In vitro Activity of Penicillins Against *Staphylococcus albus.** (28563)

JAN O. KJELLANDER,† JEROME O. KLEIN AND MAXWELL FINLAND
Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and Department of Medicine, Harvard Medical School, Boston, Mass.

Staphylococcus albus (Staph. epidermidis) is generally considered to be an organism of extremely low virulence and pathogenicity. However, it is known to produce disease, and cases of *Staph. albus* septicemia and endocarditis have been reported(1-6). In one study, the fatality rate in patients with blood cultures positive for *Staph. albus* was fairly high (16-20%) and did not seem to have changed appreciably since the introduction of antibiotics(2).

The patterns of susceptibility of *Staph. albus* to antibiotics have not been extensively studied. Strains resistant to benzylpenicillin (penicillin G) have been described by several workers(1,7-9), but little is known about their susceptibility to the new semisynthetic penicillins and the cephalosporins.

The mechanism of resistance of *Staph. aureus* to penicillin G has been well studied and many investigators have shown that naturally occurring strains produce a penicillin-destroying enzyme, penicillin-beta-lactamase, the characteristics of which have been extensively studied and recently reviewed (10). That strains of *Staph. albus* resistant to penicillin G are capable of destroying this drug was demonstrated by Bondi and Dietz (7), and it was shown by Kjellander and Finland(11,12) that such strains produce a penicillin-beta-lactamase. However, it is not known how widespread this property is among strains of *Staph. albus*.

Staph. aureus strains resistant to the new penicillinase insensitive penicillins have recently been reported(13-16); the mechanism of this resistance has been debated but most authors seem to agree that at least for the majority of strains, the resistance is inherent in the cells and is not due to production of an enzyme capable of destroying these new penicillins. Although some reports(16-21) seem to indicate that resistance to these new penicillins also occurs among strains of *Staph. albus*, this has not been specifically studied, and the mechanism of such resistance is not clear.

Studies have, therefore, been undertaken to clarify some of these points. Data on the susceptibility patterns to various antibiotics of strains of *Staph. albus* isolated from clinical material and observations on the mechanism of resistance of *Staph. albus* to the penicillins are presented here.

Materials and methods. Bacterial strains. One hundred and sixty isolates of *Staph. albus* (coagulase-negative) recovered in pure culture from blood cultures at the Boston City Hospital, were collected between Sept. 1962 and Feb. 1963; 15 additional strains that had grown in pure culture from urine, pleural or ascitic fluid or infected wounds were also included.‡ Two or more strains isolated from separate blood cultures taken at different times from the same patient were obtained in only 13 patients, in 4 of whom *Staph. albus* was considered definitely to have caused disease,—endocarditis in 3 and septicemia secondary to an infected venous cutdown site in the fourth.

Sensitivity tests.§ As in other recent studies(22,23) the tests for susceptibility of the organisms were done by the inocula-replicating method of Steers, Foltz and Graves(24) on plates of heart infusion agar (Difco, pH 7.2) into which 2-fold dilutions of antibiotics had been incorporated. The 17 antibiotics, including 10 penicillins, that were tested and their suppliers, were:

penicillin G (benzylpenicillin, Squibb)

ampicillin (α-aminobenzylpenicillin, Bristol)

* Aided by a grant from Nat. Inst. Health.

† Present address: Bacteriol. Lab., Karolinska Sjukhuset, Stockholm, Sweden.

‡ The strains were initially isolated in the Bacteriology Laboratory of Mallory Inst. of Pathology by or under the supervision of A. Kathleen Daly and Alice McDonald.

§ Done by Clare Wilcox.

propicillin (α-phenoxypropyl penicillin, Lilly)
phenoxyisobutyl penicillin (Pfizer)
methicillin (dimethoxyphenyl penicillin, Bristol)
oxacillin (5-methyl-3-phenyl-4-isoxazolyl penicillin, Bristol)
cloxacillin (5-methyl-3-*o*-chlorphenyl-4-isoxazolyl penicillin, Bristol)
nafcillin [6-(2-ethoxy-1-naphthamido penicillin), Wyeth]
diphenicillin (2-biphenylyl penicillin, Smith, Kline & French)
cephalothin (7-thiophene-2-acetamido cephalosporanic acid, Lilly)
erythromycin (Lilly)
chloramphenicol (Parke Davis)
tetracycline (Lederle)
novobiocin (Upjohn)
vancomycin (Lilly)
kanamycin (Bristol)
fusidic acid‖ (Squibb)

The minimum inhibiting concentration (MIC) was taken to be the lowest concentration of antibiotic on which there was no visible growth in 24 hours.

Tests for penicillinase production were done by the Haight and Finland(25) modification of the Gots test using *Sarcina lutea* as the indicator organism. Of the 116 strains that were positive in this test, and thus assumed to produce penicillinase, 12 (J1-J12) were selected for further studies to represent different levels of susceptibility to penicillin G and methicillin, 5 being resistant to the latter (MIC $\geq$ 12.5 μg/ml). These 12 strains were further tested as follows:

a. *Production of penicillin-beta-lactamase.* 0.1 ml amounts of 24hr, 37°C broth cultures (Brain Heart Infusion broth, Difco, pH 7.2) were used to inoculate into tubes containing 10 ml of broth with methicillin, 1.0 μg/ml. The tubes were incubated at 37°C for 6 hr and stored at −20°C until used. The tests were done by the micro-iodometric method of Novick(26) as modified by Kjellander and Finland(11).

b. *Mechanism of methicillin resistance. Effect of size of inoculum (Exp. 1).* The MIC of penicillin G and methicillin was determined by the inocula-replicating method, using as inocula serial 10-fold dilutions of 24 hr broth cultures (undiluted through 10^{-5}) with 0.85% saline as diluent. When growth occurred in discrete colonies, their numbers were recorded. The MIC of methicillin for Strain J11 was also determined in broth using 10-fold dilutions of culture as inocula into tubes of broth containing 2-fold dilutions of methicillin (Exp. 2). The tubes were incubated at 37°C for 48 hr and visible growth (turbidity) was noted; clear tubes were subcultured on antibiotic-free agar which was then incubated for 48 hours.

Strain J11 was also used in the following experiment (Exp. 3):¶ 0.1 ml amounts of serial 10-fold dilutions (10^{0}-10^{-7}) of a 24 hr broth culture were each used to make pour plate counts in agar containing 2-fold dilutions of methicillin. These plates and parallel plates of antibiotic-free agar were incubated for 48 hr at 37°C and the number of colonies counted. Only those plates with 30-300 colonies were used to calculate the number of viable units per ml. An identical series of plates were prepared using a culture that had been grown in broth containing methicillin, 1.0 μg/ml, to serve as an inducer of penicillinase.

Role of penicillinase. The effect of *Staph. albus* preparations on methicillin was tested in another series of experiments (Exp. 4). Nine methicillin-induced cultures were each centrifuged at 3000 rpm for 30 min., the sediment washed twice in Sörensen's phosphate buffer, pH 6.4, and extracted twice with acetone and twice with ether at −20°C, as described by Gilson and Parker(27). The dried cells were then resuspended in one-tenth of the original volume of buffer and kept frozen until used. These preparations were added in 0.5 ml amounts to a series of tubes each containing 500 μg of methicillin in 9.5 ml of buffer; one tube to which 0.5 ml of buffer instead of cell preparation was added served as control. Immediately after mixing, 1.0 of the contents of each tube was

‖ Referred to in this paper as an antibiotic for convenience.

¶ Carried out with the technical assistance of Mildred W. Barnes.

TABLE I. Susceptibility of *Staphylococcus albus* to 17 Antibiotics *in vitro*.

Antibiotic	No. of strains*	Minimum inhibiting concentration, μg/ml														
		>100	100	50	25	12.5	6.3	3.1	1.6	.8	.4	.2	.1	.04	≤.02	Median
Penicillin G	116R	64	1	10	5	5	3	6	5	11	6					>100.
	59S								1	0	3	3	8	18	26	0.04
Ampicillin	116R	37	18	8	5	4	6	9	10	9	8	2				50.
	59S								1	3	9	11	17	15	3	.1
α-phenoxypropyl penicillin	65R	5	8	0	8	8	3	5	9	8	8	2	1			3.1
	34S						1	0	0	1	2	13	10	6	1	.2
Phenoxyisobutyl penicillin	47R	2	0	1	1	1	4	2	10	12	11	3				.8
	18S								1	0	0	6	8	3		.1
Methicillin	116R		3	1	0	9	21	37	33	10	2					3.1
	59S					4	3	16	27	8	0	1				1.6
Oxacillin	116R	3	0	1	1	3	3	1	11	15	29	44	5			.4
	59S							2	5	2	8	36	6			.2
Cloxacillin	17R			1	1	4	0	0	2	2	5	2				.8
	10S								1	1	3	4	1			.2
Nafcillin	116R	2	1	0	0	0	0	6	20	13	65	9				.4
	59S							3	4	3	32	16	1			.4
Diphenicillin	116R			4	1	3	0	6	9	21	36	22	12	2		.4
	59S							1	4	1	13	26	12	0	2	.2
Cephalothin	116R						3	8	9	9	26	52	9			.2
	59S								1	3	8	19	22	2	6	.1
Erythromycin	34R	6	0	1	0	6	1	0	0	1	7	10	2			.4
	14S			1	0	0	0	1	0	0	2	9	1			.2
Chloramphenicol	34R	4	7	0	3	13	4	3								12.5
	14S					2	5	6	1							3.1
Tetracycline	34R	15	2	1	0	1	2	6	5	2						100.
	14S		1	3	0	0	2	2	5	1						3.1
Novobiocin	34R			2	1	1	0	0	0	0	5	25				.2
	14S			1	0	0	0	0	0	0	4	8	1			.2
Vancomycin	34R					5	11	12	6							3.1
	14S						5	7	2							3.1
Kanamycin	34R								7	10	10	7				.6
	14S								5	6	0	3				.8
Fusidic acid	34R				1	0	2	3	2	15	5	6				.8
	14S					1	1	0	0	3	5	4				.4

* R, penicillinase-producer (positive Gots test with penicillin G).
S, non " " (negative " " " " ").

withdrawn, diluted 1:10 with buffer solution, Seitz-filtered, and the filtrate promptly frozen. The remaining 9 ml were then kept in a water bath at 35°C for 24 hours, after which the same procedure of diluting and filtering was carried out. The filtrates were then assayed for methicillin content by the *S. lutea*, cylinder-plate method of Grove and Randall(28).

Finally, the 12 strains (J1-J12) were subjected to the Gots test with the following modifications: the agar contained 1.2 μg/ml of methicillin, a concentration just sufficient to inhibit the growth of the indicator strain of *S. lutea*, the test strains of *Staph. albus* were applied to the agar by the replica-inoculating apparatus and the plates incubated for 5 days before the results were recorded (Exp. 5).

Results. Susceptibility to antibiotics. The MICs of each of the antibiotics for all strains of *Staph. albus* studied are shown in Table I. Of the 175 strains, 116 (66.3%) were penicillinase-producers; these are listed separately from the nonpenicillinase producers and the median values for each group are also given. The penicillinase producing strains varied widely in susceptibility to the different antibiotics; for these strains the median MIC of penicillin G and ampicillin, both of which are rapidly degraded by penicillinase, was > 100 and 50 μg/ml, respectively. α-phenoxypropyl penicillin and phenoxyisobutyl penicillin, both of which are also quite susceptible to penicillinase, were much more active *in vitro* against these strains under the condition of these tests, their median MIC being 3.1 and 0.8 μg/ml, respectively. Moreover, the MICs of these 4 penicillins for the individual penicillinase-producing strains varied

TABLE II. Relation of MIC* to Penicillinase Activity of 12 Strains of *Staph. albus.*

Strain	MIC, μg/ml		Penicillinase activity		
	Penicillin G	Methicillin	Gots test with penicillin G	Penicillin G-β-lactamase, units/ml	Gots test with methicillin
J 1	1.56	1.56	+	.5	+
J 2	400	3.1	+	4.2	+
J 3	3200	6.2	+	16.1	—
J 4	100	12.5	+	.4	+
J 5	1.56	1.56	+	.5	+
J 6	1600	100	+	1.4	+
J 7	3.1	1.56	+	.5	+
J 8	50	100	+	.2	+
J 9	50	3.1	+	2.9	—
J10	.39	6.2	+	.3	+
J11	>3200	100	+	9.6	+
J12	400	>100	+	1.8	—

Strains J1, 2, 3, 5, 7, 9, 10 are methicillin "senitive". J4, 6, 8, 11, 12 are methicillin "resistant" (Table IV).

* Minimum inhibiting concentration.

over a much wider range than did those of nonpenicillinase producers.

If the MIC of ≥ 12.5 μg/ml is taken as the criterion for "resistant" strains, as suggested by Jevons *et al.*(15), 15 penicillinase-producing strains could be considered resistant to one or more of the following semisynthetic penicillins: methicillin, oxacillin, cloxacillin, nafcillin and diphenicillin; 3 of these strains were resistant only to methicillin, 3 others only to methicillin and cloxacillin, and 1 only to diphenicillin. For 5 of the strains the MIC was ≥ 50 μg/ml for 2 or more of these 5 penicillins. None of these strains were resistant to cephalothin. Of the 59 strains that did not produce penicillinase, 4 were resistant to methicillin (MIC = 12.5 μg/ml) and none were resistant to any of the other penicillins or cephalothin.

Only 34 strains that produced penicillinase and 14 that did not were tested with the remaining 7 antibiotics. The MIC of the former varied over a wide range of erythromycin, chloramphenicol and tetracycline, and a large proportion of them were moderately or highly resistant to these antibiotics; a few were also resistant to novobiocin. A few of the strains that did not produce penicillinase also required 12.5 or more μg/ml of one or more of these antibiotics to inhibit them. The

TABLE III. Effect of Inoculum Size on Growth of *Staph. albus* J6 on Agar Containing Penicillin G or Methicillin.

Inoculum, viable units/ml	Micrograms per ml							
	100	50	25	12.5	6.3	3.1	1.6	0.8
				Penicillin G				
460,000	+++	+++	+++	+++	+++	+++	+++	+++
46,000	0	+++	+++	+++	+++	+++	+++	+++
4,600		0	++	+++	++	+++	+++	+++
460			0	15	+++	+++	+++	+++
46				0	7	8	14	29
6					0	4	2	2
				Methicillin				
460,000	0	+++	+++	+++	+++	+++	+++	+++
46,000	0	++	14	++	++	+++	+++	+++
4,600	0	19	5	15	11	++	+++	+++
460					0	+	++	+++
46						0	22	++
6						0	5	5

Growth on heart-infusion agar with antibiotic incorporated: +++, heavy growth; ++ moderate growth; + countable (>25) colonies; numbers represent distinct colonies.

Method of Steers, Foltz and Graves(24).

TABLE IV. Effect of Inoculum Size on MIC of Penicillin G and Methicillin for 12 Strains of *Staph. albus*.

Inoculum*	Methicillin-sensitive strains							Methicillin-resistant strains					
	J1	J2	J3	J5	J7	J9	J10	J4	J6	J8	J11	J12
						M.I.C. of penicillin G, μg/ml of agar						
Undiluted	1.6	>100	>100	.8	.4	50	.4	>100	>100	25 –50	>100	>100
10^{-1}	.4	25	100	.2	.1	3.1	.2	25	100	25	>100	>100
10^{-2}	.2	.2	3.1	.1	.1	.4	.1		50	6.3 –12.5	>100	25– 50
10^{-3}	.1	.1	.2	.1	.04	.2	.1	.4	12.5– 25	.4 –12.5	6.3	6.3– 12.5
10^{-4}	.1	.1	.1	.04	.02	.2	.1	.4	3.1– 12.5	.01– .1	.8– 6.3	3.1– 6.3
10^{-5}	—	.04	.1	—	—	—	.04	—	6.3	—	.8– 1.6	3.1
						M.I.C. of methicillin, μg/ml of agar						
Undiluted	1.6	1.6	6.3	1.6	.4	1.6	1.6	12.5	100	100	100	>100
10^{-1}	1.6	1.6	6.3	1.6	1.6	1.6	1.6	12.5	100	25	3.1–100	6.3–100
10^{-2}	1.6	1.6	6.3	1.6	1.6	1.6	1.6	6.3	6.3–100	3.1	3.1	3.1– 50
10^{-3}	1.6	1.6	6.3	1.6	1.6	3.1	1.6	6.3	3.1	1.6 – 3.1	3.1	3.1
10^{-4}	1.6	3.1	6.3	1.6	1.6	3.1	3.1	6.3	3.1	3.1	3.1	1.6
10^{-5}	—	3.1	6.3	—	—	—	6.3	—	3.1	—	3.1	1.6

* Total number of viable units per ml of original broth cultures used for inocula ranged from 2.5×10^8 to 1.2×10^9 as calculated from colony counts on agar plates and an estimated 0.002 ml volume of each inoculum in the inocula-replicating method(24).

median MIC of vancomycin was 3.1 μg/ml for both producers and nonproducers of penicillinase although the MIC of 5 of the former was 12.5 μg/ml. All of the strains were sensitive to kanamycin, the MIC being ≤ 1.6 μg/ml. All but 2 of the strains (one in each group) were inhibited by ≤ 6.3 μg/ml of fusidic acid.

Mechanisms of resistance to penicillin G and methicillin. The 12 strains selected for these studies are listed in Table II, which also shows the MIC of penicillin G and methicillin, the results of modified Gots tests with these agents and the penicillin G-beta-lactamase activity of these cultures. All of the strains produced the latter enzyme, their activity varying from 0.2-16.1 units**/ml. The average activity of the methicillin-resistant strains (MIC ≥ 12.5 μg/ml) was 2.5 units as comparei with 3.5 units for the methicillin-sensitive strains. The highest activity was found in a methicillin-sensitive strain (J3), the lowest in a methicillin-resistant one (J8) and resistance to methicillin did not correlate with the beta-lactamase activity of the cultures.

Effect of inoculum size. Table III shows detailed results of the tests for susceptibility of one of the methicillin-resistant strains (J6) to that antibiotic and to penicillin G, using varying (10-fold) dilutions of culture as inocula in the agar-dilution test done with the replica-inoculating apparatus (Exp. 1). The results of similar tests of all 12 strains are summarized in Table IV and in Fig. 1. All of the strains showed an effect of inoculum size on the MIC of penicillin G; a similar effect with methicillin is noted only in the case of the 5 methicillin-resistant strains. The methicillin-sensitive strains did not show the effect with that antibiotic. Table V shows results of the test carried out with J11, a methicillin-resistant strain, in broth containing 2-fold dilutions of methicillin and inoculated with 10-fold dilutions of culture (Exp. 2). Essentially the same inoculum effect on the MIC is noted as in the agar dilution method.

** A unit of penicillinase is defined here as the amount of enzyme that catalyzes the hydrolysis of 1 μmole of penicillin G in 1 hr at 35°C and pH 6.4.

TABLE V. Effect of Inoculum Size on Bacteriostatic and Bactericidal Action of Methicillin on *Staph. albus*, Strain J11.

Inoculum, viable units/ml	Methicillin, μg/ml of broth 100	50	25	12.5	6.3	3.1	1.6	0
3,500,000	+	+	+	+	+	+	+	+
350,000	+	+	+	+	+	+	+	+
35,000	—	+	+	+	+	±	+	+
3,500	—	—	—	—	—	±	+	+
350	—	—	—	—	—	±	+	+
35	—	—	—	—	—	—	+	+

+, visible growth (turbidity) in broth after 48 hr at 37°C. ±, no visible growth (turbidity) in broth after 48 hr at 37°C, growth only after subculture on antibiotic-free agar. —, no growth in broth or after subculture on agar.

That this inoculum effect with methicillin is probably due to the presence in the original culture of a small proportion of cells that are highly resistant to methicillin is shown by the results of the experiment (Exp. 3 under *Materials and methods*) recorded in Table VI. Of the total 38 × 10^7 viable units in the control culture, with the strain grown in antibiotic-free broth and colony counts done in antibiotic-free agar, only 6200 (or 0.0017%) grew out in agar containing 128 μg/ml of methicillin. It is also seen that growth in a subinhibitory concentration of methicillin in the broth culture (1.0 μg/ml, the amount usually employed as a penicillinase inducer) subsequently enhanced the growth of these resistant cells in the agar which contained larger concentrations of methicillin, so that approximately 1 of every 4 cells in the culture (23% of the control number) grew in the presence of the highest concentration of methicillin tested, namely 128 μg/ml.

TABLE VI. Comparison of Methicillin-Resistance of Non-Induced and Methicillin-Induced *Staph. albus* (Strain J11).

Methicillin	Antibiotic-free broth culture: Viable units/ml	% of control	Culture grown in broth + methicillin, 1.0 μg/ml: Viable units/ml	% of control
0	38 × 10^7	100	52 × 10^6	100
1	26 × 10^7	68	50 × 10^6	96
2	20 × 10^4	.53	43 × 10^6	102
4	36 × 10^3	.0095	55 × 10^6	106
8	12 × 10^3	.0032	53 × 10^6	102
16	41 × 10^3	.011	51 × 10^6	98
32	23 × 10^3	.0061	55 × 10^6	106
64	82 × 10^2	.0022	20 × 10^6	38
128	62 × 10^2	.0017	12 × 10^6	23

Table VII shows the effect of the acetone-ether extracted cells on methicillin (Exp. 4). None of the 9 *Staph. albus* cell preparations tested seemed to affect the methicillin under the conditions of this experiment, there being no difference in concentration of that antibiotic after 24 hours of incubation between the control tube containing only drug and buffer and the tubes containing the drug and the cell preparations.

TABLE VII. Effect of Incubating *Staph. albus* Penicillinase* with Methicillin.

Hr of contact	Concentration of methicillin, μg/ml: Methicillin-sensitive strains J1	J2	J3	J5	Control†
0	5.1	5.1	4.7	5.1	5.1
24	3.0	3.7	2.6	2.6	2.7
	Methicillin-resistant strains J4	J6	J8	J11	J12
0	5.2	5.1	5.0	5.0	5.0
24	2.6	2.8	2.7	2.7	2.7

* Acetone-ether extracted cells in phosphate buffer, pH 6.3 at 35°C.

† Buffer with methicillin without cell preparation.

The results of the Gots tests with penicillin G and with methicillin (Exp. 5) are given in Table II. All of the strains showed a positive Gots test with penicillin G, and all but 3 of the strains (J3, J9 and J12) gave rise to satellite colonies of the indicator organisms *(S. lutea)* in the methicillin-containing agar.

Discussion. Leedom *et al.*(18) detected the presence of strains of *Staph. albus* resistant to methicillin in nasal cultures of 3 nurses and 3 patients following introduction of methicillin therapy on 2 wards of their hos-

pital. Stewart(16) was able to develop methicillin-resistant strains of *Staph. albus* readily by serial subcultures in methicillin-containing broth; such resistance developed more rapidly than in strains of *Staph. aureus*. The latter author also noted that serial isolates of *Staph. albus* obtained from 3 children in whom ventriculo-caval shunts were instituted for progressive hydrocephalus showed increasing resistance to the drug during methicillin therapy. The clinical course of these cases, including repeated demonstration of positive blood cultures for *Staph. albus* despite prolonged therapy with large doses of methicillin was described by Callaghan, Cohen and Stewart(17).

The experiences with use of methicillin aerosols in attempts to prevent crossinfections in newborn nurseries are of interest in this regard. In each of 3 such studies conducted by different groups of workers(19-21), this procedure resulted in rapid suppression and disappearance of *Staph. aureus* from the nares of the newborns; strains of *Staph. albus,* on the other hand, were eliminated with greater difficulty or not at all, or, as in the study reported by Elek and Fleming(19), a colonization with new strains of *Staph. albus* took place during the methicillin aerosol regimen.

The significance of finding strains of *Staph. albus* that are resistant to the new semisynthetic, penicillinase-insensitive penicillins is uncertain. At this hospital there has been no clear indication of increased virulence of such strains, although in one patient with *Staph. albus* bacteremia these new penicillins seemed to be only partially effective. However, there is some concern that the finding of strains of *Staph. albus* resistant to the new penicillins may portend the appearance and spread of strains of *Staph. aureus* resistant to the same penicillins. Earlier, Lepper and coworkers (29) found that following the initial introduction of spiramycin and novobiocin on a hospital ward, the first strains of staphylococci that became resistant to these antibiotics were *Staph. albus;* only later did strains of *Staph. aureus* resistant to the same drugs appear. The recent report of Jevons, Coe and Parker (15) seems to indicate that there already is a slow but steady increase in prevalence of methicillin-resistant strains of *Staph. aureus*.

The clinical use of methicillin began at Boston City Hospital in the fall of 1960. In the 2½ years since, approximately 400 patients with severe staphylococcal infections have been treated with this and others of the new penicillins. Of the more than 1200 strains of *Staph. aureus* isolated from these patients before, during and after such therapy and tested for sensitivity to these penicillins, none have been found to be resistant to methicillin or the other penicillinase-insensitive penicillins.

The results of these studies confirm previous findings(11,12) that penicillin G-resistant strains of *Staph. albus* produce a penicillin-beta-lactamase. Although only 12 of the Gots-positive strains were tested, it seems likely that at least the majority of them produce the same type of enzyme, and that the ability to do so is widespread among *Staph. albus* strains derived from clinical sources. This might explain the lack of success in penicillin treatment in some cases of *Staph. albus* bacteremia.

The mechanism of resistance to methicillin in the 5 strains studied seemed to be different from that of penicillin-G resistance. The beta-lactamase-containing cell preparations, although concentrated 10 times from the original cultures, did not affect the methicillin. Furthermore, there was no correlation between beta-lactamase activity of the whole cultures and their susceptibility to methicillin. The possibility remains that at extracellular enzyme other than beta-lactamase is produced by the resistant strains and is capable of destroying methicillin. This does not seem likely, however, since one of the methicillin-resistant strains (J12) gave a negative Gots test with methicillin.

The most likely mechanism of resistance to methicillin, therefore, is that of "inherent" or "natural" resistance. The dependence of the MIC of methicillin for the resistant strains on inoculum size shows itself not in a gradual stepwise fashion, as for penicillin G, but in one or 2 large steps from low to high values. This is shown in Fig. 1 and Table IV, and even more clearly in Tables III and V. Such a result is consistent with the presence in the original culture of a number of resistant cells.

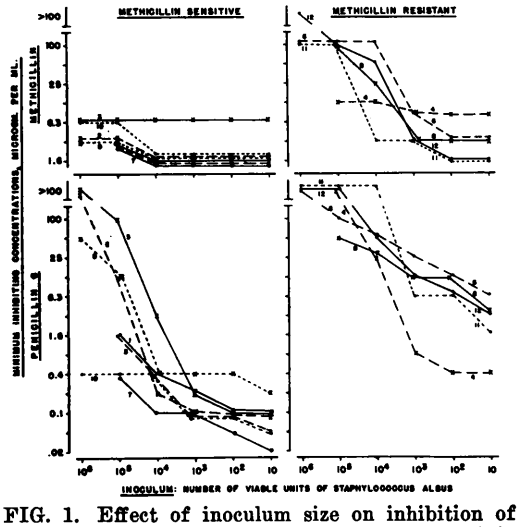

FIG. 1. Effect of inoculum size on inhibition of *Staphylococcus albus* by methicillin and penicillin G.

In the case of strain J11 this was demonstrated in the experiment recorded in Table VI.

Interpretation of the results of the Gots tests with methicillin is less clear. There seemed to be no correlation between the results of this test and the susceptibility of the strains to methicillin, nor was there any correlation between the results of this test and beta-lactamase activities of the cultures. Some unexplained factors may, therefore, be operative in this test.

Summary and conclusions. Among 175 clinical isolates of *Staph. albus* (*Staph. epidermidis*)—most of them from blood cultures—resistance (MIC $\leq$ 12.5 μg/ml) was most frequent to penicillin G (50%) and ampicillin (41%), but 10% were resistant to methicillin, 5% to oxacillin and diphenicillin, less than 2% to nafcillin and none to cephalothin. A varying number of these strains were also tested against 3 additional penicillins, 6 other antibiotics and fusidic acid; resistance was most frequent to chloramphenicol (60%), tetracycline (48%) and erythromycin (29%), none of them were resistant to kanamycin and a varying proportion were resistant to the other agents. Penicillinase-like action was demonstrated by the Haight-Finland test with 119 of the 175 strains; 12 of these strains, including 5 that were methicillin-resistant, were studied further. The 12 strains all produced a penicillin G-beta-lactamase but cell preparations from the 5 methicillin-resistant strains failed to destroy methicillin in 24 hours. There was no correlation between the MIC of methicillin and enzyme activity or results of the Haight-Finland test. Inoculum size had a marked effect on the MIC of methicillin for methicillin-resistant strains, but not for methicillin-sensitive strains. The experimental data favor the hypothesis that the inoculum effect was due to presence in the cultures of a small number of cells with inherent resistance rather than to the action of a methicillin-destroying enzyme. No methicillin-resistant strains of *Staph. aureus* have been isolated at this hospital, but the finding of methicillin-resistant strains of *Staph. albus* may portend the appearance of such strains.

1. Wallerström, A., *Nord. Med.*, 1961, v66, 1376.
2. Finland, M., Jones, W. F., Barnes, M. W., *J.A.M.A.*, 1959, v170, 2188.
3. Smith, I. M., Beals, P. D., Kingsbury, K. R., Hasenclever, H. F., *Arch. Int. Med.*, 1958, v102, 375.
4. Dowling, H. F., Lepper, M. H., Caldwell, E. R., Spies, H. W., *Medicine*, 1952, v31, 155.
5. Herbst, R. H., Merricks, J. W., *J.A.M.A.*, 1945, v127, 518.
6. Leopold, J. S., *Arch. Pediat.*, 1944, v61, 347.
7. Bondi, A., Jr., Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 55.
8. Chandler, C. A., Davidson, V. Z., Long, P. H., Monnier, J. J., *Bull. Johns Hopkins Hosp.*, 1951, v89, 81.
9. Oswald, E. J., Reedy, R. J., Randall, W. A., Welch, H., *Antibiot. Ann.*, 1953-1954, p318.
10. Pollock, M. R., *Resistance of Bacteria to the Penicillins*, CIBA Foundation Study Group No. 13, Little, Brown & Co., Boston, 1962, p56.
11. Kjellander, J. O., Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 1031.
12. ———, *New England J. Med.*, 1963, v269, in press.
13. Barber, M., *J. Clin. Path.*, 1961, v14, 385.
14. Cetin, E. T., Ang, O., *Brit. M. J.*, 1962, v2, 51.
15. Jevons, M. P., Coe, A. W., Parker, M. T., *Lancet*, 1963, v1, 904.
16. Stewart, G. T., *Brit. Med. J.*, 1961, v1, 863.
17. Callaghan, R. P., Cohen, S. J., Stewart, G. T., *ibid.*, 1961, v1, 860.
18. Leedom, J. M., Kennedy, R. P., Lepper, M. H., Jackson, G. G., Dowling, H. F., in *Antimicrobial Agents and Chemotherapy*—1961, M. Finland and G. M. Savage, Ed., Am. Soc. Microbiol., Ann Arbor, 1962, p683.

19. Elek, S. D., Fleming, P. C., *Lancet*, 1960, v2, 569.

20. Sutherland, J. M., *et al.*, *Am. J. Dis. Child.*, 1961, v102, 793.

21. Sonnenwirth, A. C., Greenman, M., Kallus, B. A., *J. Pediat.*, 1962, v61, 351.

22. Sabath, L. D., Postic, B., Finland, M., *Am. J. Med. Sci.*, 1962, v244, 484.

23. Klein, J. O., Sabath, L. D., Finland, M., *ibid.*, 1963, v245, 399.

24. Steers, E., Foltz, E. L., Graves, B. S., *Antibiot. and Chemother.*, 1959, v9, 307.

25. Haight, T. M., Finland, M., *Am. J. Clin. Path.*, 1952, v22, 806.

26. Novick, R. P., *Biochem. J.*, 1962, v83, 236.

27. Gilson, B. S. C., Parker, R. F., *J. Bact.*, 1948, v55, 801.

28. Grove, D. C., Randall, W. A., *Assay Methods of Antibiotics*, Medical Encyclopedia, Inc., New York, 1955, p14.

29. Lepper, M. H., Dowling, H. F., Jackson, G. G., Spies, H. W., Mellody, M., *Antibiot. Ann.*, 1956-1957, p640.

Received June 20, 1963. P.S.E.B.M., 1963, v113.

Studies on the Penicillinase of *Staphylococcus albus*.* (28564)

JAN O. KJELLANDER† AND MAXWELL FINLAND

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and Department of Medicine, Harvard Medical School, Boston, Mass.

It has been demonstrated(1) that a large proportion of strains of *Staphylococcus albus (Staph. epidermidis)* from various clinical sources were resistant *in vitro* to penicillin G, and that about 10% of such strains were also resistant to methicillin and to some of the other new, semisynthetic penicillins that are considered to be resistant to degradation by penicillinase. The majority of these strains produced a penicillin G-beta-lactamase, but the mechanism of their resistance to methicillin appeared to be inherent in the bacterial cells. Further studies of the properties of penicillinase of *Staph. albus* are presented here and discussed in the light of current knowledge about the penicillinase of *Staph. aureus*(2-11).

Materials and methods. Bacterial strains. The 12 strains of *Staph. albus* (J1-J12) used are the same as those included in the previous study(1); they had been obtained in pure culture from the blood of patients at the Boston City Hospital. *Staph. aureus*, BCH 60/1, the strain that was used for comparisons was isolated in 1960 from a suppurative lesion; it was phage type 80/81/KS6, produced penicillinase, and had been used in earlier studies in this laboratory(2, 12). The susceptibility of these strains to penicillin G and methicillin as determined on antibiotic-containing agar by the inocula-replicating method of Steers, Foltz and Graves(13), is shown in columns 1 and 2, respectively, of Table I.

Preparation of penicillinase. Except where otherwise indicated the strains were grown for 24 hr at 37°C in brain-heart infusion broth (Difco), pH 7.2, containing methicillin, 1.0 μg/ml, as an inducer. Three preparations of these broth cultures were tested for penicillinase activity. One consisted of the culture sediment obtained after centrifugation at 3000 rpm for 30 min; the sediment was washed twice with Sörensen's phosphate buffer, pH 6.4, and extracted twice with acetone and then twice with ether at −20°C, as described by Gilson and Parker(3). The dried residues were resuspended in the original volume of buffer solution containing 0.5% gelatin and shaken vigorously with sterile glass beads; these suspensions will be referred to as "cell preparations." The culture supernatant was divided into 2 portions, one of which was passed through a Seitz filter, the other through a Millipore filter of 0.3 μ porosity. The 3 preparations from each strain were kept frozen at 20°C until used and showed no loss of enzyme activity during the 4

* Aided by a grant from Nat. Inst. Health.

† Present address: Bacteriol. Lab., Karolinska Sjukhuset, Stockholm, Sweden.