ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1981, p. 901-910
0066-4804/81/050901-10$02.00/0

Vol. 19, No. 5

In Vitro and In Vivo Morphological Response of *Klebsiella pneumoniae* to Cefotiam and Cefazolin

MASAFUMI NAKAO, TAKESHI NISHI, AND KANJI TSUCHIYA*

Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan

Received 1 October 1980/Accepted 27 February 1981

The effect of cefotiam and cefazolin on the ultrastructure of *Klebsiella pneumoniae* DT-S in vitro and in experimental pneumonia in mice was examined by electron microscopy. The action of both cephalosporins against *K. pneumoniae* DT-S was bactericidal, and a dose response in the action was definite. At the minimal inhibitory concentration of each cephalosporin, filamentation of the cells was induced and the cytoplasm became sparse during the course of incubation. With elevation of the concentration of the cephalosporins, spheroplasts were formed; they subsequently collapsed. In the lungs of mice, the infecting organisms localized in the alveolar space, and each cell was connected by a threadlike material. A fibrous matrix, located on the cell surface of the infecting organisms, was observed in ultrathin sections. By administration of each cephalosporin to the mice, several morphological changes, similar to those noted in vitro, were observed in the infecting organisms.

Cefotiam is a cephalosporin with a potent antibacterial activity against various gram-positive and gram-negative organisms, and its activity, especially against gram-negative organisms, is superior to that of previously available β-lactam antibiotics (21, 22). The excellent in vitro and in vivo antibacterial effects of cefotiam on *Klebsiella pneumoniae* have also been reported (21, 22).

Although there are several reports on the experimental pneumonia caused by *K. pneumoniae*, only observation by light microscopy of the infecting organisms in lungs has been performed (2, 3, 12, 16–18). A few studies on the morphological response of *K. pneumoniae* exposed to β-lactam antibiotics have been presented (6, 23); however, all of them have been carried out in vitro only.

This report describes the effect of cefotiam on the morphology of *K. pneumoniae* examined in vitro and in experimental pneumonia in mice, with cefazolin used as a control cephalosporin.

MATERIALS AND METHODS

Cephalosporin. Cefotiam was prepared at Takeda Chemical Industries, Ltd., Osaka, Japan. Cefazolin (Cefamezin; Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was obtained from a commercial source.

Bacteria. *K. pneumoniae* DT-S (biotype edwardsii, capsular type 1) was maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). The minimal inhibitory concentrations of cefotiam and cefazolin against this strain were 0.1 and 1.56 μg/ml, respectively. The other characteristics of this strain have been previously detailed (12).

Culture conditions. Organisms were grown overnight at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). For in vitro experiments, the overnight culture was diluted in fresh brain heart infusion broth and cultivated at 37°C for 2 h with shaking to about 7×10^7 colony-forming units per ml cell density. For in vivo experiments, the overnight culture was processed as previously described (12).

Bactericidal activity. The killing of cefotiam and cefazolin against *K. pneumoniae* DT-S was determined in brain heart infusion broth containing fourfold serial dilutions of each cephalosporin. A sample was removed at various time intervals, and the number of colony-forming units was determined by the plate count method.

Infection. Slc:ICR male mice (4 weeks old) weighing 22 g each were infected by the aerosol method. The procedure of infection was the same as that previously reported (12).

Chemotherapy. The cephalosporins dissolved in sterile saline were administered subcutaneously four times at 30-min intervals, starting at 30 h after the infection. Doses were 80 and 20 mg/kg for cefotiam and 640 and 160 mg/kg for cefazolin. The doses of 80 mg/kg for cefotiam and 640 mg/kg for cefazolin were equipotent in that practically all of the infected mice survived when treated by the following schedule: two dosing series of the cephalosporins, each consisting of four subcutaneous injections at 30-min intervals, were made daily starting at 8 a.m. and 4 p.m., respectively, and continued for 10 days (11).

Scanning electron microscopy. In the in vitro experiments, the cells exposed to the cephalosporins were prefixed with 2.5% glutaraldehyde (electron microscopic reagent) dissolved in 0.05 M cacodylate buffer (pH 7.4) for 3 to 6 h. After washing with Ryter-Kellenberger buffer (5), the cells were postfixed with

1% osmium tetroxide (electron microscopic reagent) dissolved in Ryter-Kellenberger buffer for 2 h. In the in vivo experiments, the mice were sacrificed by bleeding at various time intervals after the start of medication, and the lungs were removed. A part of the lung in which a gross lesion developed was cut out and steeped in the fixative. Fixation was carried out under evacuated conditions during the first few minutes to remove air in the lung and to assure penetration of the fixative into the lung tissue.

After dehydration, tissue blocks were placed in gelatin capsules which were then filled with absolute ethanol and frozen with liquid nitrogen, by using a cryo-cracking apparatus (TF-1; Eiko Engineering Co., Ltd., Naka-gun, Ibaragi, Japan), and cut to obtain a plane including the lesion. All of the specimens were dried with a critical-point dryer (HCP-2; Hitachi Koki Co., Ltd., Katsuta, Japan). For the surface conductivity, specimens were mounted on aluminum stabs and sputter-coated with gold by an ion coater (IB-3; Eiko Engineering Co., Ltd.). Observation and photography were performed in a scanning electron microscope (MSM 4C-101; Akashi Seisakusho Ltd., Tokyo, Japan) operated at an acceleration voltage of 15 kV.

Transmission electron microscopy. The method of fixation of specimens for transmission electron microscopy was similar to that employed for the preparation of scanning electron microscopy samples. Fixed specimens were stained with 0.6% uranyl acetate dissolved in Ryter-Kellenberger buffer, dehydrated by a graded ethanol series, and embedded in Epok 812 by the Luft method (8). Ultrathin sections were obtained on an ultramicrotome (Ultrotome III; LKB Instruments Inc., Rockville, Md.) equipped with a diamond knife and doubly stained with uranyl acetate and lead citrate (15). Micrographs were taken with a transmission electron microscope (JEM-100B; JEOL Ltd., Tokyo, Japan) operated at 80 kV.

RESULTS

Bactericidal activity. The killing kinetics of cefotiam and cefazolin against *K. pneumoniae* DT-S in the exponential growth phase are shown in Fig. 1. The number of colony-forming units decreased slightly after the addition of 0.1 μg of cefotiam per ml. At 0.39 and 1.56 μg of the cephalosporin per ml, a distinct decrease of colony-forming units was observed through the treatment. The effect of cefazolin on this bacterial strain was also bactericidal. At a concentration of 1.56 μg of the cephalosporin per ml, the number of colony-forming units began to decrease after 1 h of the treatment. At concentrations above 6.25 μg/ml, the number of colony-forming units decreased during the first hour of the treatment. With both cephalosporins, dose response in the bactericidal activity was clearly noticed.

Morphological response in vitro. Scanning electron microscopy revealed that the untreated control cells of *K. pneumoniae* DT-S were surrounded by an amorphous material which appeared to originate from the capsule (Fig. 2). The cells became filaments after 2 h of exposure to 0.1 μg of cefotiam per ml (Fig. 3a), and at 0.39 to 1.56 μg/ml, spheroplast-like structures together with filaments were formed (Fig. 3b and c). After 4 h of exposure to cefotiam, spheroplast-like structures appeared even at a concentration of 0.1 μg/ml, and at higher concentrations, the spheres became more swollen and collapsed (Fig. 3d through f). The morphological alteration of the cells exposed to cefazolin for 2 h was almost the same as that observed with cefotiam; filaments developed at concentrations near the minimal inhibitory concentration, and spheroplast-like structures or bulges were formed as the concentration rose (Fig. 4a through c). Bulges besides filaments emerged at 1.56 μg of cefazolin per ml after 4 h of exposure, and at higher concentrations, disintegrated cell debris with vesicular conformation was observed (Fig. 4d through f).

The control cells of *K. pneumoniae* DT-S that were examined by ultrathin sectioning gave an appearance of gram-negative bacilli. The cell wall was made up of an outer triple-layered membrane and an intermediate layer of murein. In the cytoplasm, clustered ribosomes and dispersed nuclear regions were seen. After 2 h of exposure to 0.1 to 0.39 μg of cefotiam per ml, the cells became filaments, while the appearance of the cell surface profiles and the cytoplasm were relatively unchanged. At 1.56 μg of the cephalosporin per ml, prominent morphological changes occurred: many cells turned into spheroplasts, and as for the cells with bacillary form, both the outer and plasma membranes broke; additionally, the density in the cytoplasm rose, possibly by condensation of the cytoplasmic contents.

The cytoplasm of the cells became sparse, and scattered electron-dense lumps were seen after 4 h of exposure to cefotiam. Among the cells which turned into a spherical form, some possessed the outer membrane whereas others lost it. After exposure to 1.56 to 6.25 μg of cefazolin per ml for 2 h, the cells became filaments and plasmolysis occurred. In addition, the electron density in the cytoplasm rose enormously, and the condensed nuclear regions showed abnormal distribution. At 25 μg of the cephalosporin per ml, spheroplasts and disintegrated cell debris were observed. After 4 h of exposure to cefazolin, electron-dense lumps and many membranous structures were often noted in the sparse cytoplasm (data not shown).

Morphological response in vivo. Scanning electron microscopy of a cross section of a lung sample after 34 h of infection showed distribution of infecting organisms. At the central part of the lesion, the organisms localized densely in

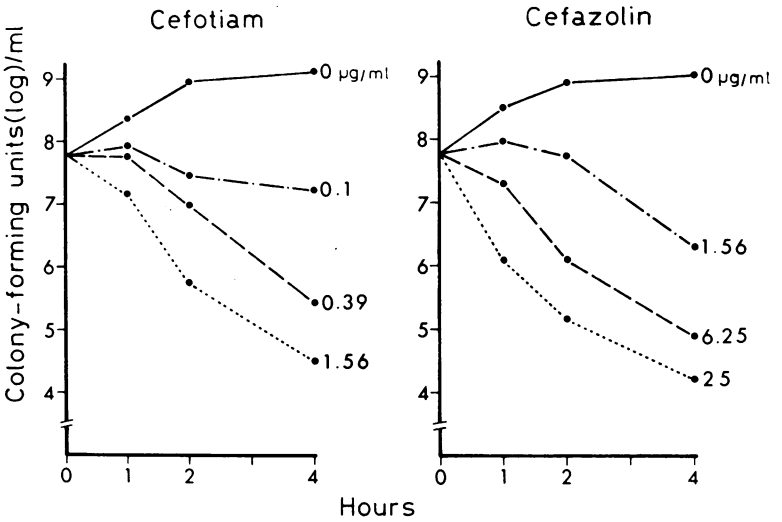

FIG. 1. *Bactericidal activity of cefotiam and cefazolin against K. pneumoniae DT-S.*

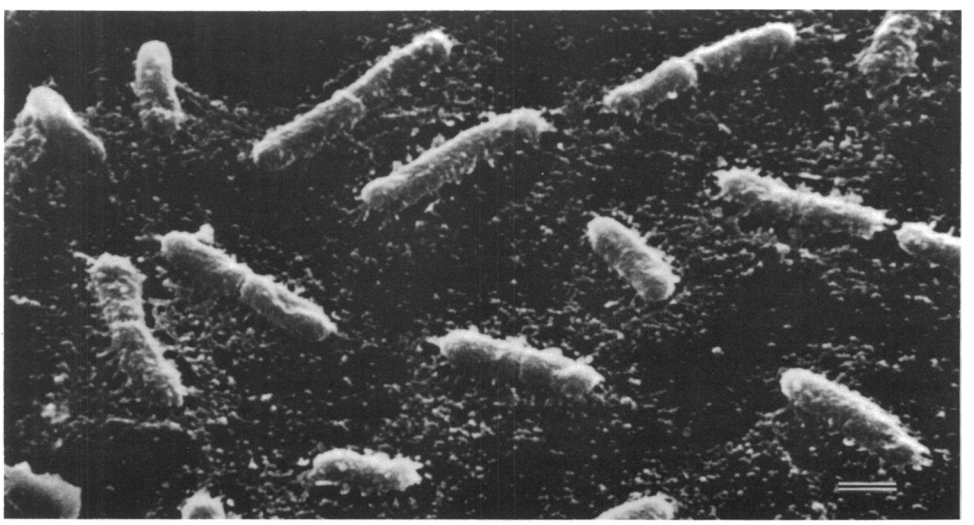

FIG. 2. *Scanning electron micrograph of control cells of K. pneumoniae DT-S. Bar = 1 µm.*

the alveolar space, and the number decreased as the distance from the center of the lesion increased. A higher magnification revealed that the infecting organisms were covered with a fibrous material and that each cell was also connected to others by this threadlike material. The organisms were localized in the alveolar space and not in the alveolar wall (Fig. 5). At 4 h after the start of medication with 80 mg of cefotiam per kg, a dose which corresponded with the chemotherapeutically effective dose, most of the organisms in the alveolar space appeared to be lysed, and the cells with bacillary form were rarely observed (Fig. 6a). At 20 mg of cefotiam per kg, which corresponded with the subeffective dose, the organisms in the alveolar space turned into filaments (Fig. 6b). The morphological response of the organisms in the alveolar space at 4 h after the start of medication with 640 mg of cefazolin per kg was similar to that noted with 80 mg of cefotiam per kg (Fig. 7a). At 160 mg of cefazolin per kg, both apparently intact and disintegrated cells were observed, but filamentous cells were not seen (Fig. 7b).

By ultrathin sectioning, localization of the infecting organisms in the lung was clearly confirmed.

The configuration characteristic to gram-neg-

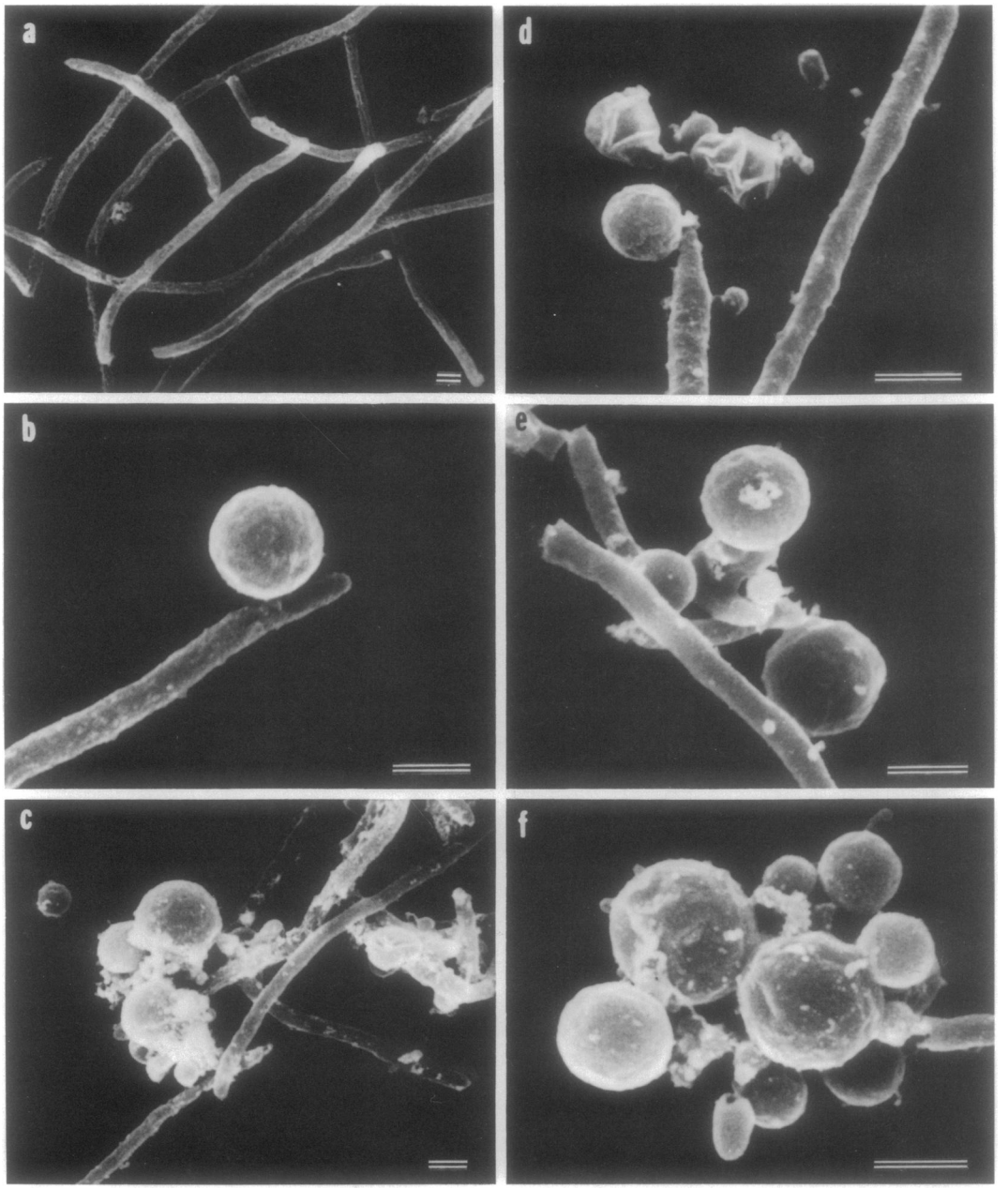

FIG. 3. *Scanning electron micrographs of K. pneumoniae DT-S exposed to cefotiam at 0.1 μg/ml, 2 h (a); 0.39 μg/ml, 2 h (b); 1.56 μg/ml, 2 h (c); 0.1 μg/ml, 4 h (d); 0.39 μg/ml, 4 h (e); and 1.56 μg/ml, 4 h (f). Bar = 1 μm.*

ative bacilli was maintained in the alveolar space. Unlike the cells cultivated in brain heart infusion broth, the cells grown in the alveolar space were often covered with a fibrous matrix (Fig. 8). Density of the extracellular hairy material differed among cells located at different alveoli in a lung, and some cells lacked this structure. At 4 h after the start of medication with 80 mg of cefotiam per kg, the cells turned into spheres and nuclear regions became sparse; in addition, plasmolysis was observed. At 20 mg of cefotiam per kg, filamentous cells possessing

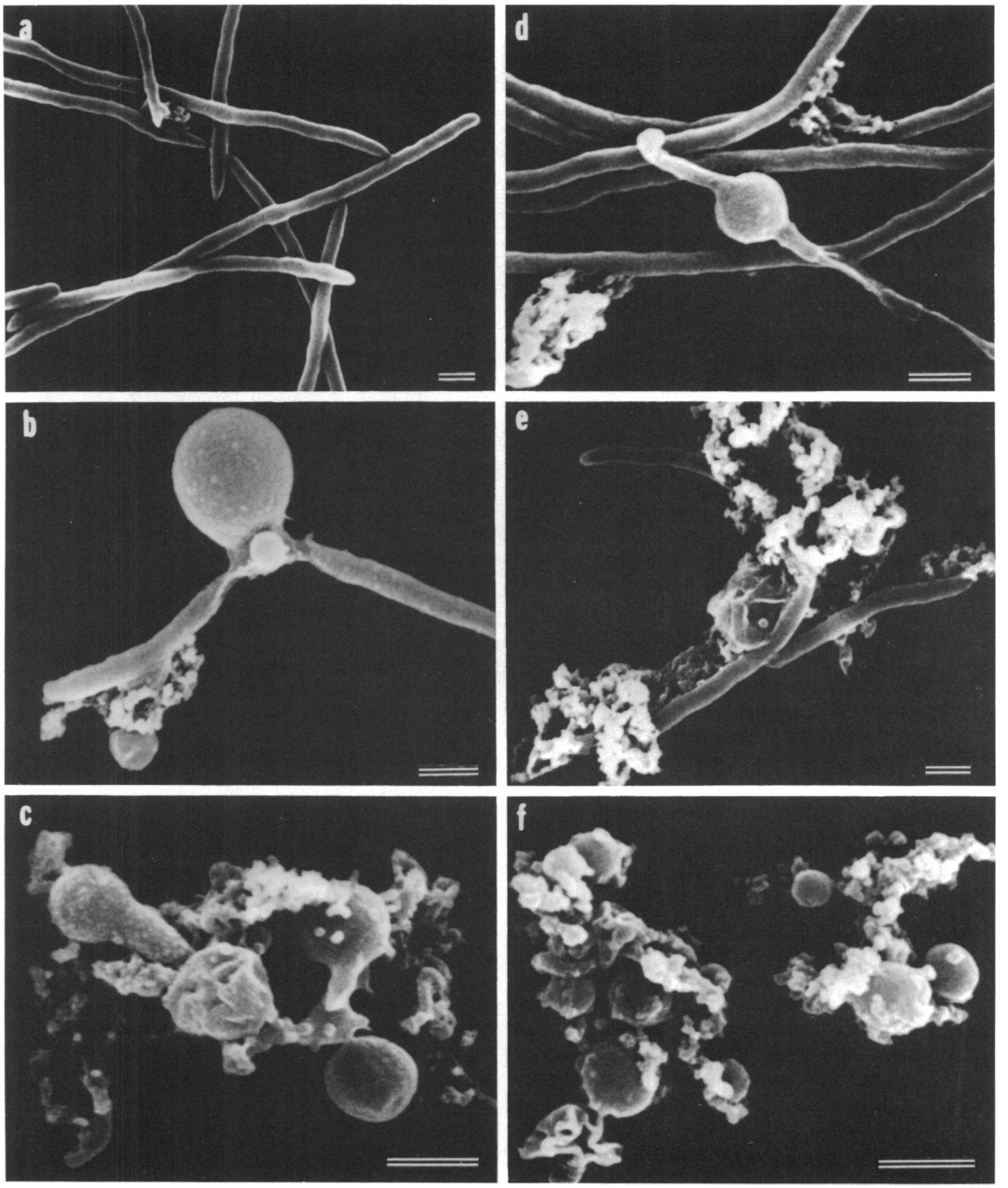

FIG. 4. *Scanning electron micrographs of K. pneumoniae DT-S exposed to cefazolin at 1.56 μg/ml, 2 h (a); 6.25 μg/ml, 2 h (b); 25 μg/ml (c); 1.56 μg/ml, 4 h (d); 6.25 μg/ml, 4 h (e); and 25 μg/ml, 4 h (f). Bar = 1 μm.*

bulges emerged and electron-dense deposits covering the cell surface were noted. At 6 h after the start of medication, spheroplasts or spherical forms were formed. The cells treated with 640 mg of cefazolin per kg also turned into spheres at 4 h after the start of medication, and the cells, which were converting into spheres, were seen at 160 mg of the cephalosporin per kg. At 6 h after the start of medication, plasmolysis was prominent in many cells (data not shown).

DISCUSSION

Cefotiam showed a potent bactericidal activity against *K. pneumoniae* DT-S in vitro and

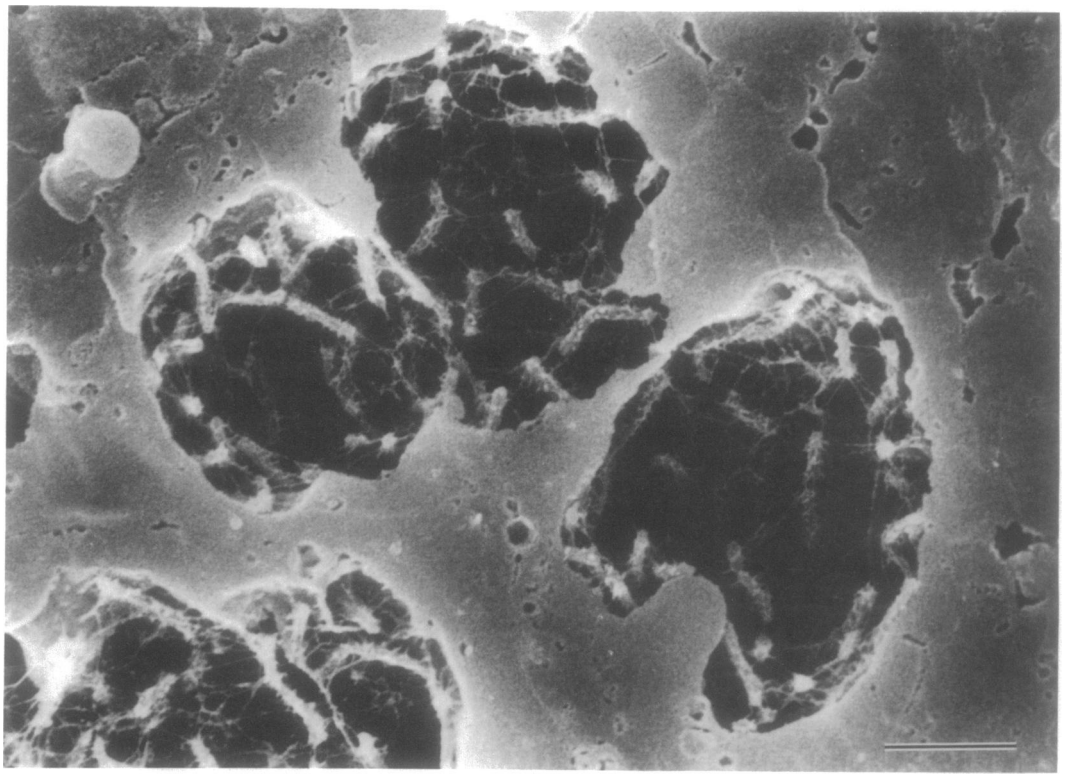

FIG. 5. *Scanning electron micrograph of cross section of the lung of a mouse after 34 h of infection with K. pneumoniae DT-S by the aerosol method. Bar = 5 μm.*

induced several morphological alterations both in vitro and in experimental pneumonia in mice. The range of concentration of cefotiam at which elongation of *K. pneumoniae* cells was induced was wider than that with cefazolin (11). The minimal inhibitory concentration of cefotiam against *K. pneumoniae* DT-S, however, was about 1/8 to 1/16 of that of cefazolin, and a similar degree of decrease of colony-forming units was brought about by cefotiam at concentrations about 1/8 to 1/16 of those of cefazolin. At concentrations above the minimal inhibitory concentrations, treatment with either cephalosporin resulted in spheroplast-like structures, which subsequently underwent collapse, and this was well reflected in the progressive decrease of the number of colony-forming units in *K. pneumoniae* DT-S exposed to these concentrations of the cephalosporins. Konno et al. (6) reported a phase-contrast microscopy study on the morphological response of *K. pneumoniae* treated with cefotiam in vitro, but the photographs they presented were those with cells observed after fairly prolonged incubation with the cephalosporin.

Transmission electron microscopy revealed several intracellular ultrastructural changes such as elevation of the electron density of the cytoplasm and abnormal distribution of the nuclear regions. When the cells were exposed to relatively high concentrations of the cephalosporins, amorphous electron-dense clusters were observed in the background; at a higher magnification, these materials appeared to be composed of ribosomes and deoxyribonucleic acid fibers flowing out of the cells by lysis. In the cells which were turned into spheres by exposure to cefotiam, an inverse correlation was often noted; the cells with apparently intact outer membrane almost lost the cytoplasmic contents, whereas the cells without outer membrane maintained them. For the present, we do not have a reasonable explanation for this somewhat contradictory phenomenon.

Scanning electron microscopy revealed that the infecting organisms in the alveolar space were connected to each other by a threadlike material, but it is uncertain whether this is of a bacterial or host origin. In the report of Bayer and Thurow (1), freeze-etching of capsulated cells of dehydrated *Escherichia coli* revealed that the capsular material became shrunken to filamentous structures that extended from one cell to the next. Therefore, the threadlike ma-

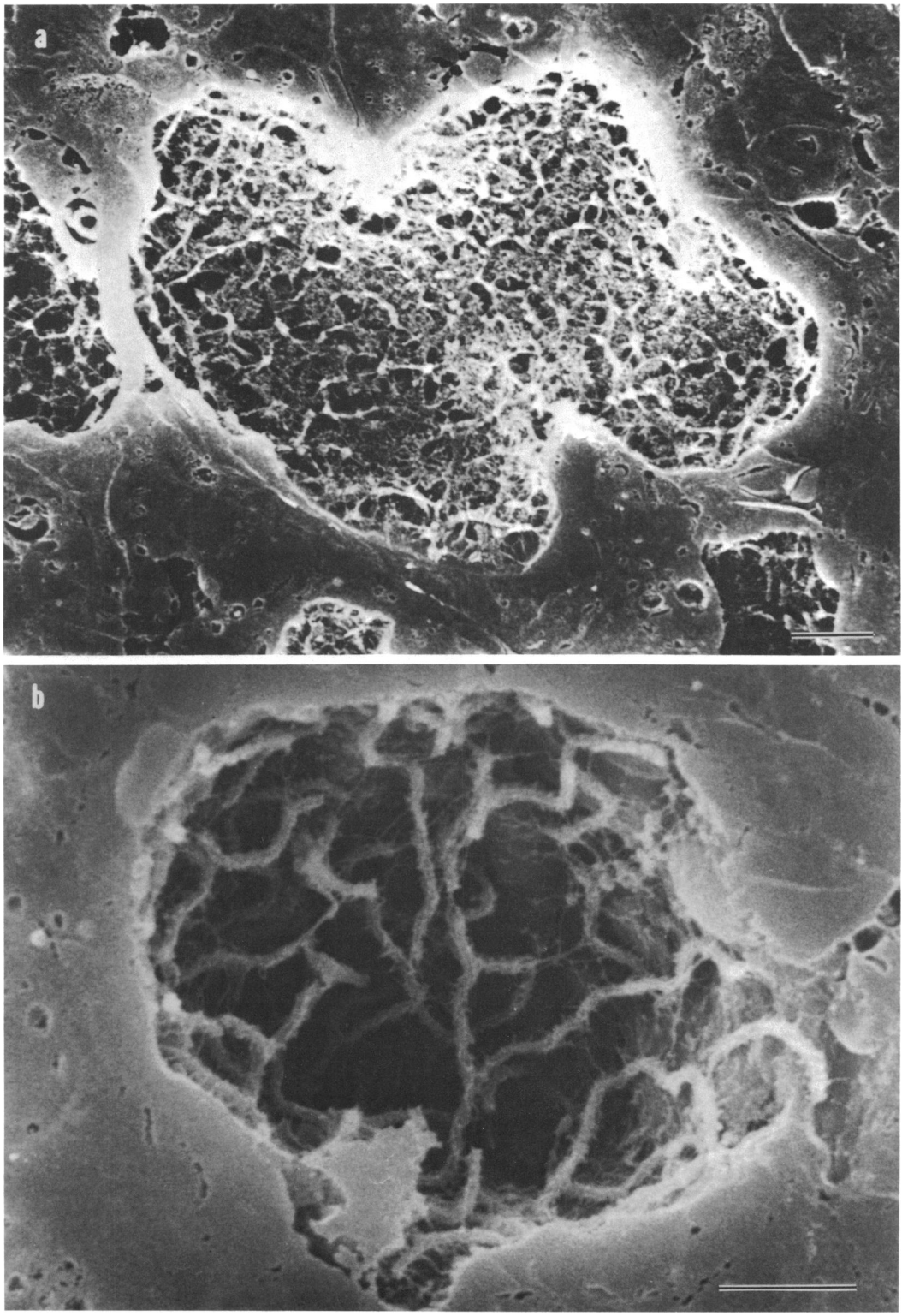

FIG. 6. *Scanning electron micrographs of K. pneumoniae DT-S in mouse lungs at 4 h after the start of medication with cefotiam at 80 mg/kg (a) and 20 mg/kg (b). Bar = 5 μm.*

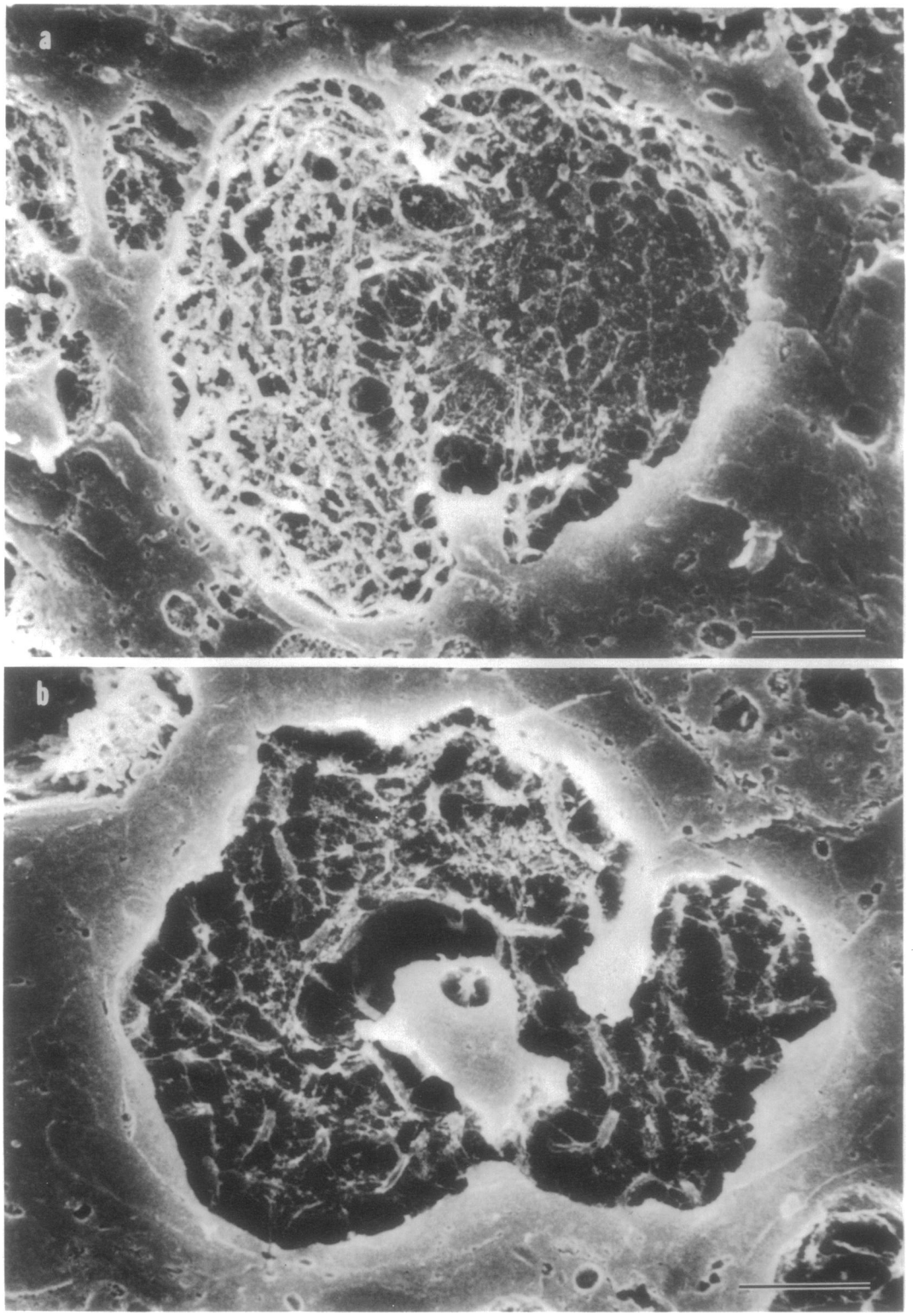

FIG. 7. *Scanning electron micrographs of K. pneumoniae DT-S in mouse lungs at 4 h after the start of medication with cefazolin at 640 mg/kg (a) and 160 mg/kg (b). Bar = 5 μm.*

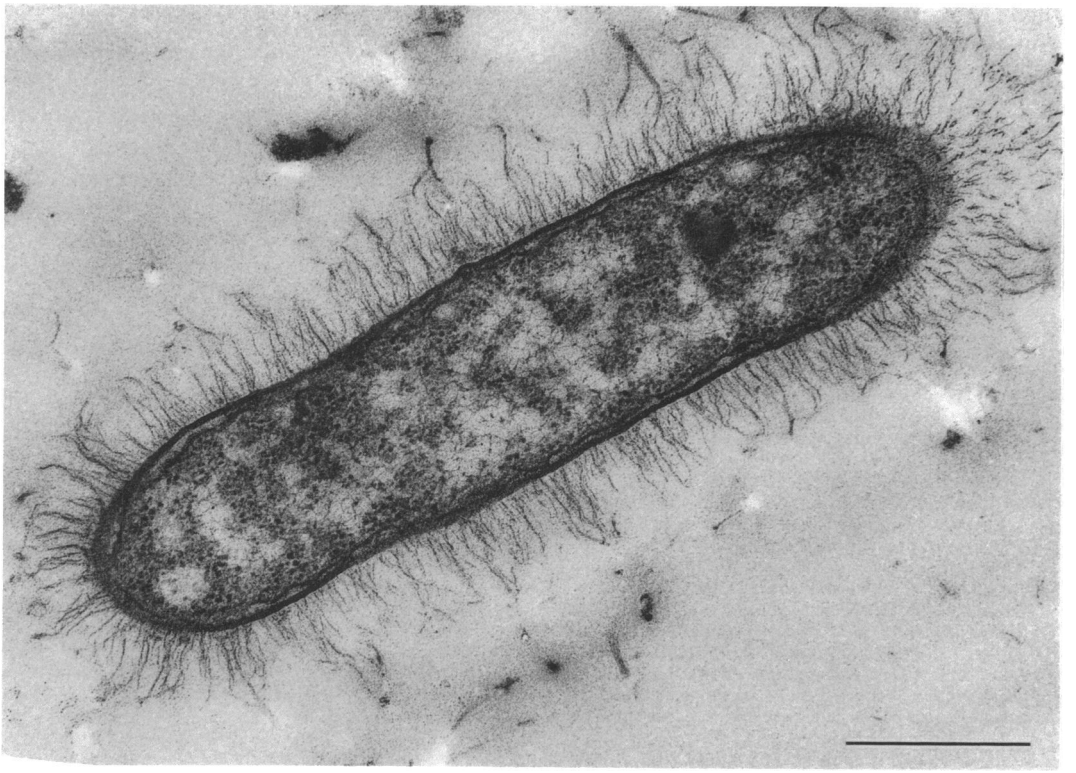

FIG. 8. *Transmission electron micrograph of K. pneumoniae DT-S in the lung of a mouse after 34 h of infection by the aerosol method. Bar = 0.5 μm.*

terial observed in this study may have resulted from capsule shrinkage induced by dehydration of the cells employed for the preparation of the electron microscopy specimens.

Springer and Roth (19, 20) examined the ultrastructure of the capsule of *K. pneumoniae* by ultrathin sectioning and freeze-etching, and proposed that the appearance of the capsule would be diversified by the difference in the procedure of dehydration and embedding. Some researchers (1, 7, 9, 19) mentioned that the fibrous conformation of capsule or slime material could be prevented from collapse by pretreating the cells with capsule- or slime material-specific antibody before the performance of dehydration. For visualization of bacterial capsules in ultrathin sections, staining with ruthenium red has been preferred. Springer and Roth (19) described that the cells of pneumococci stained with ruthenium red were surrounded by the mat-like capsule; in unstained cells, however, no such capsular materials were evident. In *K. pneumoniae* DT-S, a fibrous matrix was clearly observed on the surface of some cells in the alveolar space, even when neither ruthenium red staining nor antibody treatment was applied. In the preparation of the specimens for electron microscopy, it was difficult to collect the cells of *K. pneumoniae* DT-S by centrifugation; this might have been caused by their viscous nature. The surface of non-encapsulated gram-negative bacteria, in general, has been shown to be relatively clean by scanning electron microscopy (13). In *K. pneumoniae* DT-S, on the other hand, the cells were surrounded by an amorphous material. Therefore, this material appears to originate from the capsule.

With the infecting organisms in the lung, the morphological response demonstrated after administration of cefotiam and cefazolin was not uniform, and this seemed to be attributable to unevenness of the concentration of the cephalosporin in each alveolus. The damage of the infecting organisms by the effect of the cephalosporins was severer in those located at the center than in those located at the margin of the lesion, and this phenomenon appears to be consistent with the opinion of Halprin and McMahon (4) and May and Delves (10) that the integrity of the "blood-bronchus barrier" decreases during inflammation.

The morphological alteration of *K. pneumoniae* DT-S induced by cefotiam was similar to that induced by cefazolin with a few exceptions;

therefore, similarity of the penicillin-binding proteins, to which each cephalosporin has an affinity, is conceivable as confirmed in *E. coli* K-12 (14).

LITERATURE CITED

1. **Bayer, M. E., and H. Thurow.** 1977. Polysaccharide capsule of *Escherichia coli*: microscope study of its size, structure, and sites of synthesis. J. Bacteriol. **130**:911–936.
2. **Berendt, R. F., G. L. Knutsen, and M. C. Powanda.** 1978. Nonhuman primate model for the study of respiratory *Klebsiella pneumoniae* infection. Infect. Immun. **22**:275–281.
3. **Berendt, R. F., G. G. Long, and J. S. Walker.** 1975. Treatment of respiratory *Klebsiella pneumoniae* infection in mice and aerosols of kanamycin. Antimicrob. Agents Chemother. **8**:585–590.
4. **Halprin, G. M., and S. M. McMahon.** 1973. Cephalexin concentrations in sputum during acute respiratory infections. Antimicrob. Agents Chemother. **3**:703–707.
5. **Kellenberger, E., A. Ryter, and J. Sechaud.** 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671–678.
6. **Konno, M., K. Ubukata, H. Takahashi, M. Sawai, and K. Saito.** 1979. Morphological studies on antibacterial activities of cefotiam. Jpn. J. Antibiot. **32**:583–597.
7. **Lam, J., R. Chan, K. Lam, and J. W. Costerton.** 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. **28**:546–556.
8. **Luft, J. H.** 1961. Improvement in epoxy resin embedding method. J. Biophys. Biochem. Cytol. **9**:409–414.
9. **Mackie, E. B., K. N. Brown, J. Lam, and J. W. Costerton.** 1979. Morphological stabilization of the capsules of group B streptococci, types Ia, Ib, II, and III, with specific antibody. J. Bacteriol. **138**:609–617.
10. **May, J. R., and D. M. Delves.** 1965. Treatment of chronic bronchitis with ampicillin. Some pharmacological observations. Lancet **i**:929–933.
11. **Nishi, T., M. Nakao, and K. Tsuchiya.** 1981. Relevance of in vitro antibacterial activities of cefotiam and cefazolin to their therapeutic effects on experimental pneumonia caused by *Klebsiella pneumonia* DT-S in mice. J. Antibiotics **34**:127–135.
12. **Nishi, T., and K. Tsuchiya.** 1980. Experimental respiratory tract infection with *Klebsiellae pneumoniae* DT-S in mice: chemotherapy with kanamycin. Antimicrob. Agents Chemother. **17**:494–505.
13. **Nishino, T., Y. Utsui, N. Goto, and S. Nakazawa.** 1978. Morphological alterations in *Escherichia coli* no. 29, *Proteus morganii* no. 101, and *Serratia marcescens* T-55 exposed to CS-1170. Chemotherapy (Tokyo) **26**: 67–80.
14. **Nozaki, Y., A. Imada, and M. Yoneda.** 1979. SCE-963, a new potent cephalosporin with high affinity for penicillin-binding proteins 1 and 3 of *Escherichia coli*. Antimicrob. Agents Chemother. **15**:20–27.
15. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
16. **Sale, L., Jr., M. R. Smith, and W. B. Wood.** 1947. Studies on the mechanism of recovery in pneumonia due to Friedländer's bacillus. II. The effect of sulfonamide chemotherapy upon the pulmonary lesion of experimental Friedländer's bacillus pneumonia. J. Exp. Med. **86**:249–256.
17. **Sale, L., Jr., and W. B. Wood.** 1947. Studies on the mechanism of recovery in pneumonia due to Friedländer's bacillus. I. The pathogenesis of experimental Friedländer's bacillus pneumonia. J. Exp. Med. **86**:239–248.
18. **Smith, M. R., and W. B. Wood.** 1947. Studies on the mechanism of recovery in pneumonia due to Friedländer's bacillus. III. The role of "surface phagocytosis" in the destruction of the microorganisms in the lung. J. Exp. Med. **86**:257–266.
19. **Springer, E. L., and I. L. Roth.** 1973. The ultrastructure of the capsules of *Diplococcus pneumoniae* and *Klebsiella pneumoniae* stained with ruthenium red. J. Gen. Microbiol. **74**:21–31.
20. **Springer, E. L., and I. L. Roth.** 1973. Ultrastructure of the capsule of *Klebsiella pneumoniae* and slime of *Enterobacter aerogenes* revealed by freeze etching. Arch. Mikrobiol. **93**:277–286.
21. **Tsuchiya, K., M. Kida, M. Kondo, H. Ono, M. Takeuchi, and T. Nishi.** 1978. SCE-963, a new broad-spectrum cephalosporin: in vitro and in vivo antibacterial activities. Antimicrob. Agents Chemother. **14**:551–568.
22. **Tsuchiya, K., M. Kida, M. Kondo, H. Ono, M. Takeuchi, and T. Nishi.** 1979. Cefotiam (SCE-963), a new broad-spectrum cephalosporin: in vitro and in vivo antibacterial activities. Chemotherapy (Tokyo) **27**:73–93.
23. **Ubukata, K., H. Takahashi, M. Sawai, K. Saito, and M. Konno.** 1979. Antibacterial activities of a new cephamycin derivative cefmetazole (CS-1170). Chemotherapy (Tokyo) **27**:652–668.