Effect of Carbenicillin on *Pseudomonas aeruginosa*

SUMMARY.—*The activity of carbenicillin against 200 strains of* Pseudomonas aeruginosa *was measured by a quantitative agar dilution method. Minimal inhibitory concentrations (M.I.C.'s) for five graded inocula were measured in terms of complete inhibition (CI) and reduced growth (RG). The M.I.C. decreased progressively as inocula were reduced, median values for the 200 strains ranging from 100 to 37.5 μg. per ml. by the CI criterion, and from 75 to 25 μg. per ml. by the RG definition. Ratios of M.I.C. obtained for large and small inocula were usually small. Identical M.I.C.'s by both CI and RG criteria were most often obtained when the inoculum for the RG criterion was 1 or 2 logs higher than that for complete inhibition.*

Population analysis of 15 strains of Ps. aeruginosa *showed that one specific drug concentration usually caused a sharp drop in proportion of viable cells, ranging from 3 to 5 logs. None of the populations were completely non-viable even at 150 μg. per ml. There was evidence that the viability of different-sized populations was reduced disproportionately by carbenicillin.*

Carbenicillin 300 μg. per ml. exerted appreciable bactericidal effect against nine of 15 strains of Ps. aeruginosa *after a 24-hour contact period; after only six hours the bactericidal effect was very small.*

Quantitative sensitivity measurements for carbenicillin should include M.I.C. values for both CI and RG criteria, using a range of inocula for testing. Such M.I.C. values may well be useful in monitoring carbenicillin therapy of tissue infections.

Carbenicillin is a semi-synthetic penicillin compound, first described by Acred *et al.*[1] and by Knudsen, Rolinson and Sutherland,[2] which has significant activity against *Pseudomonas aeruginosa.* This activity is of great interest, as *Ps. aeruginosa* is frequently resistant to many of the antibiotics available for chemotherapy of infections in man.

Acred *et al.*[1] and Knudsen and his colleagues[2] measured the activity of carbenicillin against many strains of *Ps. aeruginosa in vitro* by an agar dilution method. When inocula were taken from an undiluted broth culture, the end-points in these estimations were not sharp. Minimal inhibitory concentration (M.I.C.), defined in terms of complete inhibition of growth, was often as high as 250 μg. per ml., but at several concentrations below this, growth was very fine and often included some very small colonies. Much sharper end-points were obtained when the inoculum was taken from a 10^{-2} dilution of an overnight broth culture. With this inoculum the modal M.I.C. for 111 strains was 50 μg. per ml., with a range from 12.5 to 250 μg. per ml.

The inoculum effect described by these authors was apparently not due to penicillinase activity, as carbenicillin is stable to penicillinase produced by *Ps. aeruginosa.* Neither did the resistant colonies growing in the presence of high carbenicillin concentrations appear to be of mutational origin, for when their sensitivity was retested quantitatively, they showed the same pattern as the original strain.

Brumfitt, Percival and Leigh[3] found that the minimal inhibitory concentration of carbenicillin for 94 out of 99 strains varied from less than 1.5 to 200 μg. per ml. and for many of the strains it was 50 or 100 μg. per ml. The M.I.C. was inoculum-dependent.

Jones and Lowbury,[4] who tested 106 strains of *Ps. aeruginosa* against carbenicillin, found that 68 strains had M.I.C. of 64 μg. per ml. and all but one of the remainder required either 32 or 128 μg. per ml. for inhibition. Strains made more resistant by serial subculture in increasing concentrations of carbenicillin frequently grew in the form of small colonies. Van Rooyen *et al.*[5] noted the development of small colonies in cultures made from burned patients treated with carbenicillin, and also observed poor growth of these organisms on attempted subcultivation.

In this investigation, *in vitro* measurements were made of the activity of carbenicillin on 200 strains of *Ps. aeruginosa.* The M.I.C. was defined in two ways so that both complete inhibition and reduction in growth were taken into account. The influence of inoculum size was studied by varying the inoculum over a 4-log range. In addition, 15 of the strains were subjected to more detailed study, which included (a) population analysis to show the distribution in a culture of bacteria of different degrees of resistance and (b) the bactericidal activity of carbenicillin.

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TABLE I.—MINIMAL INHIBITORY CONCENTRATIONS (μG./ML.) OF CARBENICILLIN FOR 200 STRAINS OF *PSEUDOMONAS AERUGINOSA*. M.I.C. DEFINED AS SMALLEST TESTED CONCENTRATION CAUSING COMPLETE INHIBITION OF GROWTH AFTER 24 HOURS' INCUBATION AT 37°C.

Inoculum		*Number of strains showing M.I.C. of (μg./ml.)*											
Dilution of broth culture	*Approx. cell numbers*	*6.25*	*12.5*	*18.75*	*25*	*37.5*	*50*	*75*	*100*	*125*	*150*	*>150*	*Total strains*
10^{-0}	10^{5}	0	1	1	1	5	4	62	41	24	17	44	200
10^{-1}	10^{5}	0	1	4	4	17	23	91	22	15	9	14	200
10^{-2}	10^{4}	1	4	12	21	41	41	54	10	4	2	10	200
10^{-3}	10^{3}	3	5	26	37	47	28	38	4	3	3	6	200
10^{-4}	10^{2}	4	11	39	43	38	20	31	4	2	4	4	200

MATERIALS AND METHODS

The 200 strains of *Ps. aeruginosa* were all isolated from infective processes in patients, mostly urinary tract or wound infections. Each strain was isolated from a different patient. They were identified as belonging to the genus *Pseudomonas* by motility, gram-stain, morphology, catalase and oxidase activity, and their oxidative attack on glucose. Identification as *Ps. aeruginosa* was made on the basis of positive reactions for arginine dihydrolase and oxidation of gluconate, growth on TTC medium, and failure to hydrolyze starch. Many strains were subjected to pyocine typing and most of these were recognized as belonging to one of the types described by Gillies and Govan.[6]

Carbenicillin was supplied by Ayerst, McKenna & Harrison Limited in the form of a powder with an activity of 766 μg. per mg. For sensitivity testing it was incorporated in tryptose agar in 10 concentrations ranging from 6.25 to 150 μg. per ml. The carbenicillin agar was poured in square plastic plates marked with a 36-square grid. These plates were stored at 4° C. and used usually within one and always within two weeks of preparation. For measurement of M.I.C., strains were grown on tryptose agar overnight at 37° C. and then subcultured to tryptose broth which was also incubated overnight. Serial (tenfold) dilutions of the broth were then made to 10^{-4} in quarter-strength Ringer's solution. Samples of the dilutions and of the undiluted broth were transferred by means of a multiple inoculator to a series of carbenicillin plates and to a control tryptose agar plate without any drug. The inocula consisted of about 0.001 ml. of the broth dilution, so that the number of bacteria placed on the agar ranged from about 10^{6} to 10^{2}. One strain was therefore represented by five graded inocula, and six strains could be inoculated in this way on one set of plates. The plates were incubated for 24 hours and then read by naked eye for evidence of growth or inhibition. The M.I.C. was defined in two ways: (a) the lowest tested concentration of drug causing complete inhibition of growth and (b) the lowest tested concentration of drug causing reduction of growth, in terms of growth on the control plate. For each strain, M.I.C.'s were recorded according to both definitions for all inocula tested.

For the population analyses, viable counts were performed by the method of Miles, Misra and Irwin[7] on overnight broth cultures of 15 strains, all of different pyocine type, using plain tryptose agar as control medium, and tryptose agar containing different concentrations of carbenicillin, again ranging from 6.25 to 150 μg. per ml. Plates were incubated for 24 hours at 37° C.

For measurement of bactericidal activity, each of the 15 strains subjected to population analysis was first grown in tryptose broth overnight, after which 2.5 ml. of a 10^{-3} dilution of this broth was transferred to 22.5 ml. of tryptose broth in a flask. The flask was incubated at 37° C. for four hours, at which time a viable count was performed, again by the method of Miles, Misra and Irwin. Four samples were then removed from this flask, and to these samples was added either broth or carbenicillin solution to give final carbenicillin concentrations of 0, 75, 150 and 300 μg. per ml. The final volume of each culture was 5 ml. Incubation was continued at 37° C. Samples (0.2 ml.) were removed from each broth culture at 2, 4, 6 and 24 hours and subjected to viable counts, using tryptose agar as plating medium. Colonies on these plates were counted after a further 24-hour incubation period. Bactericidal activity was measured in terms of $\log_{10}$ reduction in viable count compared to the initial count performed after the four-hour incubation period in the flask.

TABLE II.—MINIMAL INHIBITORY CONCENTRATIONS (μG./ML.) OF CARBENICILLIN FOR 200 STRAINS OF *PSEUDOMONAS AERUGINOSA*. M.I.C. DEFINED AS SMALLEST TESTED CONCENTRATION CAUSING REDUCTION OF GROWTH AFTER 24 HOURS' INCUBATION AT 37°C.

Inoculum		*Number of strains showing M.I.C. of (μg./ml.)*											
Dilution of broth culture	*Approx. cell numbers*	*6.25*	*12.5*	*18.75*	*25*	*37.5*	*50*	*75*	*100*	*125*	*150*	*>150*	*Total strains*
10^{-0}	10^{6}	1	2	8	7	34	35	85	10	5	4	9	200
10^{-1}	10^{5}	4	4	12	26	52	43	46	2	4	2	5	200
10^{-2}	10^{4}	7	4	32	34	53	27	31	3	2	3	4	200
10^{-3}	10^{3}	9	13	50	43	35	18	23	2	3	2	2	200
10^{-4}	10^{2}	12	32	53	36	26	20	12	3	3	2	1	200

TABLE III.—RATIOS OF MINIMAL INHIBITORY CONCENTRATIONS OF CARBENICILLIN FOR INOCULA TAKEN FROM UNDILUTED BROTH AND 10^{-4} DILUTION OF BROTH

	Number of strains showing ratio within range	
Ratio range	*Complete inhibition*	*Reduction of growth*
1.0—1.67.....	24	50
2.0—2.67.....	36	68
3.0—3.67.....	33	27
4.0.....	36	27
5.0—6.0.....	22	15
>6.0.....	9	3
NR*.....	40	10
Total strains	200	200

*No ratio obtainable.

TABLE IV.—RELATIONSHIP BETWEEN COMPLETE INHIBITION AND REDUCED GROWTH M.I.C.'S OF CARBENICILLIN FOR UNDILUTED INOCULA OF 200 STRAINS OF *PSEUDOMONAS AERUGINOSA*

Complete inhibition	*Numbers of strains showing reduced growth M.I.C.'s of (μg./ml.)*											
M.I.C. (μg./ml.)	*>150*	*150*	*125*	*100*	*75*	*50*	*37.5*	*25*	*18.75*	*12.5*	*6.25*	*Total strains*
>150	9	2	3	4	21	2	3	0	0	0	0	44
150		2	0	3	10	2	0	0	0	0	0	17
125			2	3	13	4	2	0	0	0	0	24
100				1	17	14	7	1	1	0	0	41
75					23	13	17	3	5	1	0	62
50						0	3	1	0	0	0	4
37.5							2	1	2	0	0	5
25								1	0	0	0	1
18.75									0	0	1	1
12.5										1	0	1
6.25											0	0
												200

RESULTS

The results of the M.I.C. measurements are presented in Tables I and II. Table I shows the distribution of M.I.C.'s for the 200 strains using the criterion of complete inhibition of growth, and Table II shows the distribution of M.I.C.'s based on reduction of growth. In both cases, results are shown for inocula varying from an undiluted overnight broth culture to a 10^{-4} dilution of this culture. The number of organisms placed on the agar, corresponding to these dilutions, varied from around 10^6 to approximately 10^2.

The salient points emerging from these tables are:

(a) Whether the M.I.C. was defined in terms of complete inhibition or reduction of growth, there was a progressive decrease of M.I.C. as the inoculum diminished. The gradient of decrease became less steep when the inoculum was small; i.e., the M.I.C.'s showed little change when the inoculum fell from 10^3 to 10^2 bacteria.

(b) Median M.I.C. values for the 200 strains were, by the complete inhibition definition, 100 μg. per ml. for the undiluted inoculum and 75, 50, 37.5 and 37.25 μg. per ml. for successive tenfold dilutions of the overnight broth. Median M.I.C.'s using the reduced growth criterion were, respectively, 75, 50, 37.5, 25 and 25 μg. per ml. The M.I.C. was, therefore, lower for many strains when defined in terms of reduced growth than when based on complete inhibition of growth.

Table III shows the distribution of M.I.C. ratios for inocula taken from the undiluted and 10^{-4} dilutions of broth cultures, for both complete inhibition and reduced growth criteria of measurement. For some strains no ratio was obtainable, as either the undiluted inoculum showed M.I.C. greater than 150 μg. per ml. or the M.I.C. for the 10^{-4} inoculum was smaller than 6.25 μg. per ml. For both methods of reading the ratio for most strains did not exceed 4 and for very few strains was the ratio higher than 6. In general the ratios were lower for the reduced growth criterion (the median ratio was 2 for 190 strains, as opposed to 3 for the 160 strains by the complete inhibition criterion).

Table IV shows that very many of the strains showing M.I.C. from 75 to above 150 μg. per ml. for complete inhibition of an undiluted inoculum showed M.I.C. of 75 μg. per ml. when inhibition of the same inoculum was measured in terms of growth reduction. Use of this latter

TABLE V.—RELATIONSHIP BETWEEN NUMBERS OF STRAINS SHOWING VARIOUS M.I.C.'S OF CARBENICILLIN WHEN MEASURED BY COMPLETE INHIBITION (10^{-2} INOCULUM) AND REDUCED GROWTH (UNDILUTED INOCULUM)

	Minimal inhibitory concentrations of carbenicillin (μg./ml.)											
	>150	*150*	*125*	*100*	*75*	*50*	*37.5*	*25*	*18.75*	*12.5*	*6.25*	*Total strains*
Number of strains showing M.I.C.....												
Complete inhibition (10^{-2} inoculum).....	10	2	4	10	54	41	41	21	12	4	1	200
Reduced growth (undiluted inoculum).....	9	4	5	11	84	35	34	7	8	2	1	200

TABLE VI.—NUMBER OF STRAINS (OUT OF 200) SHOWING IDENTICAL M.I.C.'S BY VARIOUS PAIRED CRITERIA

Comparisons				
Reading method	*Inoculum*	*Reading method*	*Inoculum*	*Number of strains (out of 200) showing identical M.I.C.*
RG	10^{-2}	CI	10^{-3}	128
RG	10^{-3}	CI	10^{-4}	123
RG	10^{-2}	CI	10^{-4}	117
RG	10^{-1}	CI	10^{-2}	107
RG	10^{-1}	CI	10^{-3}	98
RG	10^{0}	CI	10^{-1}	87
RG	10^{0}	CI	10^{-2}	84
RG	10^{-3}	CI	10^{-3}	80
RG	10^{-1}	CI	10^{-4}	78
RG	10^{-2}	CI	10^{-2}	74
RG	10^{-4}	CI	10^{-4}	70
RG	10^{0}	CI	10^{-3}	52
RG	10^{0}	CI	10^{-4}	42
RG	10^{0}	CI	10^{0}	41
RG	10^{-1}	CI	10^{-1}	34

RG = reduced growth.
CI = complete inhibition.
Inoculum expressed as dilution of overnight broth culture.

TABLE VII.—POPULATION ANALYSIS OF 15 STRAINS OF *PSEUDOMONAS AERUGINOSA* ON AGAR CONTAINING CARBENICILLIN IN CONCENTRATIONS RANGING FROM 0 TO 150 μG. PER ML.

Strains	*Log₁₀ viable count per ml. of 24-hour broth culture on agar containing carbenicillin concentrations (μg./ml.) of*										
	0	*6.25*	*12.5*	*18.75*	*25*	*37.5*	*50*	*75*	*100*	*125*	*150*
1925	8.92	9.07	8.77	9.03	9.15	8.96	8.52	8.92	8.82	8.88	8.96
170	9.30	9.58	9.47	9.58	9.43	6.22	NC	5.05	NC	3.78	3.54
25	9.22	9.26	8.98	7.85	NC	4.61	4.28	3.56	3.68	2.88	NC
67	9.68	9.55	9.71	9.55	9.58	9.48	NC	4.24	4.20	3.55	3.38
10	9.37	9.32	9.18	NC	NC	NC	4.60	3.43	3.00	2.82	1.92
89	9.45	9.41	9.40	9.49	9.47	9.51	9.50	4.00	3.51	3.26	3.03
1927	9.56	9.68	9.71	9.63	9.69	NC	NC	4.67	4.03	3.63	3.57
2413	9.99	10.04	10.03	9.93	9.90	NC	NC	NC	NC	NC	NC
13980	10.18	10.21	10.29	NC	NC	5.38	NC	NC	NC	NC	NC
1720	8.82	8.92	8.81	8.24	5.61	5.00	NC	2.88	2.76	2.71	2.41
12815	8.87	8.89	NC	NC	4.40	3.83	3.20	2.73	NC	2.58	2.54
180	9.34	9.38	9.45	NC	5.97	4.10	3.62	3.82	3.57	NC	NC
2796	9.59	9.60	9.51	6.51	5.87	NC	5.43	NC	NC	NC	NC
142	>10.0	>10.0	>10.0	>10.0	NC	6.18	5.32	5.21	5.04	5.06	4.97
86	8.69	8.78	8.68	8.71	8.54	5.37	4.43	3.45	3.20	2.63	2.30

NC = not countable.

criterion tended to unify these strains and eliminate differences due to very slight growth at the higher concentrations.

Acred *et al.*[1] found that sharper end-points were obtained when the inoculum was taken from a 10^{-2} dilution of a broth culture instead of from an undiluted one. It was of interest, therefore, to compare the M.I.C.'s obtained with a 10^{-2} dilution (complete inhibition) with those obtained with an undiluted inoculum (reduced growth).

Table V shows the results of this comparison, which, at first sight, indicates that the grouping of M.I.C.'s is similar in the two instances. Differences of some magnitude are, however, apparent at the 75 and 25 μg. per ml. levels.

The principle of comparison of M.I.C.'s with different inocula and criteria of inhibition was extended to other pairs of observations. The numbers of strains giving identical M.I.C.'s by the reduced growth criteria with one inoculum and by the complete inhibition definition with identical or smaller inocula are shown in Table VI. The numbers are arranged in decreasing order of frequency of identical M.I.C.'s. The closest matches were seen when the reduced growth M.I.C. using a 10^{-2} inoculum was compared with the complete inhibition M.I.C. for the 10^{-3} inoculum and for the reduced growth (10^{-3} inoculum) compared with complete inhibition (10^{-4} inoculum). In these instances 128 and 123 strains, respectively, out of the 200 showed identical M.I.C.'s. There was frequent agreement between the two types of M.I.C. when the inoculum for the reduced growth criterion was 1 or 2 logs higher than that for complete inhibition. The two M.I.C.'s were less frequently identical when the inocula were the same or when they differed by 3 or 4 logs.

The results of the population analyses performed on 15 strains of different pyocine types are presented in Table VII. These results are expressed in terms of the $\log_{10}$ of the number of organisms per ml. of the culture which were able to grow on agar containing different concentrations of carbenicillin. The figures were derived from colony counts, and with 12 of the 15 strains it was not possible to obtain counts at all concentrations because of the nature of the growth on the agar. At these concentrations (NC in the table) counts were impossible either because the highest dilution of culture showing growth presented a fine haze of growth or very small colonies too numerous to count, or if discrete colonies were formed they were extremely small, and varied in size down to the point of virtual imperceptibility. With some of the strains, the non-countable growth occurred over a zone of drug concentrations just below a concentration which reduced growth by a factor of 4 or 5 logs, e.g. strains 10, 1927, 13980 and 12815. With other strains (25, 67, 1720, 180, 142) this "zone" was represented by one concentration only. Strains 170, 25, 13980 and 180 showed a second zone of non-countability at higher drug concentrations, but in three of these cases the end of this zone could not be determined using this particular range of concentrations. Strain 12815 showed an apparent second zone at 100 μg. per ml., but this is difficult to explain because of the uniformity of counts at 75, 125 and 150 μg. per ml. Strains 170, 1720 and 2796 showed a substantial drop in count at concentrations lower than those presenting non-countable growth. The first two of these strains showed a further drop in viable count at drug concentrations higher than those causing non-countable growth. Strains 1925, 89 and 86 gave countable growth at all concentrations tested; in the case of strain 1925 this was due to a high degree of resistance.

In the bactericidal tests, the $\log_{10}$ starting population per ml. immediately before addition of carbenicillin ranged from 6.51 to 7.74.

Carbenicillin in concentrations of 75, 150 or 300 μg. per ml. exerted no appreciable bactericidal effect on any of the 15 strains during the first six hours after its addition to the cultures. In most cases the viable population remained approximately constant, indicating a bacteriostatic effect. The control culture showed an increase in $\log_{10}$ viable population during this time, ranging from 0.66 to 1.88.

After 24 hours' exposure to carbenicillin some bactericidal activity was seen for most of the strains, though the effect was rarely dramatic. A concentration of 75 μg. per ml. of carbenicillin produced less than a 1 log kill in 13 strains and between 1 and 2 log kill in the other two; the 150 μg. per ml. concentration produced less than 1 log reduction in viable population in 10 strains, between 1 and 2 log reduction in four and more than a 2 log

TABLE VIII.—BACTERICIDAL ACTIVITY OF CARBENICILLIN FOR 15 STRAINS OF *PS. AERUGINOSA*. REDUCTION OF VIABLE COUNT IN BROTH CULTURES AFTER EXPOSURE TO VARIOUS CARBENICILLIN CONCENTRATIONS

Strain	*Starting population (log10 organisms/ml.)*	*Log10 population/ml. after 24 hours exposure to carbenicillin (μg./ml.)*				*Log10 reduction * in viable population by carbenicillin (μg./ml.)*		
		0	*75*	*150*	*300*	*75*	*150*	*300*
1925	6.86	9.84	7.85	7.86	4.97	—	—	1.89
170	7.06	9.21	7.51	7.29	7.26	—	—	—
25	7.74	9.09	7.18	7.18	6.02	—	—	1.72
67	7.38	9.61	7.68	6.30	6.30	—	1.08	1.08
10	6.76	9.48	7.72	6.80	6.13	—	—	0.63
89	6.51	9.26	5.48	4.98	2.88	1.03	1.53	3.63
1927	7.28	9.09	7.31	6.54	6.08	—	0.74	1.20
2413	7.20	9.08	7.24	7.30	6.68	—	—	—
13980	6.74	9.70	7.06	5.70	5.54	—	1.04	1.20
1720	6.95	9.10	6.86	6.86	6.10	—	—	0.85
12815	6.74	9.18	7.03	5.85	3.94	—	0.89	2.80
180	6.85	9.55	7.11	4.35	<1.92	—	2.50	>4.93
2796	7.13	9.35	5.40	5.57	5.54	1.73	1.56	1.59
142	7.49	9.55	6.98	7.22	6.63	—	—	0.86
86	7.38	9.57	7.72	7.30	6.90	—	—	—

*Log10 reductions of less than 0.6 have been disregarded in this part of the table.

kill in the remaining one strain; when the cultures were exposed to 300 μg. per ml. of carbenicillin, six strains showed less than a 1 log kill, six showed reduction in population between 1 and 2 logs, and one strain each showed respectively, 2 to 3 log kill, 3 to 4 log kill, and over 4 log kill. The results are summarized in Table VIII. Log reductions of less than 0.6 were disregarded.

DISCUSSION

Carbenicillin exerted a significant antibacterial effect on most of 200 strains of *Pseudomonas aeruginosa* in this series. Nearly all the strains were inhibited by 150 μg. per ml. or less of the drug at some inoculum level. Four strains (by the complete inhibition criterion) and one strain (by the reduced growth definition) were resistant to 150 μg. per ml. even when a 10^{-4} dilution of overnight broth was used for inoculum.

The minimal inhibitory concentration was clearly influenced by inoculum size and by the method of reading. The magnitude of the inoculum effect can be judged from the ratios of M.I.C.'s using undiluted broth and 10^{-4} dilutions as inoculum. These ratios ranged from 1 to 16 with a median of 3 when the complete inhibition definition was used, and from 1 to 10 with a median of 2 by the reduced growth definition. The median ratios are small, suggesting that in most instances the inoculum effect with carbenicillin is not due to destruction of the drug by a penicillinase-like enzyme. However, it is possible that strains showing an M.I.C. ratio of the order of 12 to 16 may elaborate a small quantity of such an enzyme.

M.I.C.'s defined in terms of complete inhibition (CI) were, for identical inocula, higher than those based on reduced growth (RG). But especially with the heavier inocula, these higher M.I.C.'s were often due to a slight degree of growth persisting at the three or four tested concentrations immediately below the M.I.C.; this growth usually consisted of a fine haze, sometimes surmounted by a few discrete but very small colonies.

This residual growth was not evident with smaller inocula, an observation consistent with the finding of Acred *et al.*[1] that sharper end-points were obtained by using a 10^{-2} dilution of an overnight broth culture as inoculum instead of an undiluted culture. Results obtained in the present work suggest that defining the M.I.C. in terms of reduced growth may cause a sharpening of end-point similar to that obtained by decreasing the inoculum and still measuring by complete inhibition. Although the overall M.I.C. patterns were similar with undiluted inoculum (RG) and 10^{-2} inoculum (CI), only 84 strains showed identical M.I.C.'s by these two criteria. A similar number of strains (87) showed identical M.I.C.'s when undiluted inocula (RG) and 10^{-1} inocula (CI) were compared. Table VI shows that CI and RG M.I.C.'s were closest when the inoculum for the former reading was 1 or 2 logs lower.

Complete inhibition M.I.C.'s may be unrealistic in that the completeness of the inhibition refers only to the sample taken and may not be reproducible for another sample which might yield a few resistant cells. Also, complete inhibition implies a total suppression of growth, whereas growth might occur on an agar surface for a few generations without becoming evident macroscopically.

Reduced growth as a basis for measuring the M.I.C. has the advantage that it usually reflects the behaviour of most of the population, and provides a value which is not influenced by a minority of resistant cells. This principle was utilized in a study conducted by the British Medical Research Council,[8] in measuring sensitivity of tubercle bacilli to isoniazid, and by Mitchison and Monk[9] in estimating the sensitivity of these organisms to para-amino salicylic acid. A minority resistant flora is of course important if it consists of resistant mutants which are likely to be selected by the antibiotic *in vivo*, but the sensitivity of the remaining large population should be important clinically if the tissue defences can destroy the few resistant organisms before selection by the antibiotic allows these variants to develop a large population of their own. At any one time a homogeneously resistant population, and one which is only partially resistant

by virtue of a few variant cells, must have different clinical significance.

In this context the degree of growth reduction becomes important. Reduced growth may be obvious enough if progressively less dense growth appears on a series of carbenicillin plates containing increasing drug concentrations, but such observations are somewhat subjective. Reduction of growth can in principle be measured quantitatively, in terms of viable counts on media containing different antibiotic concentrations, which indicate the proportion of a population capable of growth in the presence of such concentrations. Population analyses of this type have been carried out, for instance by Chabbert[10] for oxacillin and cephaloridine and by Seligman[11] for methicillin.

The quantitative growth reduction experiments reported in this investigation (Table VII) showed that with most strains tested there was a sharp drop in viable cell population at one specific drug concentration. This drop was of the order of 3, 4 or 5 logs. Increasing the carbenicillin concentration further usually caused a second smaller decrease in viable count, but none of the populations were completely eliminated even at 150 μg. per ml. Besides showing the falsity of the complete inhibition criterion for M.I.C., this finding indicated that the original populations contained a small number of bacteria resistant to the highest concentrations tested. The nature of these resistant organisms must remain in some doubt, however, because of the observation of Acred and his colleagues that colonies growing on agar containing high concentrations of carbenicillin consisted of populations which, on retesting, gave the same pattern of resistance as the original population.

With most of the 15 strains analyzed quantitatively in this investigation, viable counts on agar containing graded carbenicillin concentrations were somewhat vitiated by the occurrence of non-countable growth at concentrations just below those causing a 3 to 5 log drop in count (Table VII). Although a certain dilution of the culture gave numerous colonies (small but insufficiently discrete for counting), the next higher dilution showed no growth. At this antibiotic concentration, therefore, production of visible growth on the agar depended upon a certain minimum number of cells in the inoculum. The effect is different from that seen in systems involving β-lactamase enzymes and appropriate penicillins, where, both with *Staphylococcus aureus* and β-lactamase-forming gram-negative bacilli, confluent growth arising from a heavy inoculum is often complemented by complete absence of growth from an inoculum 1 log smaller. What the NC results do imply, however, is that carbenicillin causes a disproportionate reduction in different inocula, so that an M.I.C. value based on reduced growth must be stated for each inoculum tested. In this respect the reduced growth criterion is at no advantage or disadvantage in comparison with the complete inhibition definitions of M.I.C. An additional implication is that the principle of using a standard inoculum for M.I.C. measurements, as recommended by the World Health Organization[12] for the performance of the broth dilution procedures, is liable to give an incomplete picture of the response of an organism to carbenicillin, if not to other antibiotics which may, on analysis, be found to behave similarly. The differing ratios of M.I.C. found for undiluted and 10^{-4} inocula (Table III) show that use of a standardized inoculum does not permit prediction of the response of larger ones.

For clinical purposes, the value of M.I.C. determinations will depend upon the type of infection to be treated. After a dose of 1.0 g. carbenicillin given intramuscularly, Acred *et al.*,[1] Brumfitt, Percival and Leigh,[3] and Smith and Finland[13] found drug concentration in excess of 1000 μg. per ml. in the urine. From the range of M.I.C.'s found by various authors, and also in this investigation, attainment of effective urinary concentrations would present no problem, so that the only justification for performing quantitative sensitivity measurements on strains of *Ps. aeruginosa* infecting the urinary tract would be to detect highly resistant variants selected by carbenicillin. Jones and Lowbury[4] found an increase in M.I.C. for one strain of 128 to 2048 μg. per ml. after repeated subculture in subinhibitory concentrations of carbenicillin, but such resistance may not arise *in vivo* if infecting organisms are exposed to high carbenicillin concentrations at the outset of treatment.

Should resistant strains become prevalent in hospitals as a result of frequent use of the drug, M.I.C. determinations might become mandatory even for control of urinary tract infections.

Peak concentrations of carbenicillin attained in the serum after intramuscular administration of 1.0-g. doses has varied over the approximate range 14 to 48 μg. per ml.,[1, 3, 13] the higher values being achieved with the aid of probenecid. Such levels are unlikely, on the basis of M.I.C. determinations made in this and other investigations, to produce a significant therapeutic effect in tissue infections. Brumfitt and his colleagues reported that carbenicillin was in some instances disappointing in the treatment of non-urinary infections. Intravenous injections of 1 g. carbenicillin will produce peak levels above 100 μg. per ml., and have been recommended for tissue infections by Knudsen and his colleagues.[2] Van Rooyen *et al.*[5] obtained striking therapeutic results in patients with burns infected by *Ps. aeruginosa* by means of intravenous carbenicillin.

In view of the serum levels attainable, it would seem advisable to measure the M.I.C. of infecting strains, even though the dose of carbenicillin may presumably be increased considerably without risk of toxic reactions.

The bactericidal effect of carbenicillin demonstrated in this investigation was disappointing. Even with a concentration of 300 μg. per ml., most of the 15 strains tested showed less than a 2 log kill over a 24-hour period, and very little reduction in population during the first six hours of exposure to the drug. Acred *et al.*[1] found that a starting population of 10^6 cells per ml. exposed to a concentration of 50 μg. per ml. showed a 2 log kill in seven hours followed by a rise to about 3×10^7/ml. in 24 hours. A concentration of 250 μg. per ml. produced a 1.9 log kill in four hours and a 2.5 log kill in seven hours, followed by a small increase to 3×10^4/ml. by 24 hours. In the present work the starting population was

somewhat higher, ranging from 3 $\times$ 10^6/ml. to 5 $\times$ 10^7/ml., so possibly the main bactericidal effect occurred beyond the preliminary six-hour observation period. Obviously, more observations should be made between 6 and 12 hours of incubation. Comparison of the present results with those of Acred and his colleagues demonstrates, however, that conditions of starting inoculum and incubation time may have a decisive effect on the bactericidal activity of carbenicillin. How far a bactericidal action is necessary for controlling *Ps. aeruginosa* infections is not yet established; van Rooyen *et al.*[5] mention, in their report, one patient whose burns were grafted successfully after carbenicillin therapy, although small numbers of *Ps. aeruginosa* persisted in cultures from the burned areas.

Further work is necessary to explore the nature of the resistant colonies growing on agar containing high concentrations of carbenicillin, and the significance of the disproportionate reduction of different-sized inocula. Moreover, it is not yet clear whether "complete inhibition" or "reduced growth" M.I.C.'s correlate better with clinical response. In the meantime, there would appear to be some advantage, for the quantitative measurement of the activity of carbenicillin on *Ps. aeruginosa,* in expressing the M.I.C. as the smallest concentration of drug causing both complete inhibition and reduction in growth of a series of inocula ranging from samples of undiluted broth culture through a range of tenfold dilutions extending to 10^{-4}. The report on the M.I.C. should be completed by including the median M.I.C.'s by both criteria and for the complete range of inocula, for a series of strains isolated from patients in the hospital concerned. One important advantage of this approach is that quantitative sensitivity determinations would become more comparable from one laboratory to another; among the numerous values determined would be one or more which could be validly compared with observations in other laboratories.

All the M.I.C. determinations performed in this work were carried out on solid media; because of the ability of such media to show small numbers of resistant cells and to differentiate between a totally resistant population and a predominantly sensitive population containing a minority of resistant organisms, there would appear to be no advantage in performing M.I.C. estimations in fluid media. However, the results shown here have demonstrated that quantitative expression of the activity of carbenicillin on *Ps. aeruginosa* is not simple, and they raise the question whether similar complexities are inherent in any bacterium-antibiotic relationship.

Résumé

Nous avons mesuré l'activité de la carbénicilline contre 200 souches de Pseudomonas aeruginosa *par une méthode quantitative de dilution sur agar. Les concentrations inhibitrices minima (CIM) pour cinq inocula gradués ont été mesurés et exprimés soit en inhibition complète (IC) soit en pousse réduite (PR). La CIM a diminué progressivement à mesure que les inocula étaient réduits, les valeurs moyennes pour les 200 souches variant de 100 à 37.5 mcg par ml pour le critère IC et de 75 à 25 mcg par ml pour le critère PR. Les rapports entre les CIM obtenues pour les inocula forts et faibles étaient généralement peu élevés. La plupart du temps, on a obtenu des CIM identiques suivant critères IC et PR, quand le critère PR était de 1 à 2 logs plus élevé que pour l'inhibition complète.*

L'analyse de populations de 15 souches de Ps. aeruginosa *a montré qu'une concentration spécifique du médicament a provoqué généralement une chute brusque dans la proportion des cellules viables, variant de 3 à 5 logs. Cependant aucune des populations microbiennes n'était complètement non viable même à la concentration de 150 mcg par ml. On a constaté que la viabilité de populations de différentes grandeurs était réduite de façon disproportionelle par la carbénicilline.*

La carbénicilline à la concentration de 300 mcg par ml a exercé un effet bactéricide contre neuf des 15 souches de Ps. aeruginosa *après un contact de 24 heures; après un contact de six heures seulement, l'effet bactéricide était très faible.*

La mesure de la sensibilité quantitative de la carbénicilline devrait porter sur les valeurs de CIM, tant pour le critère IC que pour le PR, et utiliser une gamme graduée d'inocula. Ces valeurs de CIM peuvent être utiles pour contrôler le traitement à la carbénicilline des infections tissulaires.

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