explained by a single charge difference.

The hydrolytic activities of the neutral β -lactamases produced by *Pseudomonas aeruginosa* NCTC 8203 and NCTC 10490 for different penicillins are related to the degree of the hydrophobic nature of these antibiotics i.e. (cephaloridine > benzylpenicillin > ampicillin). The least hydrophobic antibiotic, carbenicillin, is not hydrolysed although the enzyme inhibitory

activities of carbenicillin and cloxacillin suggest that this category of enzymes must associate in some way with both hydrophilic and hydrophobic penicillins. The basic enzymes from *P. aeruginosa* 69/3425 and 69/4992 both hydrolyse carbenicillin, though less rapidly than other antibiotics tested. This type of enzyme could be responsible for the marked carbenicillin resistance exhibited by these strains of *P. aeruginosa*.

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