ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1977, p. 541-552

Vol. 11, No. 3
Printed in U.S.A.

Comparison of the Effects of Mecillinam and 6-Aminopenicillanic Acid on *Proteus mirabilis*, *Escherichia coli*, and *Staphylococcus aureus*

VICTOR LORIAN* AND BARBARA ATKINSON

Division of Microbiology and Epidemiology, Department of Pathology, The Bronx-Lebanon Hospital Center, Bronx, New York 10456, and The Albert Einstein College of Medicine, Bronx, New York 10461

Received for publication 20 July 1976

Single strains of *Proteus mirabilis*, *Escherichia coli*, and *Staphylococcus aureus* were grown on filter membranes placed on agar containing concentration series of mecillinam (FL 1060), 6-aminopenicillanic acid (6-APA), or ampicillin. *P. mirabilis* and *E. coli* were also exposed to combinations of mecillinam or 6-APA with ampicillin. Colony-forming units were counted, and cells were examined by interference phase-contrast and transmission electron microscopy. Mecillinam and 6-APA were very effective in reducing the viability of the two gram-negative species, but they were less effective against *S. aureus*. Combinations of mecillinam and 6-APA with ampicillin acted synergistically against the gram-negative bacilli. When the antibiotics were presented consecutively, their effects on viability were usually no greater than the effects of the individual antibiotics acting alone. When *P. mirabilis* and *E. coli* were exposed to mecillinam alone or in combination with ampicillin, the cells became rounded. 6-APA alone or in combination with ampicillin produced elongated polymorphic cells in these species. The most unusual morphological effects were ultrastructural. Mecillinam and, to a lesser extent, 6-APA produced inward growth of numerous pairs of trilamellar membranous structures within the cells. It is possible that these membranes represent the growth initiation of aberrant cross walls. Both mecillinam and 6-APA produced multiple, thick cross walls in *S. aureus*.

Mecillinam (FL 1060), a β-amidinopenicillanic acid derivative, differs from the other known penicillanic acid derivatives in its antibacterial spectrum (1, 7, 19, 23) and in its mode of action (16). Mecillinam is very active against *Escherichia coli* and other gram-negative rods, and it acts synergistically with ampicillin (1, 7, 23). Whereas subinhibitory concentrations of most penicillins turn *E. coli* and other gram-negative bacilli into filaments (2-4, 14), mecillinam produces ovoidal forms and no filaments (6, 17, 21). Subinhibitory concentrations of 6-aminopenicillanic acid (6-APA) also produce ovoidal forms in *E. coli* and *Proteus mirabilis* (14, 21), and 6-APA acts synergistically with ampicillin (L.D. Sabath, personal communication).

The present paper reports the effects of mecillinam and 6-APA on the morphology and viability of *E. coli*, *P. mirabilis*, and *Staphylococcus aureus*. Combinations of mecillinam or 6-APA with ampicillin were also tested on *P. mirabilis* and *E. coli*.

MATERIALS AND METHODS

Strain 209P (FDA) of *S. aureus*, strain K-12 of *E. coli*, and a strain of *P. mirabilis* isolated from urine (9) were grown for 24 h in Trypticase soy broth (BBL) at 37°C. The minimum inhibitory concentrations (MIC) of 6-APA, mecillinam, and ampicillin were determined for each strain by using a twofold agar dilution method (22).

Trypticase soy agar plates (BBL) containing concentration series of single drugs were prepared. For each organism, the drug concentration ranged, in a series of twofold dilutions, from 4× the MIC to 1/128 of the MIC. Mecillinam and 6-APA were also tested in combination with ampicillin for their effects on *P. mirabilis* and *E. coli*. Filter membranes (no. PHWP047SO, Millipore Corp., Bedford, Mass.) were placed on Trypticase soy agar, prewarmed for 1 h at 37°C, and inoculated with 0.1 ml of a 1:10 Trypticase soy broth dilution of a 24-h broth culture of one of the organisms. The inoculated membranes were incubated on drug-free agar at 37°C for 90 min. They were then transferred to agar containing the antibiotics and incubated at 37°C for another 6 h. Some membranes, after exposure to mecillinam, were transferred back onto drug-free agar and incubated at 37°C for an additional 4 h. Inoculated control membranes were transferred from drug-free agar to drug-free agar at the same time that the test membranes were transferred from drug-free agar to agar containing antibiotic.

In an effort to examine the nature of the synergistic effect, each component of the combination treatment was presented consecutively. *P. mirabilis* and

E. coli were exposed to ampicillin for 1 h and then to 6-APA or mecillinam for 5 h. The reverse sequences were also carried out; that is, both species were exposed to 6-APA or mecillinam for 1 h and then to ampicillin for 5 h.

To select the samples appropriate for ultrastructural examination, the organisms on each membrane were inspected by interference phase-contrast microscopy, and the number of colony-forming units (CFU) per membrane was counted. One concentration of mecillinam and one concentration of 6-APA that produced representative morphological changes in *P. mirabilis* were selected and one concentration of mecillinam that produced morphological changes in *S. aureus* was selected; the organisms grown on these membranes were examined by electron microscopy. The bacterial cells were fixed for 1 h in 5% glutaraldehyde in 0.067 M phosphate buffer, pH 7.4. They were centrifuged, rinsed in buffer, and fixed for 30 min in 1% osmic acid in chromate buffer, pH 7.4. The bacteria were then treated with 0.5% uranyl and lead salts. Details of the CFU counting and the electron microscopy fixation procedures were described previously (11).

RESULTS

The viability curves for *P. mirabilis* and *E. coli* treated with decreasing concentrations of mecillinam, 6-APA, ampicillin, and combinations of mecillinam and 6-APA with ampicillin are shown in Fig. 1. *S. aureus* viability in the presence of mecillinam and 6-APA is shown in Fig. 2. There were large quantitative differences between the drug concentrations and the magnitude of the viability responses of *E. coli* and *P. mirabilis*.

When *P. mirabilis* was grown on Trypticase

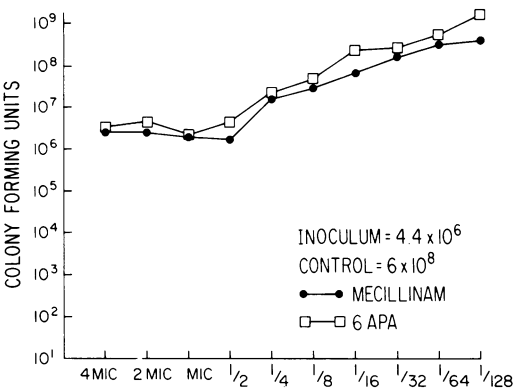

FIG. 2. *Effect of mecillinam and 6-APA on the viability of S. aureus. The MICs were: mecillinam, 100 μg/ml; 6-APA, 25 μg/ml.*

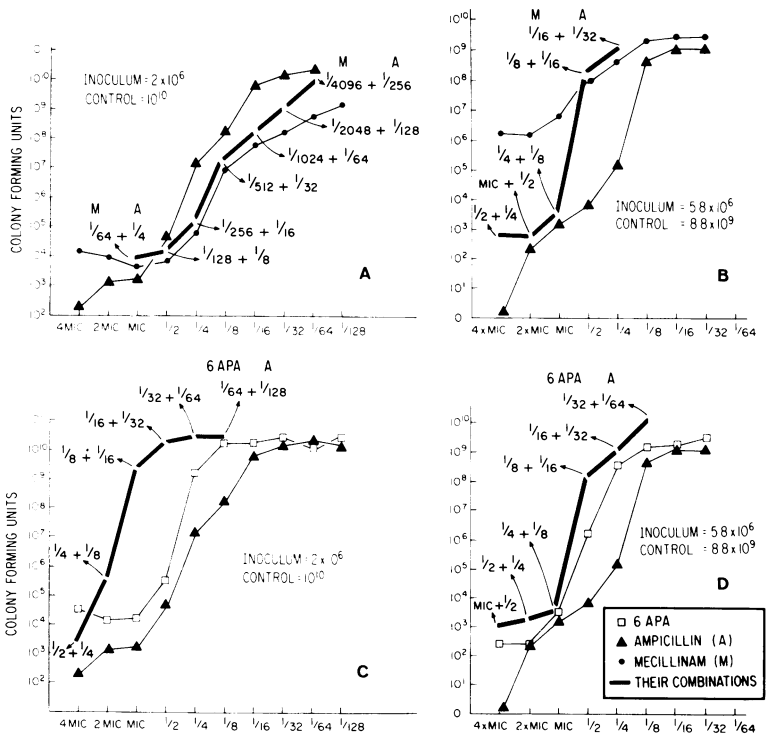

FIG. 1. *Effect of mecillinam, 6-APA, and their combinations with ampicillin on the viability of P. mirabilis (A and D) and E. coli (B and C). The MICs were: P. mirabilis to ampicillin, 3 μg/ml; 6-APA, 100 μg/ml; mecillinam, 800 μg/ml; E. coli to ampicillin, 12 μg/ml; 6-APA, 100 μg/ml; mecillinam, 0.5 μg/ml.*

soy agar containing 1/8 of the MIC of mecillinam, the CFU per membrane were 1.7×10^7; CFU per membrane for the control were 10^{10}. Interference phase-contrast microscopy showed that this treatment turned *P. mirabilis* into round cells 7 to 12 μm in diameter (Fig. 3). The reversibility of this response was indicated when *P. mirabilis* exposed to 1/8 of the MIC of mecillinam for 6 h was transferred to drug-free agar, incubated for 4 h at 37°C. The number of CFU increased to 10^8, and the organisms returned to rodlike forms.

P. mirabilis grown on Trypticase soy agar containing 1/4 of the MIC of 6-APA for 6 h had a CFU count of 1.6×10^9; the control had a CFU count of 10^{10}. Although some organisms resem-

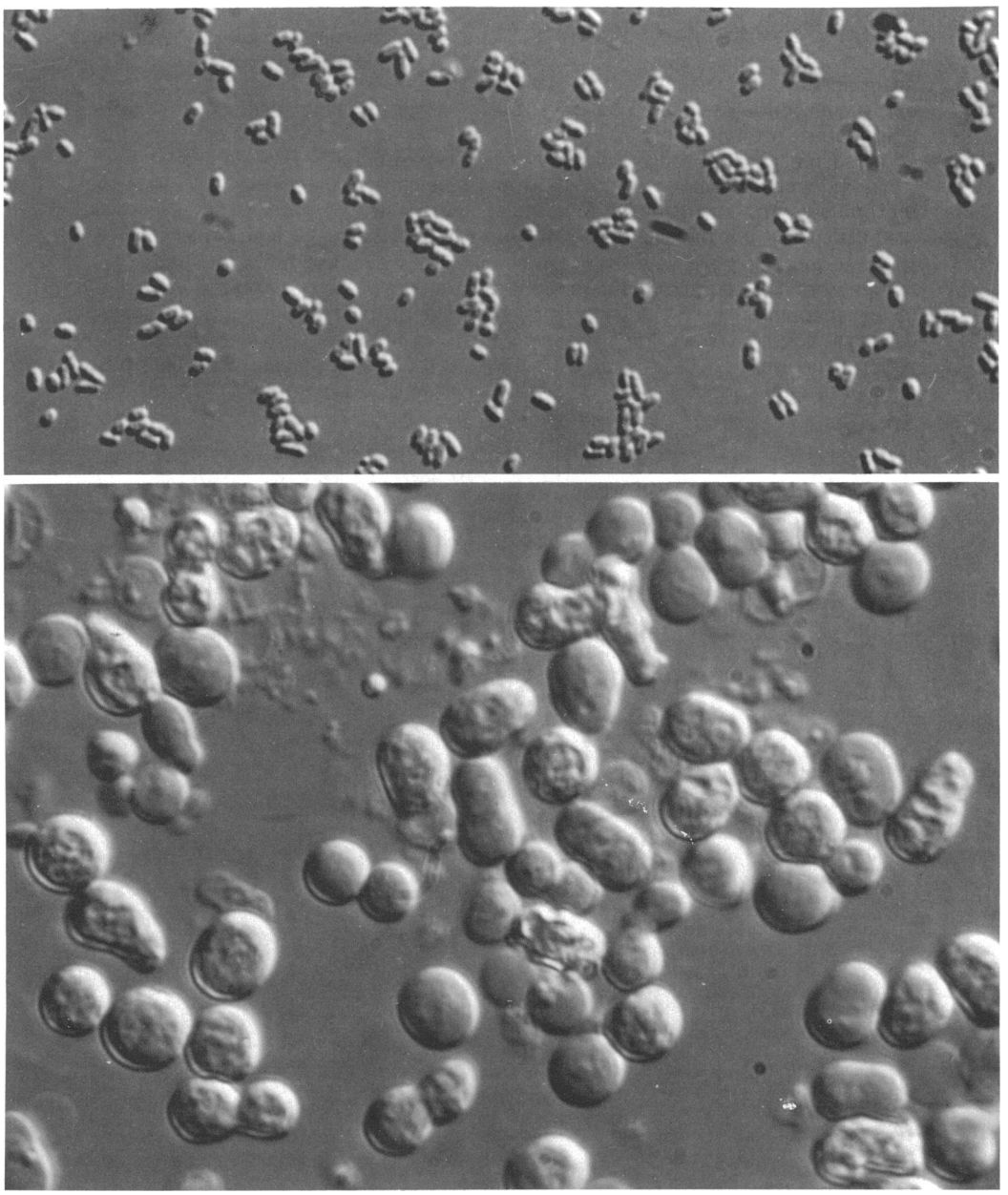

FIG. 3. *(Top) P. mirabilis grown on drug-free agar. (Bottom) Agar containing 1/8 of the MIC (100 μg/ml) of mecillinam produced round and ovoid cells. Interference phase contrast (×1,200).*

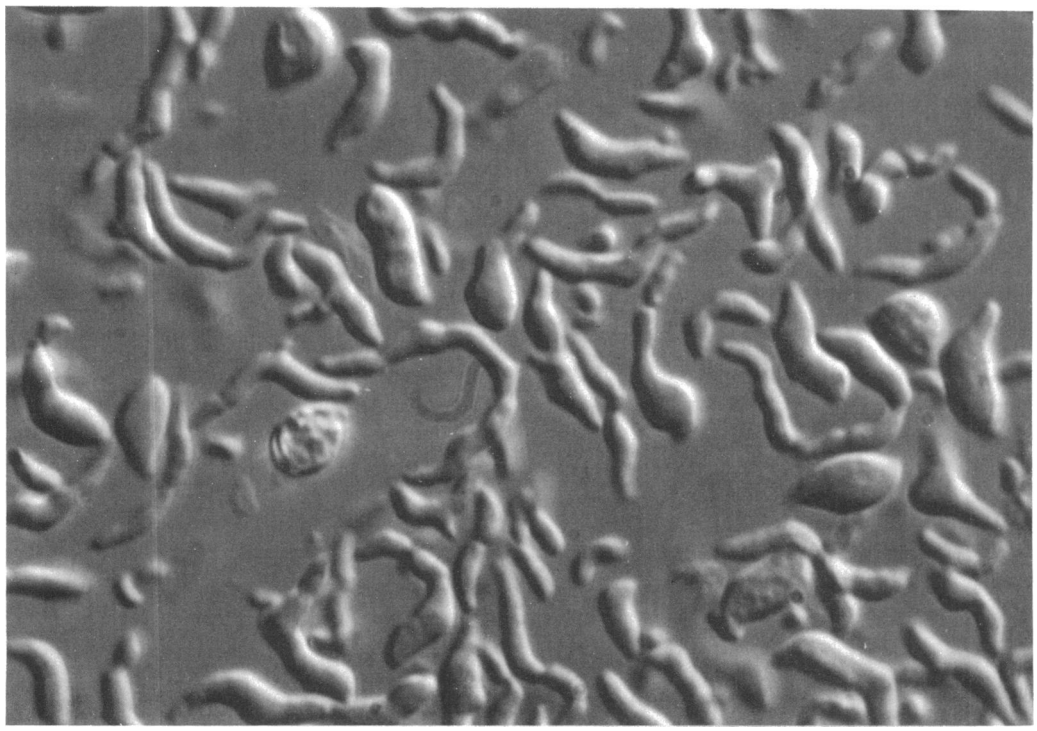

FIG. 4. *P. mirabilis grown on agar containing 1/4 of the MIC of 6-APA (25 μg/ml) produced elongated polymorphic cells and some round cells. Interference phase contrast (×1,162).*

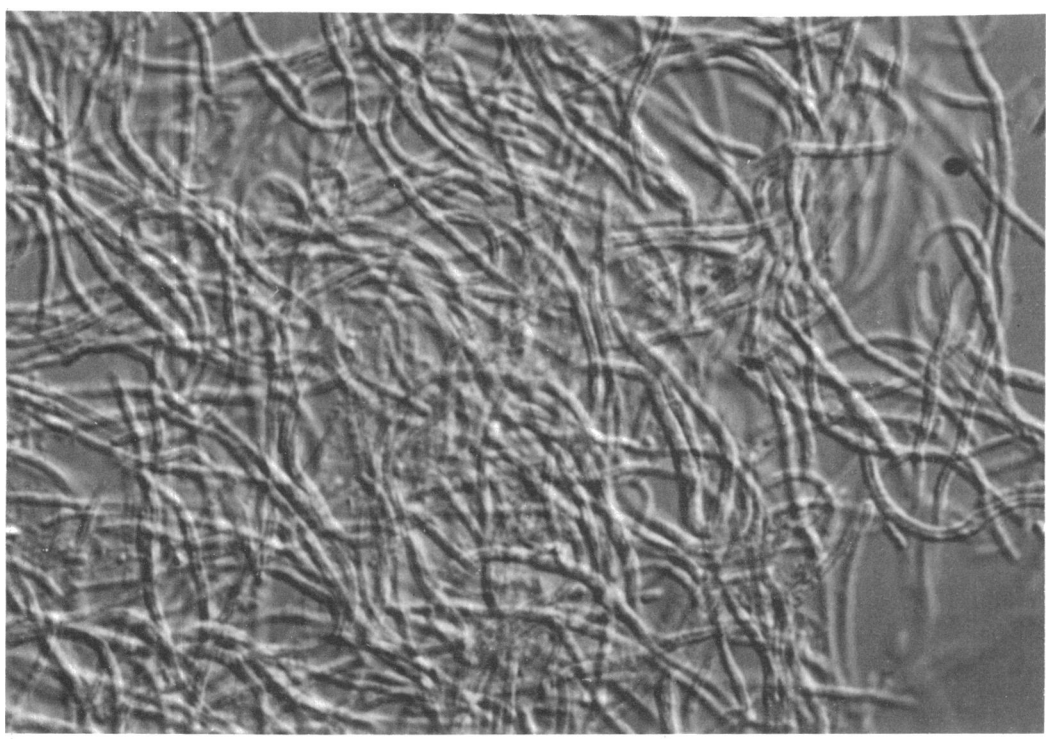

FIG. 5. *P. mirabilis grown on agar containing 1/4 of the MIC (0.75 μg/ml) of ampicillin produced filaments. Interference phase contrast (×1,175).*

bled the round cells produced by mecillinam, most 6-APA-treated bacilli became elongated polymorphic cells 8 to 15 μm long and 2 to 5 μm wide (Fig. 4).

Ampicillin treatment (1/4 of the MIC) of *P. mirabilis* reduced CFU per membrane to 1.5×10^7. Figure 5 shows the filaments produced by this treatment. The morphology of *P. mirabilis* exposed to ampicillin in combination with mecillinam or 6-APA resembled round cells or elongated polymorphic cells, respectively.

The morphological responses of *E. coli* to all three antibiotics and to the ampicillin combinations with mecillinam and 6-APA were very similar to the responses of *P. mirabilis* discussed above.

The *S. aureus* grown on Trypticase soy agar containing 1/4 of the MIC of mecillinam or 6-APA produced 2×10^7 or 1.4×10^7 CFU/membrane, respectively. Compared with a CFU count of 6×10^8 for the control, this indicated a weak antibacterial effect. Both drugs produced cells with irregular forms (Fig. 6).

The synergistic effect of combining mecillinam with ampicillin was striking for *P. mirabilis*. Viability was reduced to 1 log below control levels when 1/2,048 of the mecillinam MIC was combined with 1/128 of the ampicillin MIC. These concentrations were also the lowest that produced morphological changes (Table 1). The synergistic effect was also seen with *E. coli* (Table 1, Fig. 1).

Consecutive exposure of *P. mirabilis* or *E. coli* to ampicillin/6-APA or ampicillin/mecilli-

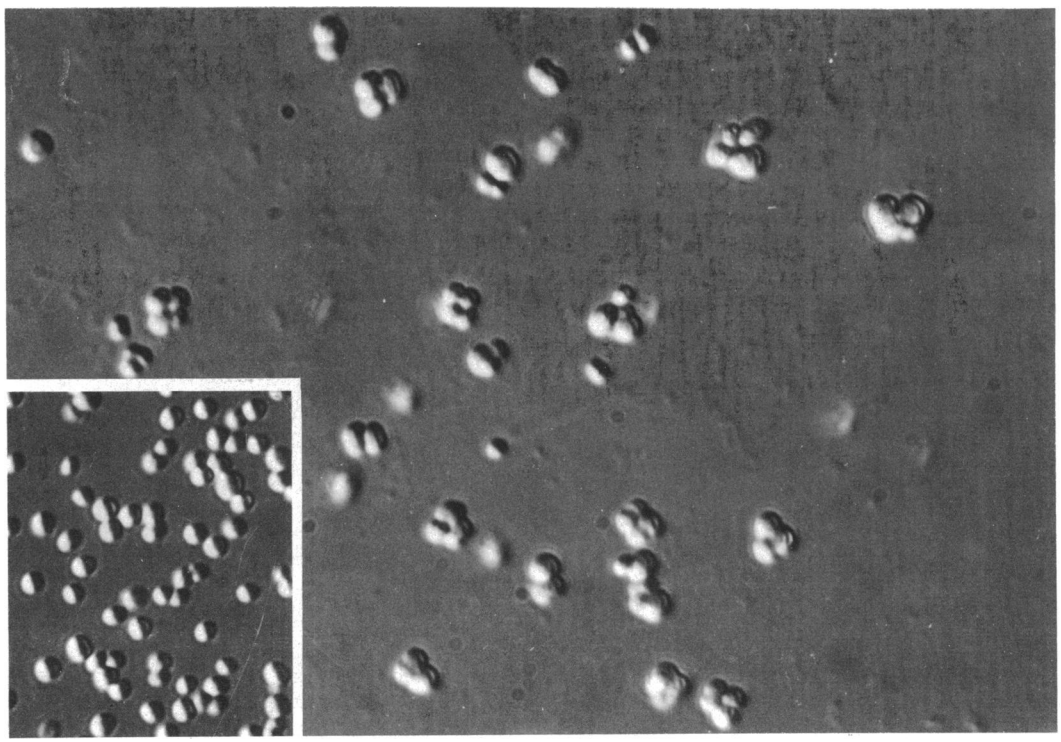

FIG. 6. *S. aureus grown on agar containing 1/4 of the MIC (25 μg/ml) of mecillinam produces cells with irregular forms. Inset is control grown on drug-free agar. Interference phase contrast (×1,200).*

TABLE 1. *Lowest concentration of drug to produce a change in morphology*

Species	Mecillinam		6-APA		Ampicillin		Ampicillin (am) plus mecillinam (mec)		Ampicillin (am) plus 6-APA (ap)	
	μg/ml	Fraction of MIC	μg/ml	Fraction of MIC	μg/ml	Fraction of MIC	μg/ml	Fraction of MIC	μg/ml	Fraction of MIC
P. mirabilis	0.80	1/1,024	6.2	1/16	0.38	1/8	0.022 am	1/128	0.38 am	1/8
							0.400 mec	1/2,048	25.00 ap	1/4
E. coli	0.05	1/8	12.5	1/8	1.50	1/8	0.75 am	1/16	0.75 am	1/16
							0.05 mec	1/8	12.50 ap	1/8
S. aureus	6.20	1/16	0.75	1/16						

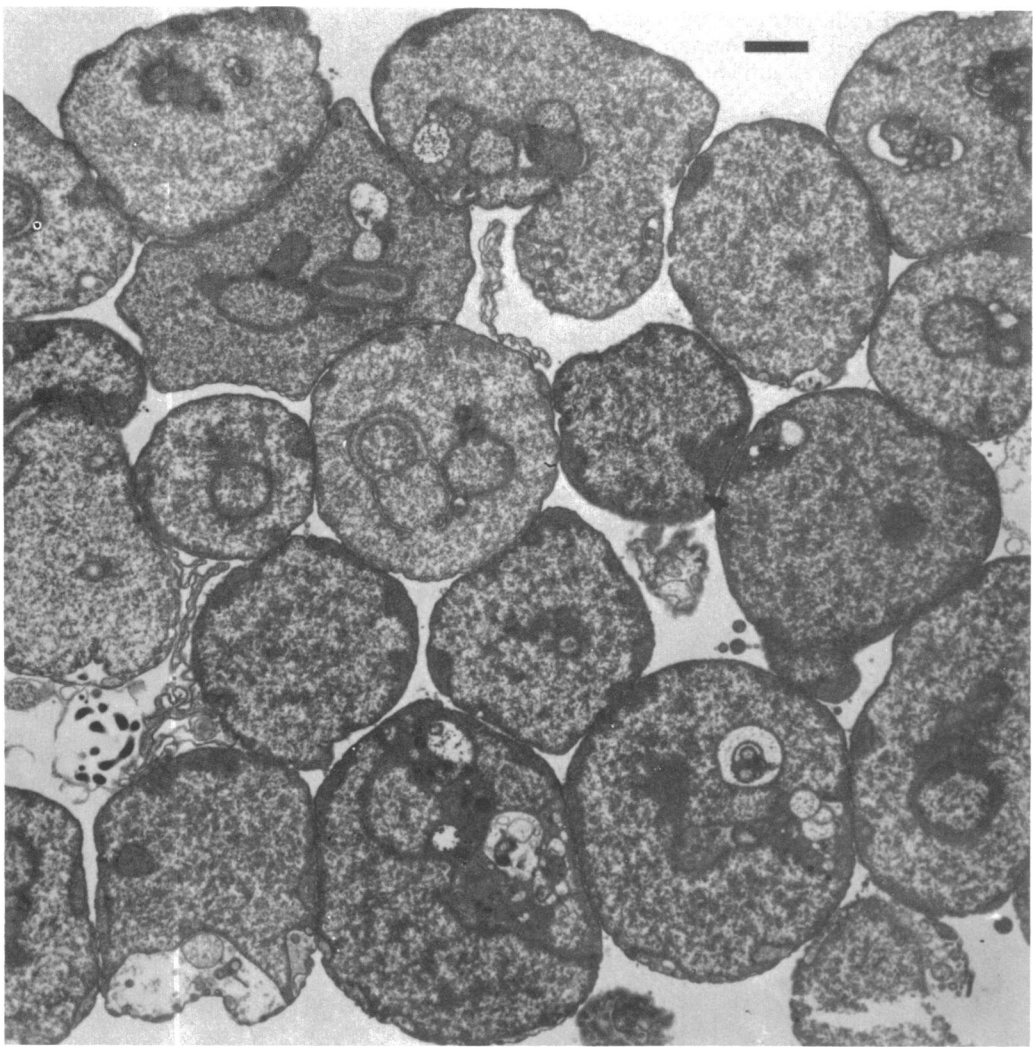

FIG. 7. *P. mirabilis grown on agar containing 1/8 of the MIC (100 μg/ml) of mecillinam. Most cells contain some membranous structures (×8,100). Bar, 1 μm.*

nam did not produce a synergistic decrease in viability. Table 2 shows that the viability effects were, in every case except one, equal to or less than the viability reductions produced by the drugs separately. The one exception, 1-h exposure to mecillinam and 5-h exposure to ampicillin, produced the same response as the response seen after exposure to the mecillinam/ampicillin combination, that is, a 5-log reduction compared to the control.

Electron microscopy of *P. mirabilis* exposed to mecillinam at a concentration of 1/8 of the MIC showed round cells with cell walls that appeared as a dense layer of 10 to 15 nm. Most of the cells contained paired trilamellar membranes, some of which appeared to be membranous intrusions of the peripheral cytoplasmic membrane (Fig. 7 and 8). Many of the membranes were surrounded by clusters of ribosomes in greater density than in the rest of the cell (Fig. 9). Electron microscopy of *P. mirabilis* exposed to 6-APA showed elongated cells of various irregular shapes. The cell wall was of normal appearance; some cells contained membranous structures (Fig. 10 and 11).

Electron microscopy of the filaments induced in *P. mirabilis* by ampicillin showed a normal cell wall and some decrease in density of the ribosomes compared with the control. The effect has been described previously (15).

TABLE 2. *Effect of consecutive exposure of P. mirabilis and E. coli to mecillinam or 6-APA and ampicillin*

Species	Exposure				CFU/membrane
	1 h		5 h		
	Antibiotic	μg/ml	Antibiotic	μg/ml	
P. mirabilis					
Inoculum, 1.7×10^7 per membrane	Mecillinam	6.2	Ampicillin	0.38	2.8×10^5[a]
Control at 1.5 h, 3.2×10^7 per membrane	6-APA	25	Ampicillin	0.38	3.9×10^9
Control at 7.5 h (1.5 + 6), 1.6×10^{10} per membrane	Ampicillin	0.38	Mecillinam	6.2	5.5×10^8[b]
	Ampicillin	0.38	6-APA	25	6×10^9
E. coli					
Inoculum, 7.0×10^6 per membrane	Mecillinam	0.1	Ampicillin	1.5	2.7×10^8
Control at 1.5 h, 2.5×10^7 per membrane	6-APA	25	Ampicillin	1.5	1.8×10^9
Control at 7.5 h (1.5 + 6), 4.5×10^{10} per membrane	Ampicillin	1.5	Mecillinam	0.1	7.5×10^8
	Ampicillin	1.5	6-APA	25	4.2×10^9

[a] Round cells with irregular surfaces.
[b] Round cells with smooth surfaces.

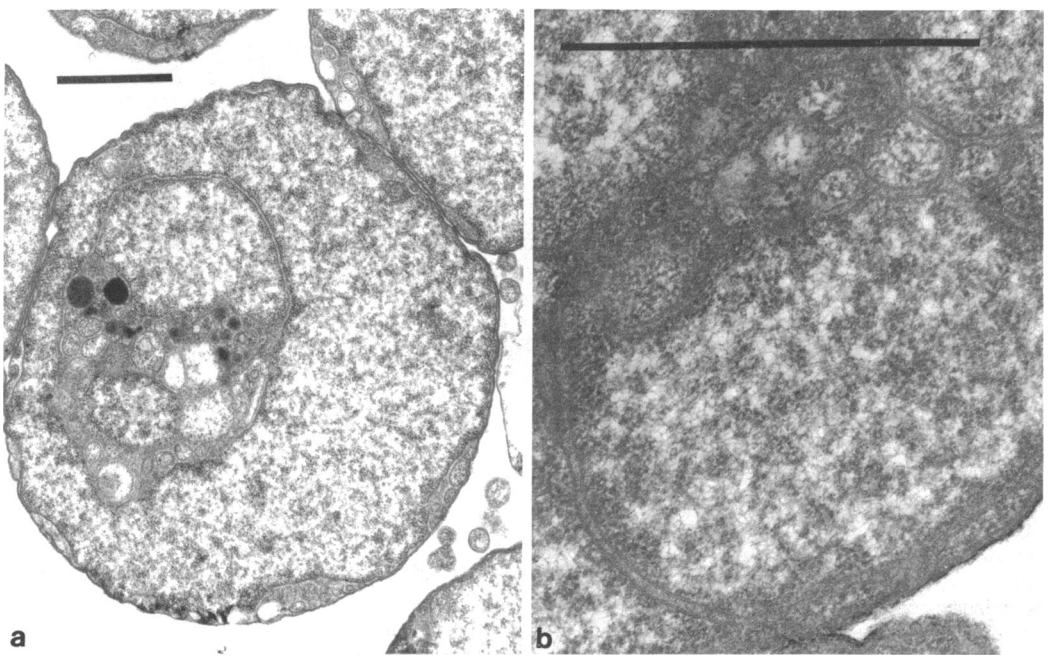

FIG. 8. *P. mirabilis grown on agar containing 1/8 of the MIC (100 μg/ml) of mecillinam. (a) Membranes are surrounded by a dense zone of ribosomes. Some opaque material as well as some vacuoles are present (×14,880). (b) A pair of intruding membranes, which appear to be a continuation of the peripheral cytoplasmic membrane, are surrounded by a dense zone of ribosomes (×55,800). Bar, 1 μm.*

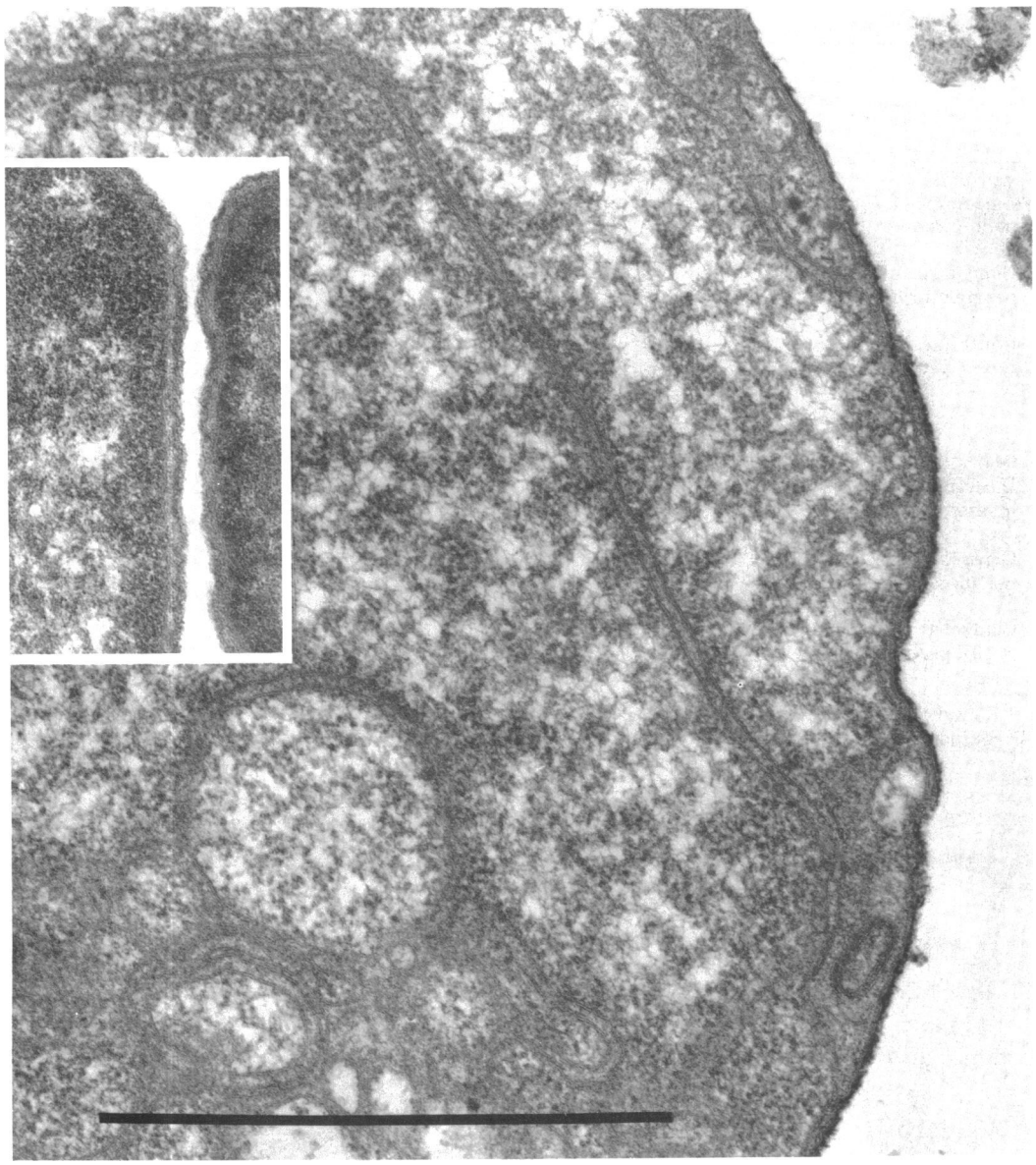

FIG. 9. *P. mirabilis grown on agar containing 1/8 of the MIC (100 μg/ml) of mecillinam. A pair of membranes surrounded by ribosomes traverses the cell in a septum-like manner (×77,400). Inset is control grown on drug-free agar. Bar, 1 μm.*

On electron microscopy, *S. aureus* exposed to mecillinam or 6-APA had changed into large cells of 1.5 to 3.5 μm in diameter. These cells were crisscrossed by thick cross walls 0.1 to 0.4 μm wide (Fig. 12).

DISCUSSION

The synergism of mecillinam with β-lactam antibiotics against *Enterobacteriaceae* has been well documented (1, 7, 23). The results of the present study show that for the strain of *P. mirabilis* investigated, the lowest active synergistic combination of mecillinam and ampicillin (as a fraction of the MIC) involves lower concentrations of mecillinam than have been reported previously. In fact, the lowest mecillinam concentration that, in combination with ampicillin, produced an effect on the structure

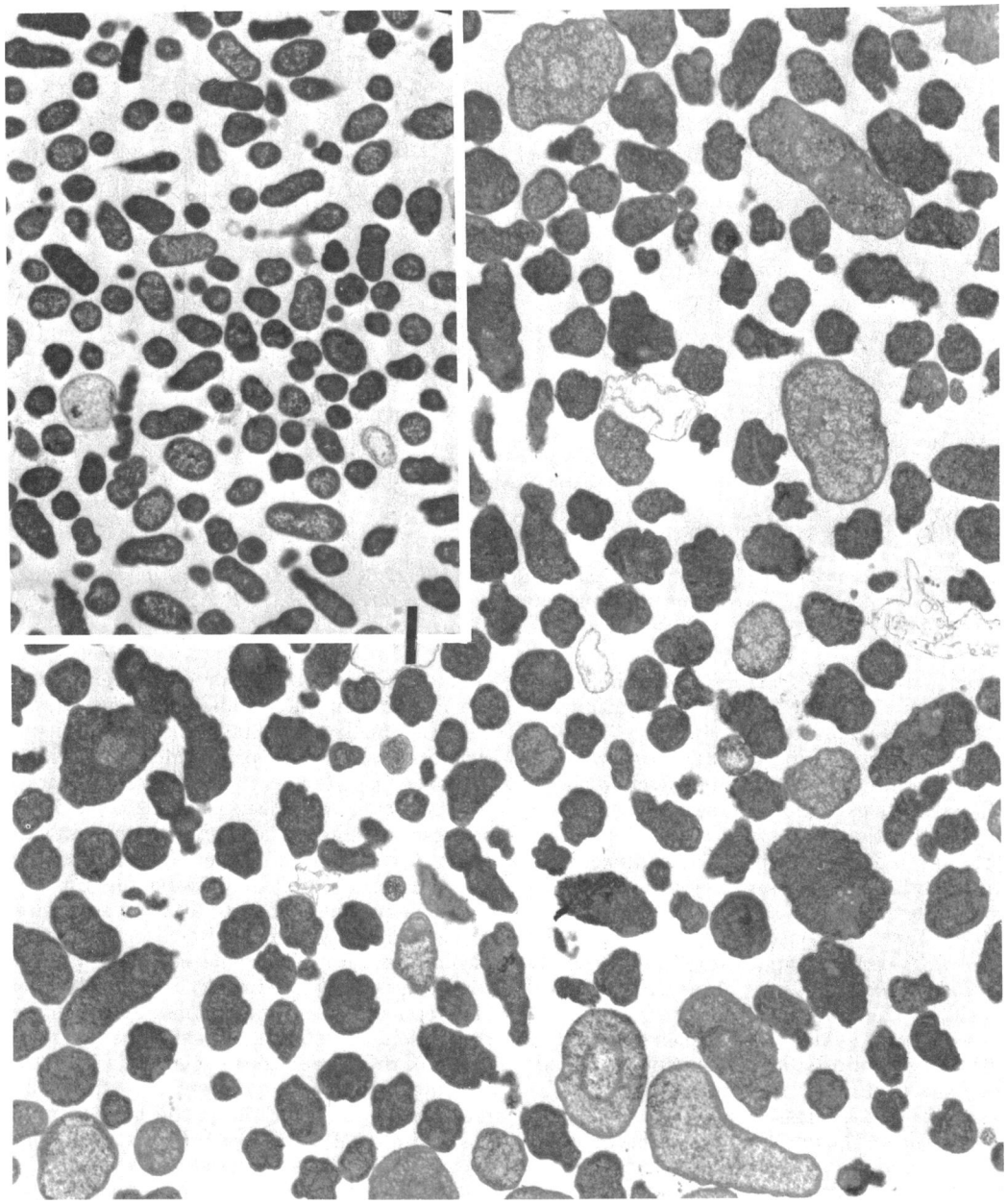

FIG. 10. *P. mirabilis grown on agar containing 1/4 of the MIC (25 μg/ml) of 6-APA. Polymorphic cells, some of which contain membranous structures. Inset is control grown on drug-free agar. (×7,515) Bar, 1 μm.*

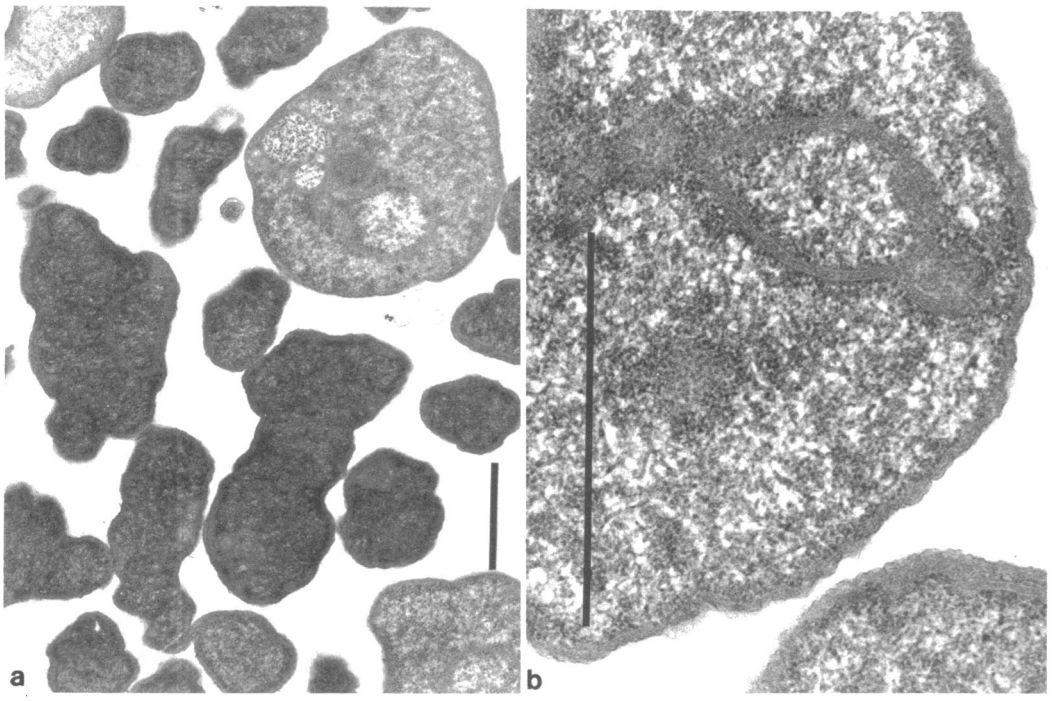

FIG. 11. *P. mirabilis grown on agar containing 1/8 of the MIC (12.5 μg/ml) of 6-APA. (a) One oval cell and other polymorphic cells. Two cells contain some membranous structure (×13,920). (b) A normal cell wall and a membranous structure (×52,200). Bar, 1 μm.*

and growth rate of *P. mirabilis* was the smallest fraction of the MIC of any drug ever investigated in this laboratory (10, 11, 13, 14).

The consecutive exposure of *P. mirabilis* and *E. coli* to the synergistically effective drugs had, with one exception, the same or less effect on viability that each drug acting separately had. This indicates that the simultaneous action of both drugs is necessary to produce the synergistic effect.

The effects of β-lactam antibiotics on the morphology of *E. coli* appear to be related to the affinity of these drugs for three different proteins (20). The morphological changes induced by mecillinam, 6-APA, and ampicillin in the *P. mirabilis* strain used in the present study suggest that a similar mechanism may also be present in this species.

It has been shown that the exposure of *S. aureus* to subinhibitory concentrations of penicillin or oxacillin leads to the formation of large cells containing many thick cross walls (11, 12). In this study, subinhibitory concentrations of mecillinam and 6-APA had the same effect. Because the structural effects of mecillinam and 6-APA are the same as those of penicillin, the mode of action on *S. aureus* may also be similar to that of penicillin.

Polymyxin is known to produce alterations of the cytoplasmic membrane in gram-negative bacteria (5). In this study, the most unusual structural alterations produced by mecillinam and, to some extent, by 6-APA were the pairs of trilamellar membranes inside the cells. Many of these membranes appeared to grow inward as an intrusion from the periphery of the cytoplasmic membrane. One might suggest that these membranes are deteriorating structures inside a dying organism. However, the quantity of membrane in some cells was greater than can be accounted for by the sloughing off of cytoplasmic membrane alone. Furthermore, the regional clustering of ribosomes in the vicinity of the membranes suggested a recent or ongoing transfer of proteins into functional membranes (8, 18). Finally, the 1-log increase in CFU after 4 h of growth on drug-free agar showed that these organisms are not dying cells. It is possible that these paired membranes represent the growth initiation of aberrant cross walls. No other β-lactam antibiotic has shown this effect.

ACKNOWLEDGMENTS

We wish to thank Herbert Dembitzer for the electron microscopy and for his help in interpreting the micrographs

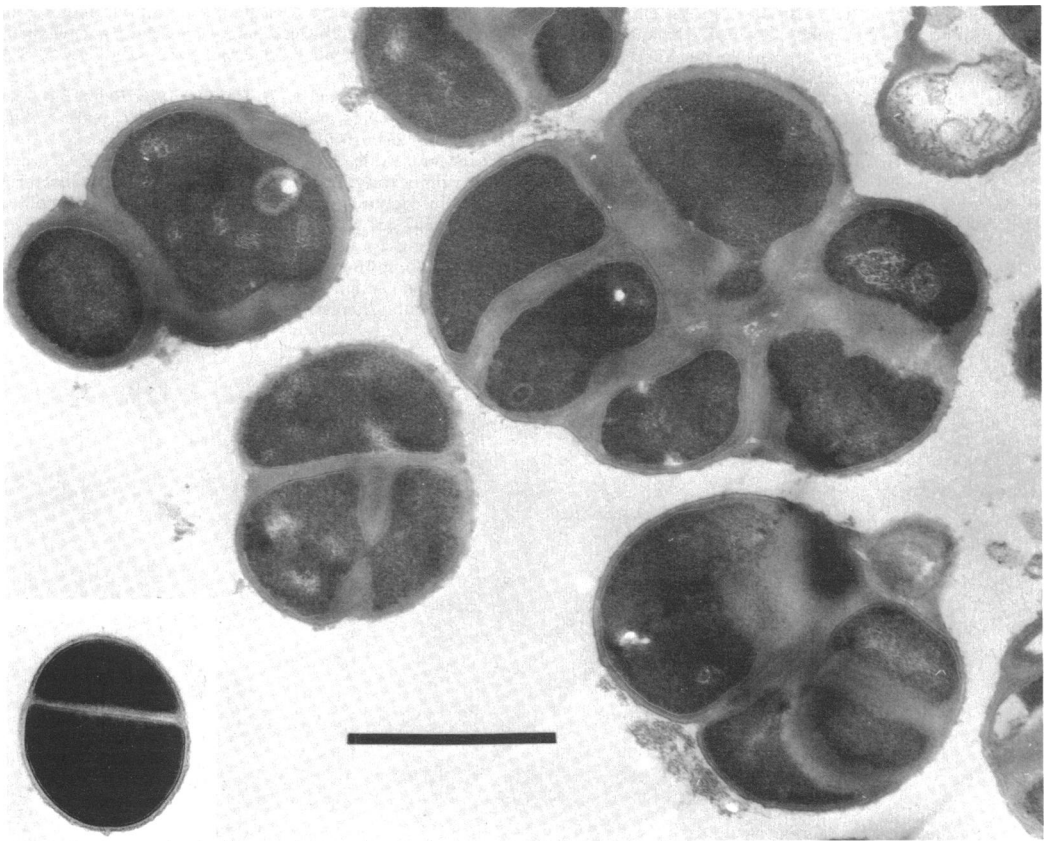

FIG. 12. *S. aureus grown on agar containing 1/4 of the MIC (200 μg/ml) of mecillinam produced large cells containing multiple, wide cross walls (×21,160). Inset is control grown on drug-free agar. Bar, 1 μm.*

and R. G. E. Murray and Milton Salton for their suggestions regarding the membranous structures seen in the electron micrographs.

LITERATURE CITED

1. **Baltimore, R. S., J. O. Klein, C. Wilcox, and M. Finland.** 1976. Synergy of mecillinam (FL 1060) with penicillins and cephalosporins against *Proteus* and *Klebsiella*, with observations on combinations with other antibiotics and against other bacterial species. Antimicrob. Agents Chemother. 9:701-705.
2. **Duguid, J. P.** 1946. The sensitivity of bacteria to the action of penicillin. Edinburgh Med. J. 53:401-412.
3. **Fleming, A., A. Voureka, I. R. H. Kramer, and W. H. Hughes.** 1950. The morphology and motility of *Proteus vulgaris* and other organisms cultured in the presence of penicillin. J. Gen. Microbiol. 4:257-269.
4. **Gardner, A. D.** 1940. Morphological effects of penicillin on bacteria. Nature (London) 146:837-838.
5. **Gilleland, H. E., Jr., and R. G. E. Murray.** 1976. Ultrastructural study of polymyxin-resistant isolates of *Pseudomonas aeruginosa*. J. Bacteriol. 125:267-281.
6. **Greenwood, D., and F. O'Grady.** 1973. FL1060: a new beta-lactam antibiotic with novel properties. J. Clin. Pathol. 26:1-6.
7. **Gruneberg, E., R. Cleeland, G. Beskid, and W. F. DeLorenzo.** 1976. In vivo synergy between 6β-amidinopenicillanic acid derivatives and other antibiotics. Antimicrob. Agents Chemother. 9:589-594.
8. **Leive, L. (ed.).** 1973. Bacterial membranes and walls. M. Dekker, Inc., New York.
9. **Lennette, E. H., E. H. Spaulding, and J. P. Truant (ed.).** 1974. Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
10. **Lorian, V.** 1975. Some effects of subinhibitory concentrations of antibiotics on bacteria. Bull. N.Y. Acad. Med. 51:1046-1055.
11. **Lorian, V.** 1975. Some effects of subinhibitory concentrations of penicillin on the structure and division of staphylococci. Antimicrob. Agents Chemother. 7:864-870.
12. **Lorian, V., and B. Atkinson.** 1976. Effects of subinhibitory concentrations of antibiotics on cross walls of cocci. Antimicrob. Agents Chemother. 9:1043-1055.
13. **Lorian, V., and B. Poppola.** 1972. The effect of nitrofurantoin on the morphology of gram negative bacilli. J. Infect. Dis. 125:187-189.
14. **Lorian, V., and L. D. Sabath.** 1972. Penicillins and cephalosporins: differences in morphologic effects. J. Infect. Dis. 125:560-564.
15. **Lorian, V., L. D. Sabath, and M. Simonescu.** 1975. Decrease in ribosomal density of *Proteus mirabilis* exposed to subinhibitory concentrations of ampicillin or cephalothin (38888). Soc. Exp. Biol. Med. 149:731-735.

16. **Matsuhashi, S., K. Tatsuyuki, P. M. Blumberg, P. Linnett, E. Willoughby, and J. L. Strominger.** 1974. Mechanism of action and development of resistance to a new amidino penicillin. J. Bacteriol. 117:578–587.
17. **Melchior, N. H., J. Blom, L. Tybring, and A. Birth-Andersen.** 1973. Light and electron microscopy of the early response of *Escherichia coli* to a 6 beta-amidinopenicillanic acid (FL 1060). Acta Pathol. Microbiol. Scand. 81:393–407.
18. **Mindich, L.** 1973. *In* L. Leive (ed.), Bacterial membranes and walls, p. 1–27. Marcel Dekker, Inc., New York.
19. **Neu, H.** 1976. Mecillinam, a novel penicillanic acid derivative with unusual activity against gram-negative bacteria. Antimicrob. Agents Chemother. 9:793–799.
20. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K 12. Proc. Natl. Acad. Sci. U.S.A. 72:2999–3003.
21. **Spratt, B. G., and A. B. Pardee.** 1975. Penicillin binding proteins and cell shape in *E. coli*. Nature (London) 254:516–517.
22. **Steers, E., E. L. Folz, and B. S. Graves.** 1959. Inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. 9:307–311.
23. **Tybring, L., and N. H. Melchior.** 1975. Mecillinam (FL 1060), a 6β-amidinopenicillanic acid derivative: bacterial action and synergy in vitro. Antimicrob. Agents Chemother. 8:271–276.