Sensitivity of penicillinase-forming strains of *Staphylococcus aureus* and of their penicillinase-negative variants to cephaloridine, cephalothin, methicillin, and benzylpenicillin

J. H. HEWITT AND M. T. PARKER

From the Cross-Infection Reference Laboratory, Colindale, London

SYNOPSIS Twenty-eight penicillinase-forming cultures of *Staphylococcus aureus* and their penicillinase-negative variants were examined for resistance to benzylpenicillin, methicillin, cephalothin, and cephaloridine. The results supported the view that cephaloridine was more easily destroyed by staphylococcal penicillinase than was cephalothin.

In our tube-dilution tests, the minimum inhibitory concentration (M.I.C.) of cephaloridine for methicillin-sensitive cultures was never as high as some of the values reported by other workers who used apparently comparable methods. This was probably due to small differences in technique. The M.I.C. is an unsatisfactory measure of the antibiotic sensitivity of an organism which produces an enzyme which destroys the antibiotic.

Methicillin-resistant strains of *Staph. aureus* have an intrinsic resistance of 'heterogenous' type also to benzylpenicillin, cephalothin, and cephaloridine.

When cephaloridine was introduced in 1964 it was thought to be a promising antibiotic for the treatment of infections with penicillin-resistant strains of *Staphylococcus aureus* in patients who could not be given methicillin because they were allergic to all the penicillins (Mugleton, O'Callaghan, and Stevens, 1964; *British Medical Journal*, 1964). It appeared to have a somewhat greater activity against staphylococci than cephalothin, and had the additional advantage that its activity was not impaired by serum (Barber and Waterworth, 1964).

The results of *in vitro* sensitivity tests by a variety of methods gave rise to differences of opinion on the probable effectiveness of cephaloridine in the treatment of infections with *Staph. aureus* strains that formed large amounts of penicillinase. Some workers reported that all *Staph. aureus* strains were sensitive to low concentrations of the antibiotic (Mugleton *et al.*, 1964; Murdoch, Speirs, Geddes, and Wallace, 1964; Thornton and Andriole, 1966; Vymola and Hejzlar, 1966). Others found that a proportion of penicillinase-forming cultures, when tested with large numbers of staphylococci, were resistant to con-

centrations of antibiotic higher than were easily attainable in the body (Ridley and Phillips, 1965; Benner, Bennett, Brodie, and Kirby, 1965). Barber and Waterworth (1964) had observed a several-fold increase in the minimum inhibitory concentration (M.I.C.) of cephaloridine for some strains of *Staph. aureus* when the size of the inoculum was increased 500 times, and attributed this to destruction of the antibiotic by staphylococcal penicillinase. Cephalothin, on the other hand, was less susceptible to inactivation by penicillinase-forming staphylococci (Benner *et al.*, 1965).

Cross resistance between methicillin and the cephalosporins has also been reported (Barber and Waterworth, 1964; Benner *et al.*, 1965; Ridley and Phillips, 1965), and Baudens, Gerbaud, and Chabbert (1965) observed that penicillinase-negative variants of methicillin-resistant staphylococci retained their resistance to cephalothin.

We report our experience with testing the resistance of 28 penicillin-resistant strains of *Staph. aureus* to benzylpenicillin, methicillin, cephalothin, and cephaloridine. Penicillinase-negative variants were selected from each of the cultures, and were tested similarly,

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to determine the part played by staphylococcal penicillinase in their resistance to the four antibiotics.

MATERIALS AND METHODS

CULTURES Twenty-eight epidemiologically distinct penicillinase-forming strains of *Staph. aureus* were chosen to include some that were resistant only to penicillin, some that were also resistant to antibiotics unrelated to penicillin (multiple-resistant staphylococci), and some that were resistant to methicillin. Penicillinase-negative variants were obtained from each of them by the method of Dyke, Jevons, and Parker (1966). The phage-typing pattern of each penicillinase-negative variant was indistinguishable from that of its parent strain. When the original culture was resistant to one or more antibiotics unrelated to penicillin, loss of penicillinase was not accompanied by the loss of resistance to any of the other antibiotics.

The amount of penicillinase produced by the original cultures under standard conditions of induction was measured by the method of Richmond, Parker, Jevons, and John (1964), and the result expressed in units which related it to the penicillinase production of a standard culture.

The Oxford (Heatley) strain of *Staph. aureus* (N.C.T.C. No. 6571) was used as a control organism in all determinations of M.I.C.

TESTS FOR ANTIBIOTIC RESISTANCE The following tests were applied to all the penicillinase-forming cultures.

1 Resistance to streptomycin, tetracycline, chloramphenicol, erythromycin, oleandomycin, and novobiocin was determined on nutrient agar plates with Oxoid Multodisks (code no. 11-14c).

2 Disc test for resistance to methicillin (Jevons, Coe, and Parker, 1963) Blotting-paper discs, 6 mm. in diameter and each containing 10 µg. methicillin, were placed on nutrient agar plates previously inoculated with drops of undiluted six-hour nutrient broth cultures which were spread over sectors of the plate. The zone of inhibition was measured, and the presence or absence of

colonies within the zone was recorded, after incubation for 18 hours at 37°C.

3 Test for methicillin resistance on salt agar (Barber, 1964) Small drops (about 0.002 ml.) of undiluted six-hr. broth cultures were placed with a mechanical applicator on the surface of plates of nutrient agar to which 5% w/v sodium chloride and 10 µg./ml. methicillin had been added. The viable count of the inoculum was about 10⁸. If any growth appeared after 18 hours at 37°C. the culture was considered to be methicillin resistant, but most resistant strains gave confluent growth.

From the results of these tests, the cultures were placed in one of three categories (see Table I).

A METHICILLIN RESISTANT, MULTIPLE-ANTIBIOTIC RESISTANT Cultures which showed some, though sometimes slight, reduction in the radius of the zone of inhibition around a methicillin disc and grew on the methicillin-salt agar plate. Confirmation that these cultures possessed 'heterogeneous' resistance to methicillin (Chabbert and Baudens, 1962; Baudens *et al.*, 1965), that is to say, consisted of individuals differing greatly in their resistance to the antibiotic, was obtained in the course of this work, when it was found that all of them, and no others in the series, grew in 42 hours in the presence of at least 25 µg./ml. methicillin (see Sutherland and Rolinson, 1964).

B METHICILLIN SENSITIVE, MULTIPLE-ANTIBIOTIC RESISTANT Cultures which were resistant to one or more antibiotics not related to penicillin, but gave a zone of inhibition exceeding 8 mm. in radius around a methicillin disc, and did not grow on the methicillin-salt plate, or in broth containing 25 µg./ml. methicillin after incubation for 42 hours.

C RESISTANT ONLY TO PENICILLIN

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF ANTIBIOTICS

The antibiotics used—benzylpenicillin (Glaxo), methicillin (Celbenin, Beecham Research Laboratories), cephalori-

TABLE I
ANTIBIOTIC RESISTANCE CATEGORIES OF 28 STRAINS OF *STAPHYLOCOCCUS AUREUS*

Test	Category		
	A	B	C
	<i>Methicillin-resistant, Multiple-antibiotic Resistant</i>	<i>Methicillin-sensitive, Multiple-antibiotic Resistant</i>	<i>Resistant Only to Penicillin</i>
Resistant to one or more antibiotics other than penicillin ¹ in test with Multodisk	Yes	Yes	No
Disc test for methicillin resistance: radius of zone of inhibition (mm.)	3-8 ²	8.5-12.5 ²	11.5-12.5
Growth on agar containing 10 µg./ml. methicillin and 5% added NaCl	Yes	No	No
Tube-dilution test, M.I.C. of methicillin ≥ 50 µg./ml. after 42 hours' incubation	Yes	No	No

¹Streptomycin, chloramphenicol, tetracycline, erythromycin, oleandomycin, or novobiocin.

²Category A: colonies usually present within zone of inhibition.

Category B: colonies occasionally present around periphery of zone of inhibition.

dine (Glaxo 87/4), and cephalothin (Glaxo 87/1)—were pure materials provided by the manufacturers. For each experiment, a weighed amount of antibiotic was used to prepare doubling dilutions in nutrient broth (Oxoid No. 2).

Most of the determinations of the M.I.C. were made by a tube test. A standardized suspension of staphylococci (volume 0.02 ml.) was added to 1 ml. of a dilution of antibiotic in nutrient broth. Parallel tests were carried out with all four antibiotics on the same day, and with inocula from the same standardized suspension. The M.I.C. was recorded, after incubation at 37°C. for 24 hours and for 42 hours, as the lowest concentration of antibiotic in which there was no visible growth.

Inocula were prepared by making dilutions in nutrient broth of a six-hour broth culture, and were standardized by determining the optical density on a Unicam SP 600 spectrophotometer at a wavelength of 675 m μ . When necessary, cultures were diluted in nutrient broth to give suspensions of a similar optical density (0.100).

Surface viable counts on blood agar plates were also carried out on about one in two of the standardized suspensions. The results in 36 tests indicated that the mean inoculum size was 1.5×10^6 colony-forming units in 0.02 ml. where 1.0×10^6 was intended (range $0.5 - 3.2 \times 10^6$; S.D. $\pm 0.34 \times 10^6$). The inoculum size recorded in the experimental section is that inferred from the optical density.

In experiments on the effect of the size of the inoculum on the M.I.C., five serial 10-fold dilutions were made from the original broth culture which had been standardized by measurement of the optical density, and the inoculum size was verified by surface viable count. For each culture, all M.I.C. determinations were made with all the antibiotics in the same experiment.

Some tests were done by a method in which the antibiotic was diluted in nutrient agar. Agar plates were prepared by adding 1 ml. quantities of antibiotic dilution to 50 ml. molten nutrient agar (Oxoid nutrient broth no. 2 + 1.2% New Zealand agar) at 45–50°C. Two plates were prepared from each batch of medium and were dried for one hour at 37°C. Broth culture suspensions of staphylococci were placed as drops on the surface of the plate, and the size of the inoculum was in each case checked by performing a viable count. Inocula were placed on the plates as drops either with a mechanical applicator (volume 0.002 ml.: approximate area 7 sq. mm.) or from a standard pipette (volume 0.02 ml.: approximate area 80 sq. mm.). The plates were incubated at 37°C. for 24 hours. The lowest concentration of antibiotic which reduced the growth from confluent to less than 20 colonies, and the lowest concentration which gave complete absence of growth, were recorded.

RESULTS

MINIMUM INHIBITORY CONCENTRATION OF BENZYL-PENICILLIN, METHICILLIN, CEPHALOTHIN AND CEPHALORIDINE FOR PENICILLINASE-FORMING CULTURES
Table II shows the M.I.C.s of the antibiotics for the

28 penicillinase-forming cultures, and for the Oxford staphylococcus, in a tube-dilution test with an inoculum of 10^6 organisms, read after incubation for 24 hours.

With benzylpenicillin, the range of M.I.C.s observed was very wide. The organisms giving low results were generally methicillin-sensitive strains which had low penicillinase factors determined by the method of Richmond *et al.* (1964), but the correspondence was not absolute.

Most of the penicillinase-producing but methicillin-sensitive cultures were rather more resistant to methicillin than the Oxford staphylococcus, but the difference was with one exception only two- to four-fold. The distinction between methicillin-sensitive and methicillin-resistant cultures could not, however, be made clearly by this test (see Dyke, Jevons, and Parker, 1966); two of the strains considered on other grounds to be methicillin-resistant had a lower M.I.C. than one of those considered to be sensitive.

With cephalothin, no methicillin-sensitive organism had an M.I.C. exceeding eight times that of the Oxford staphylococcus, *i.e.*, greater than 1.6 μ g./ml., but seven of the nine methicillin-resistant organisms had M.I.C.s in the range 3.1–25 μ g./ml.

The M.I.C. of cephaloridine for methicillin-sensitive, penicillinase-forming staphylococci showed rather more scatter, and over half of the multiple-resistant strains (category B) appeared to be 16 to 32 times more resistant than the Oxford staphylococcus, but the absolute values for the M.I.C. appeared to be considerably lower than those recorded by several other workers who used inocula in the range 10^5 – 10^6 . For example, Benner *et al.* (1965), who used a tube-dilution method, and Ridley and Phillips (1965), who used a plate-dilution test, both found a proportion of cultures with M.I.C.s up to 25 μ g./ml.

Table II also shows, in parenthesis, the results of re-reading the tests after incubation for a further 18 hours. The M.I.C. of all four antibiotics for the methicillin-resistant organisms had increased considerably at 42 hours; with benzylpenicillin all, and with methicillin all but one, were 200 μ g./ml. or more; with cephalothin the M.I.C.s were in the range 12.5–50 μ g./ml. The least increase was with cephaloridine, and the highest M.I.C. at 42 hours was 12.5 μ g./ml. With the methicillin-sensitive cultures, little increase in the M.I.C. of methicillin occurred on further incubation, and the increase in the M.I.C.s for cephalothin and cephaloridine was, on average, only two- to fourfold.

The results shown in Table II represent the results of single determinations. With cephalothin and cephaloridine all the tests were done at least twice, and duplicate results, apart from those with

TABLE II
MINIMUM INHIBITORY CONCENTRATION OF BENZYLPENICILLIN, METHICILLIN, CEPHALOTHIN, AND
CEPHALORIDINE FOR 28 PENICILLINASE-PRODUCING CULTURES OF *STAPHYLOCOCCUS AUREUS*
AND THE OXFORD *STAPHYLOCOCCUS* IN A TUBE-DILUTION TEST

Category	No. Tested	Benzylpenicillin (μg./ml.)													Methicillin (μg./ml.)												
		0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.25	12.5	25	50	100	200	>200	1.6	3.1	6.25	12.5	25	50	100	200	>200			
A	9										2		3 (1)	4 (8)				2	3	4			(1)	(3)	(5)		
B	13					1	1		1	1			2 (3)	3 (3)	4 (5)	2	4 (5)	6 (7)	1 (1)								
C	6					1		1	1	1	1				1 (1)		3	3 (5)	1 (1)								
Oxford	1	1 (1)														1											

Inoculum 10^4 organisms.

Results read at 24 hours and (in parentheses) at 42 hours.

Category A, methicillin resistant.

B, methicillin sensitive; multiple antibiotic resistant.

C, methicillin sensitive; resistant only to penicillin.

Oxford N.C.T.C. No. 6571.

methicillin-resistant strains, never showed more than a two-fold difference from the figures quoted.

Penicillinase-forming staphylococci thus appeared to be somewhat more resistant to methicillin and to both cephalosporins than was the Oxford staphylococcus, particularly if they were multiple antibiotic-resistant. Those that were methicillin-resistant were in general considerably more resistant to all three of the other antibiotics, but could be distinguished clearly from methicillin-sensitive organisms only after incubation for 42 hours. Although some of the multiple-resistant staphylococci, which might be expected to include a number of cultures capable of producing large quantities of penicillinase, were relatively more resistant to cephaloridine than to cephalothin, the M.I.C. values we observed were considerably lower than those reported by some workers. Further investigations were therefore carried out to explore the relation of the M.I.C. to the size of the inoculum and to other conditions of the test.

EFFECT OF INOCULUM SIZE ON THE MINIMUM INHIBITORY CONCENTRATION IN THE TUBE DILUTION TEST At an early stage of this work, it had been found necessary to standardize the inoculum size precisely in order to obtain reproducible results with cephaloridine in the tube-dilution test, because relatively small changes over part of the range of dilutions often resulted in large differences in M.I.C.

Table III shows results of tube-dilution tests with all four antibiotics carried out with 10-fold differences of inocula over the range 10^1 to 10^7 organisms. Five penicillinase-producing strains and the Oxford staphylococcus were used, and all tests

with one organism were carried out with the same dilutions of a single culture. The organisms included one methicillin-resistant strain (category A), two multiple-resistant strains (category B), and two strains resistant only to penicillin (category C). One strain each from categories B and C produced large amounts of penicillinase (factor = > 0.9). The results are expressed as a ratio

M.I.C. observed
M.I.C. of the Oxford staphylococcus with inoculum 10^6 organisms

and the rate of increase of this ratio with increasing inoculum size was used as a measure of the 'inoculum effect'. The M.I.C. of the Oxford staphylococcus was relatively independent of the size of the inoculum, and in no case showed an increase exceeding four-fold between 10^1 and 10^6 .

With benzylpenicillin, all five cultures showed a large inoculum effect, usually most marked at the upper end of the range (10^4 - 10^7). The magnitude of this effect was not very closely correlated with the penicillinase factor determined by the method of Richmond *et al.* (1964), but the conditions under which penicillinase is produced in the two tests are somewhat different.

The methicillin-resistant culture also showed a considerable inoculum effect with methicillin, cephaloridine, and cephalothin, but the four methicillin-sensitive cultures showed little change in M.I.C. of methicillin or cephalothin over the range of inocula employed. With cephaloridine, however, the situation was different; the M.I.C. for the methicillin-sensitive cultures showed relatively little

TABLE II—continued

MINIMUM INHIBITORY CONCENTRATION OF BENZYL PENICILLIN, METHICILLIN, CEPHALOTHIN, AND CEPHALORIDINE FOR 28 PENICILLINASE-PRODUCING CULTURES OF *STAPHYLOCOCCUS AUREUS* AND THE OXFORD *STAPHYLOCOCCUS* IN A TUBE-DILUTION TEST

Cephalothin ($\mu\text{g./ml.}$)									Cephaloridine ($\mu\text{g./ml.}$)								
0.2	0.4	0.8	1.6	3.1	6.25	12.5	25	50	0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.25	12.5
			2	2	3	1	1	(3)						1	4	3	1
						(1)	(5)								(1)	(4)	(4)
	4	4	5							2	2	1	6	2			
	(2)	(5)	(3)	(3)					(1)	(3)			(1)	(1)	(6)	(1)	
	2	3	1								4	1	1				
		(3)	(2)	(1)							(4)	(1)		(1)			
1									1								
	(1)									(1)							

Inoculum 10^6 organisms.

Results read at 24 hours and (in parentheses) at 42 hours.

Category A methicillin resistant.

B methicillin sensitive; multiple antibiotic resistant.

C methicillin sensitive; resistant only to penicillin.

Oxford N.C.T.C. No. 6571.

TABLE III

EFFECT OF INOCULUM SIZE ON THE MINIMUM INHIBITORY CONCENTRATION OF BENZYL PENICILLIN, METHICILLIN, CEPHALOTHIN, AND CEPHALORIDINE FOR FIVE PENICILLINASE-PRODUCING CULTURES OF *STAPHYLOCOCCUS AUREUS*

Culture No.	Category	Penicillinase Factor	Inoculum (No. of Organisms)						
			10^1	10^3	10^5	10^6	10^8	10^9	10^7
Benzylpenicillin									
1	A	0.81	64	128	128	512	1,024	>4,096	—
2	B	1.08	<4	16	16	16	64	4,096	—
3	B	0.48	16	32	64	128	1,024	>4,096	—
4	C	0.99	<4	8	8	64	256	1,024	—
5	C	0.45	—	4	4	8	8	32	256
Methicillin									
1	A	0.81	—	2	4	8	8	>128	>128
2	B	1.08	—	4	4	4	4	4	4
3	B	0.48	—	1	1	1	2	2	4
4	C	0.99	—	1	1	2	2	2	2
5	C	0.45	—	1	1	1	2	2	2
Cephalothin									
1	A	0.81	—	2	4	8	8	16	32
2	B	1.08	—	4	4	4	8	8	8
3	B	0.48	—	2	2	2	2	2	4
4	C	0.99	—	1	1	2	2	4	8
5	C	0.45	—	—	<1	2	2	2	2
Cephaloridine									
1	A	0.81	—	4	8	8	16	32	256
2	B	1.08	—	4	4	<4	8	8	32
3	B	0.48	—	2	2	2	2	4	8
4	C	0.99	—	2	2	2	2	4	64
5	C	0.45	—	2	2	2	2	2	4

Results are expressed as the ratio of the M.I.C. observed to the M.I.C. of the same antibiotic for the Oxford staphylococcus with an inoculum of 10^6 organisms (see Table II). 24 hr. readings. Category, see Table I.

alteration over the range 10^1 to 10^6 , but increased considerably between 10^6 and 10^7 . This was most apparent with the two cultures which produced large amounts of penicillinase, with which there was a four- to eightfold increase (Fig. 1). Even with an inoculum of 10^7 organisms, however, neither of the two cultures with high penicillinase factors gave a M.I.C. exceeding $3.12 \mu\text{g./ml.}$

MINIMUM INHIBITORY CONCENTRATION OF CEPHALORIDINE DETERMINED BY DIFFERENT METHODS To explain the rather low M.I.C.s of cephaloridine observed in the previous experiment, we repeated the test on a selection of organisms by several different methods. Ridley and Phillips (1965) used a plate-dilution method, with an inoculum calculated to be between 10^5 and 10^6 organisms from an overnight

FIG. 1 Effect of size of inoculum on the minimum inhibitory concentration of cephaloridine for five penicillinase-producing cultures of *Staphylococcus aureus* and the Oxford staphylococcus in a tube dilution test read after incubation for 24 hours. In parentheses, the penicillinase factor of the corresponding culture.

broth culture, and Benner and his colleagues (1965) used a tube-dilution test with an inoculum of 10^6 organisms, also grown overnight.

Five penicillin-resistant cultures were used together with the Oxford staphylococcus. Three had been used in the previous experiment (nos. 1, 2, and 4) and two were methicillin-resistant (nos. 1 and 6). The M.I.C. was determined by a plate-dilution method with two inocula, one of 10^5 organisms in the form of small drops, of about 7 sq. mm. area, applied with a mechanical applicator, and the other of 10^6 organisms in a drop of about 80 sq. mm. area. The experiment was performed twice, with inocula from six-hour and from 24-hour broth cultures. A tube-dilution test with an inoculum of 10^6 organisms from the six-hour broth cultures was carried out at the same time.

The results are shown in Table IV. There was little difference between the results obtained with inocula from six-hour and 24-hour broth cultures. On the whole, however, rather higher M.I.C.s were obtained by the plate test than by the tube test with comparable inocula. The difference was small if the end-point in the plate-dilution test was taken as the lowest concentration of antibiotic which reduced growth from confluent to less than 20 colonies. When, however, the end-point was complete absence of growth, as employed by Ridley and Phillips (1965), the M.I.C.s observed were $\times 2$ to $\times 8$ greater than those obtained in the tube-dilution test, even with the methicillin-sensitive organisms.

In a few tests in which inocula of 10^6 organisms

TABLE IV
MINIMUM INHIBITORY CONCENTRATION OF CEPHALORIDINE FOR FIVE PENICILLINASE-PRODUCING CULTURES OF *STAPHYLOCOCCUS AUREUS* DETERMINED BY SEVERAL DIFFERENT METHODS

Culture No.	Resistance Category (see Table I)	Age of Culture (hr.)	M.I.C. (μ g./ml.) by		
			Plate Dilution Method		Tube-dilution Method
			Inoculation with 10^5 Organisms	Inoculation with 10^6 Organisms	Inoculation with 10^6 Organisms
1	A	6	6.25 (12.5)	6.25 (12.5)	6.25
		24	6.25 (12.5)	12.5 (25)	
6	A	6	6.25 (12.5)	12.5 (25)	6.25
		24	6.25 (12.5)	6.25 (25)	
2	B	6	1.6 (1.6)	3.1 (6.25)	0.8
		24	1.6 (1.6)	3.1 (6.25)	
7	B	6	0.8 (0.8)	1.6 (3.1)	0.8
		24	0.8 (1.6)	3.1 (6.25)	
4	C	6	0.4 (0.4)	0.4 (1.6)	0.4
		24	0.4 (0.4)	0.8 (1.6)	
	Oxford	6	0.05 (0.1)	0.05 (0.1)	0.05
		24	0.05 (0.1)	0.05 (0.1)	

The Oxford staphylococcus is included as a control. All results read at 24 hr.

Plate-dilution method: first figure is the lowest concentration which reduced growth to 20 colonies or less; in parentheses is the lowest concentration on which there was no growth.

FIG. 2 Relation of minimum inhibitory concentration of benzylpenicillin, methicillin, cephalothin, and cephaloridine for penicillinase-forming cultures of *Staphylococcus aureus* and for their penicillinase-negative variants.

Results expressed as the ratio $\frac{\text{M.I.C. of original culture}}{\text{M.I.C. of its penicillinase-negative variant}}$ M.I.C.s, determined by tube-dilution tests with an inoculum of 10^6 organisms, read after incubation for 24 hours. ● Ratio obtained with one culture.

were deposited on a small area of the plate with the mechanical applicator, no M.I.C. exceeding $6.25 \mu\text{g./ml.}$ was obtained. It seemed, therefore, that the high results obtained by Ridley and Phillips (1965) were not due to the fact that in their tests the organisms were concentrated on a small area of the plate.

INTRINSIC RESISTANCE TO PENICILLINS AND CEPHALOSPORINS OF THE PENICILLINASE-NEGATIVE VARIANTS
Minimal inhibitory concentrations of the four antibiotics for the penicillinase-negative variants were determined with inocula of 10^6 after 24 and 42 hours' incubation (Table V).

TABLE V
MINIMUM INHIBITORY CONCENTRATION OF BENZYL PENICILLIN, METHICILLIN, CEPHALOTHIN, AND CEPHALORIDINE FOR PENICILLINASE-NEGATIVE VARIANTS OF 28 *STAPHYLOCOCCUS AUREUS* CULTURES AND THE OXFORD STAPHYLOCOCCUS IN A TUBE-DILUTION TEST

Category	No. Tested	Benzylpenicillin (μg./ml.)											Methicillin (μg./ml.)									
		0.02	0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.25	12.5	25	1.6	3.1	6.25	12.5	25	50	100	200	> 200	
A	9			1	1	1		2	1	2	1 (7)	(2)		1	2	2		2	2	(1)	(8)	
B	13	2	6 (1)	4 (6)	1 (4)	(2)							1	12 (5)	(8)							
C	6	3	2 (1)	1 (2)	(3)								1	3 (4)	2 (2)							
Oxford	1		1 (1)										1		(1)							

Inoculum 10^6 organisms. Results read at 24 hr. and (in parentheses) at 42 hr.
Category: see Table I.

The M.I.C. at 24 hours of all four antibiotics for the penicillinase-negative variants of methicillin-resistant strains covered a wide range. Some cultures appeared in this test to be no more resistant than some of the methicillin-sensitive organisms. Indeed, one of the cultures had a M.I.C. for benzylpenicillin only twice that of the Oxford staphylococcus. In all cases, however, incubation for a further 18 hours altered the position completely. Every one of the cultures had a M.I.C. at 42 hours of at least 12.5 $\mu\text{g.}$ benzylpenicillin, 200 $\mu\text{g.}$ methicillin, 25 $\mu\text{g.}$ cephalothin, and 6.25 $\mu\text{g.}$ cephaloridine. This we interpret as evidence that all of the methicillin-resistant cultures had an intrinsic and 'heterogeneous' resistance to all four antibiotics, and consisted of a population which included a minority of cells capable of growing slowly in the presence of considerable quantities of each antibiotic.

At 24 hours, some of the penicillinase-negative variants of the methicillin-sensitive cultures (B and C) appeared to be a little more resistant to all four antibiotics than was the Oxford staphylococcus, but the difference was not great, and never exceeded a four-fold difference. Further incubation up to 42 hours resulted in little increase of M.I.C. It is clear, therefore, that though some of these organisms may have a slight intrinsic resistance to penicillins and cephalosporins, there is little or no 'heterogeneity' within the cultures with respect to this resistance.

At an early stage in the work, before standardized inocula had been introduced, the M.I.C. of cephaloridine and cephalothin for penicillinase-negative variants of methicillin-sensitive cultures had been found to vary little with increase in inoculum size in the range 10^5 to 10^7 . Further inoculum-size experiments confirmed that, with these antibiotics, varia-

tion in inoculum from 10^4 to 10^7 organisms made no more than a fourfold difference in M.I.C. by the tube dilution method.

ROLE OF PENICILLINASE IN RESISTANCE TO THE FOUR ANTIBIOTICS The M.I.C. of each culture was compared with the M.I.C. of its penicillinase-negative variant, thus giving an estimate of the part played by penicillinase in apparent resistance of the organisms to each antibiotic. The results are summarized in Fig. 2, which shows the ratio

$$\frac{\text{M.I.C. of original culture}}{\text{M.I.C. of its penicillinase-negative variant}}$$

as determined in tube-dilution tests with inocula of 10^6 organisms, when readings were made after 24 hours. The magnitude of this ratio is a measure of the stability of the respective antibiotics in the presence of staphylococcal penicillinase. Ratios with benzylpenicillin covered a wide range, and exceeded 1,000 for nearly half the cultures. With methicillin the median value of the ratios was 1, and no value exceeded 8, and with cephalothin the ratios were only a little higher. With cephaloridine, on the other hand, the median value was 8, and some of the cultures had ratios as high as 32-64. This was consistent with the view that cephaloridine was considerably less stable in the presence of penicillinase than were cephalothin and methicillin.

DISCUSSION

Workers who have used large inocula have found that the M.I.C.s of cephaloridine for penicillin-resistant strains of *Staph. aureus* are scattered over a wide range. The fact that some cultures can grow in

TABLE V—continued

MINIMUM INHIBITORY CONCENTRATION OF BENZYL PENICILLIN, METHICILLIN, CEPHALOTHIN, AND CEPHALORIDINE FOR PENICILLINASE-NEGATIVE VARIANTS OF 28 *STAPHYLOCOCCUS AUREUS* CULTURES AND THE OXFORD *STAPHYLOCOCCUS* IN A TUBE-DILUTION TEST

Cephalothin (μg./ml.)										Cephaloridine (μg./ml.)									
0.2	0.4	0.8	1.6	3.1	6.25	12.5	25	50	<50	0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.25	12.5	
		4	2	1		2		(2)	(4)	(3)		2	1	3	1	1	1	(1)	(8)
3	6 (3)	4 (7)	(3)								4	9 (6)	(7)						
1	5 (2)	(4)									2 (2)	4 (3)	(1)						
1	(1)										1	(1)							

Inoculum 10⁶ organisms. Results read at 24 hr. and (in parentheses) at 42 hr. Category: see Table I.

the presence of considerable amounts of cephaloridine is generally attributed to the destruction of the antibiotic by staphylococcal penicillinase. Cephalothin, on the other hand, appears to be considerably more resistant to this enzyme.

We agree with these conclusions, although the M.I.C.s of cephaloridine in our tests were considerably lower than some of those recorded by other observers. This we attribute to relatively small differences in technique.

Most of the 'inoculum effect' on the M.I.C. of cephaloridine for active producers of penicillinase is in the upper part of the range of inocula (Table III). Unless great care is taken in the standardization of the number of organisms used in the tube-dilution test, the M.I.C. for a large inoculum of an active producer of penicillinase may vary as much as four- to eightfold on repeated examination. When carefully standardized inocula were used, the results depended to some extent on the method of testing and on the way in which the end-point was read (Table IV). Benner *et al.* (1965) drew attention to the fact that, in pour plates containing sufficient cephaloridine to inhibit most of the bacterial population, a small proportion of cells survived and formed colonies, although the survivors appeared to have the same susceptibility as the parent strain. If, in an agar-dilution test, the end-point is taken as complete absence of growth, the M.I.C. may be several times greater than that observed in a tube-dilution test with a similar inoculum.

The conclusion we draw from our observations is not that *Staph. aureus* strains which produce much penicillinase are really more 'sensitive' to cephaloridine than had been supposed by the workers who observed some higher M.I.C. values, but that the M.I.C. is unlikely to be of much value in predicting

the effectiveness of treating staphylococcal infections with cephaloridine. If resistance to an antibiotic is due to tolerance, it may be relatively easy to relate the conditions of an *in vitro* test to the expected action of an antibiotic in a staphylococcal lesion. If, however, it is due to the destruction of an antibiotic by the organism, and particularly if the destructive enzyme is inducible, it is very difficult to decide which *in vitro* conditions of testing are relevant. Little is known about the formation of penicillinase by *Staph. aureus* in the body, but it appears to be influenced not only by the properties of the organism but also by the nature of the lesion (Eyckmans and Tompsett, 1965; Eyckmans and Hook, 1966). Unfortunately the conditions under which cephaloridine might have been of greatest use, *i.e.*, in the treatment of serious infections with multiple-resistant 'hospital' staphylococci, are those in which there are the greatest doubts about its probable effect, because these strains are the ones most likely to produce large amounts of an efficient penicillinase (Richmond *et al.*, 1964; Dyke and Richmond, 1967). In any event, it is quite clear that cephaloridine should not be used alone for the treatment of generalized staphylococcal infections due to penicillinase-producing organisms (Burgess and Evans, 1966; *British Medical Journal*, 1967), and that its use in combination with other antibiotics should be controlled by tests of bactericidal action.

Routine disc-sensitivity tests are of limited value in predicting the usefulness of cephaloridine for the treatment of infections due to penicillinase-producing staphylococci. The Association of Clinical Pathologists (Barber and Stokes, 1966) recommend the use of a 25 μg. cephaloridine disc for sensitivity tests, but point out the difficulty of interpreting the results with penicillinase-producing staphylococci. With

such discs we find that a clear distinction can be made between penicillinase-producing and penicillinase-negative cultures. However, to subdivide the penicillinase producers, on the basis of the diameter of the zones of inhibition, into sensitive, moderately resistant, and resistant to cephaloridine, is misleading. Narrowing of the zone of inhibition may be due to the production of penicillinase, or to the presence of 'heterogeneous', *i.e.*, methicillin, resistance, or to both, and the diameter of the inhibition zone is in each case greatly affected by the size of the original inoculum.

Observations on the penicillinase-negative variants (Table V and Fig. 2) support the view that the resistance of methicillin-sensitive, penicillinase-forming strains of *Staph. aureus* to cephalosporins is due mainly to the action of the penicillinase. However, some of these variants, particularly those obtained from multiple-resistant staphylococci, did appear also to possess, by comparison with the Oxford staphylococcus, a slightly increased intrinsic resistance to all penicillins and cephalosporins (Dyke, Jevons, and Parker, 1966). That this property was qualitatively different from the 'heterogeneous' resistance of the methicillin-resistant strains (Baudens *et al.*, 1965) is shown by the relatively slight increase in M.I.C. of the antibiotics for penicillinase-negative variants of the methicillin-sensitive organisms when the tubes were incubated for a second day (Sutherland and Rolinson, 1964).

Several workers have observed that methicillin-resistant cultures of *Staph. aureus* have an increased resistance to cephaloridine and cephalothin. Penicillinase-negative variants of methicillin-resistant staphylococci have an intrinsic resistance to benzylpenicillin which is of the 'heterogeneous' type (Parker and Jevons, 1964; Baudens *et al.*, 1965). The behaviour of the penicillinase-negative variants of nine distinct methicillin-resistant strains of *Staph.*

aureus indicates that these strains are also tolerant for cephaloridine and cephalothin. The increase in M.I.C. on incubation for a second day suggests that the resistance of these strains to the action of both penicillins and cephalosporins is of the 'heterogeneous' type.

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