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MORPHOLOGICAL CHANGES IN GRAM-NEGATIVE BACILLI EXPOSED TO CEPHALOTHIN

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ABSTRACT

CHANG, TE-WEN (Tufts University School of Medicine, Boston, Mass.), AND LOUIS WEINSTEIN. Morphological changes in gram-negative bacilli exposed to cephalothin. J. Bacteriol. **88**:1790–1797. 1964.—Exposure of gram-negative bacteria to cephalothin (7-[thiophene-2-acetamido]-cephalosporanic acid) revealed the formation of long filaments and large bodies, which were capable of reverting to normal cells when removed from contact with the drug. The degree of morphological change was found to be related to the concentration of antibiotic in which the organisms were suspended. The large bodies were altered by contact with solutions of varying osmolarity. Different species showed variation in the ability to develop large bodies. A relationship between antibiotic sensitivity and the capacity to resist morphological alteration was observed. Though most sensitive gram-negative bacilli were strikingly changed by exposure to cephalothin, naturally resistant ones were unaffected. Organisms made drug-resistant in vitro underwent changes in cellular form which were qualitatively the same but less intense than those which developed in parent strains originally sensitive to cephalothin.

Spheroplast production, by exposure of gram-negative organisms to certain antimicrobial agents, and other unfavorable conditions, is well known. Penicillin has been found to induce this morphological change in strains of *Proteus* (Dienes, 1949; Fleming et al., 1950), *Aerobacter aerogenes* (Gebicki and James, 1960), *Escherichia coli* (Lederberg and Clair, 1958), and other bacteria (Dienes and Weinberger, 1951; Lederberg, 1956). The same effect has been noted with 6-amino penicillanic acid (Hugo and Russell, 1960). This compound is related chemically to 7-amino cephalosporanic acid, the basic constituent of cephalosporin C; thus, the possibility that the latter might also cause spheroplast formation has been investigated, and it has been shown that it does produce this phenomenon in *Vibrio cholerae* (*V. comma*) (Bond, Brimblecombe, and Codner, 1962). Cephalothin is the sodium salt of 7-(thiophene-2-acetamido)-cephalosporanic acid, a broad-spectrum antibiotic effective against both gram-positive and gram-negative organisms (Chang and Weinstein, 1963). The purpose of the present study was to extend the observation to a number of gram-positive and gram-negative bacteria, to attempt to correlate the antimicrobial activity of cephalothin with its capacity to induce morphological alterations, and to describe some of the characteristics of the altered cells.

MATERIALS AND METHODS

Paper discs containing 25, 30, 250, and 2,500 μg of cephalothin were employed in these studies. The 30-μg discs were supplied by Eli Lilly & Co., Indianapolis, Ind. The others were made in the following manner: Sterile paper discs were saturated with 0.3 ml of various quantities of cephalothin, and allowed to stand at room temperature until they were dry. They were used on the same day during which they were prepared.

The solid medium employed was Heart Infusion Agar (Difco), to which either 5% sterile defibrinated horse blood or 20% horse serum was added. Heart Infusion Broth (Difco) containing 20% sucrose served as the liquid medium.

Blood-agar plates were inoculated with 0.5 ml of an 18-hr culture of the organism being studied, and the excess fluid was removed. They were then allowed to stand at room temperature for 1 hr; discs containing various quantities of cephalothin were placed on the surface, and the plates were then incubated overnight, or for different periods of time, depending on the nature of the experiment. Impression smears were made by applying a sterile cover slip, with slight pressure, to the agar surface, so that one edge crossed the midline of the drug-containing disc, and then pulling it off without sliding. The smears

were dried in air, fixed in absolute alcohol for 30 min, stained with Giemsa stain in buffered distilled water for 2 hr, dipped in absolute methyl alcohol, washed immediately in tap water, and allowed to dry.

Replicate plating was carried out by means of a round stamp of approximately the same size as the agar plate. A piece of sterile filter paper was attached to the surface of the stamp, and material was transferred from cultures to sterile medium.

The organisms used in this study were recently isolated strains, except *Salmonella*, *Shigella*, and *V. cholerae* (*V. comma*), which were stock cultures.

RESULTS

Morphological changes after cephalothin treatment. Blood-agar plates were inoculated with broth suspensions of *Proteus vulgaris* and *Vibrio comma*. Four discs, each of which contained 30 μg of cephalothin, were placed on the surfaces of the cultures, and impression smears were made at hourly intervals for 7 hr. Similar changes were observed in both organisms. The bacillary form of *P. vulgaris* increased first in size, more in length than in width. Elongation continued to take place without division so that, at the end of 4 hr, the impression preparation was covered with filamentous forms (Fig. 1), and the entire edge of the zone of clearing, which was now evident, had a hairy appearance. One (rarely two or three) swelling or bulb then appeared at one end (or some other site) of the filaments, and rapidly increased in size and number. Within 2 hr, the entire edge of the clear zone was packed with these large bodies (Fig. 2). The long filamentous forms then faded and disappeared. At this time, three morphologically distinct layers, starting from the edge of the clear area, were readily distinguishable; the inner layer consisted of large bodies, the middle one of filamentous forms, and the outer one of elongated rods. Two types of large bodies were apparent; one was small, dense, and lacked internal structure; the other was large, lightly stained, and contained either vacuoles or reticulum. As time progressed, the separate zones widened peripherally so that, after 18 hr, they were no longer distinct. Inside the edge of the clear zone, there was a small number of large bodies which became more and more numerous toward the edge, which consisted of a pure layer of these forms. Farther toward the periphery, the large bodies were much less numerous, and the long filaments became predominant; these gradually decreased in size until they finally assumed the typical rod shape. The heavy growth outside of the clear zone was made up of both large bodies and filaments.

V. comma underwent the same morphological alterations as *P. vulgaris*. However, the changes started at the end of 2 hr, and were completed by the end of 5 hr. The omission of blood from the medium, or the addition of 20% horse serum, was without effect on large bodies or filament formation. When penicillin discs (500 μg) were used in place of cephalothin, identical morphological changes were observed.

Reversion of large bodies to rod form. *Proteus* spreads on blood-agar plates; therefore, only *V. comma* was studied. The methods used were the same as those described above. After 18 hr of incubation, the antibiotic discs were removed aseptically; replicate transfers were carried out, and impression smears made at hourly intervals from these subcultures. Zero-hour specimens showed three layers of morphological transformation, the innermost layer consisting of large bodies; this area was the one studied. No visible changes were noted at the end of 1 hr. By the second hour (Fig. 3), however, marked changes in the appearance of the cells were observed. The large bodies began to extrude single or multiple processes, and assumed a cylindrical shape with one or many branches. The branching forms divided rapidly, and the usual rod forms of *V. comma* were apparent after 3 hr (Fig. 4). With the exception of lightly stained reticulated forms, all of the large bodies reverted to the original rod-shaped form.

Effect of antibiotic concentration on morphological changes. The constant presence of large bodies in the innermost layer, long filamentous forms in the middle layer, and short forms in the outer layer of the clear zone around the antibiotic-containing disc suggested the possibility that the type of cellular change which appeared was related to the concentration of antibiotic to which the organisms were exposed. To test this hypothesis, varying dilutions of cephalothin were made in Beef Heart Infusion (Difco), containing 20% sucrose, and inoculated with an equal volume of 10^{-2} dilution of an 18-hr culture of *V. comma*. The cultures were incubated at 37 C for 7 hr, after which stained and unstained preparations were examined. Large bodies were

FIG. 1. *Long filaments derived from Proteus vulgaris after exposure to cephalothin; the early lesion.*

found in cultures in which drug concentrations ranged from 25 to 100 μg/ml (Fig. 5), long filamentous forms in those in which 5 to 10 μg/ml of cephalothin were present, and normal-appearing curved rods in media to which 5 μg/ml of antibiotic had been added. The cephalothin sensitivity of the initial strain was 25 μg/ml.

Reaction of large bodies and long forms to changes in osmolarity. To test the reaction of the altered forms to changes in osmolarity, freshly prepared impression smears were made from cultures of cephalothin-exposed V. *comma*. These were treated with two drops of distilled water, or 0.85% NaCl solution. After standing at room temperature for 30 min, the fluids were poured off; the cover slips were allowed to dry in the air, and then fixed, stained, and mounted. Saline treatment did not destroy the dense or

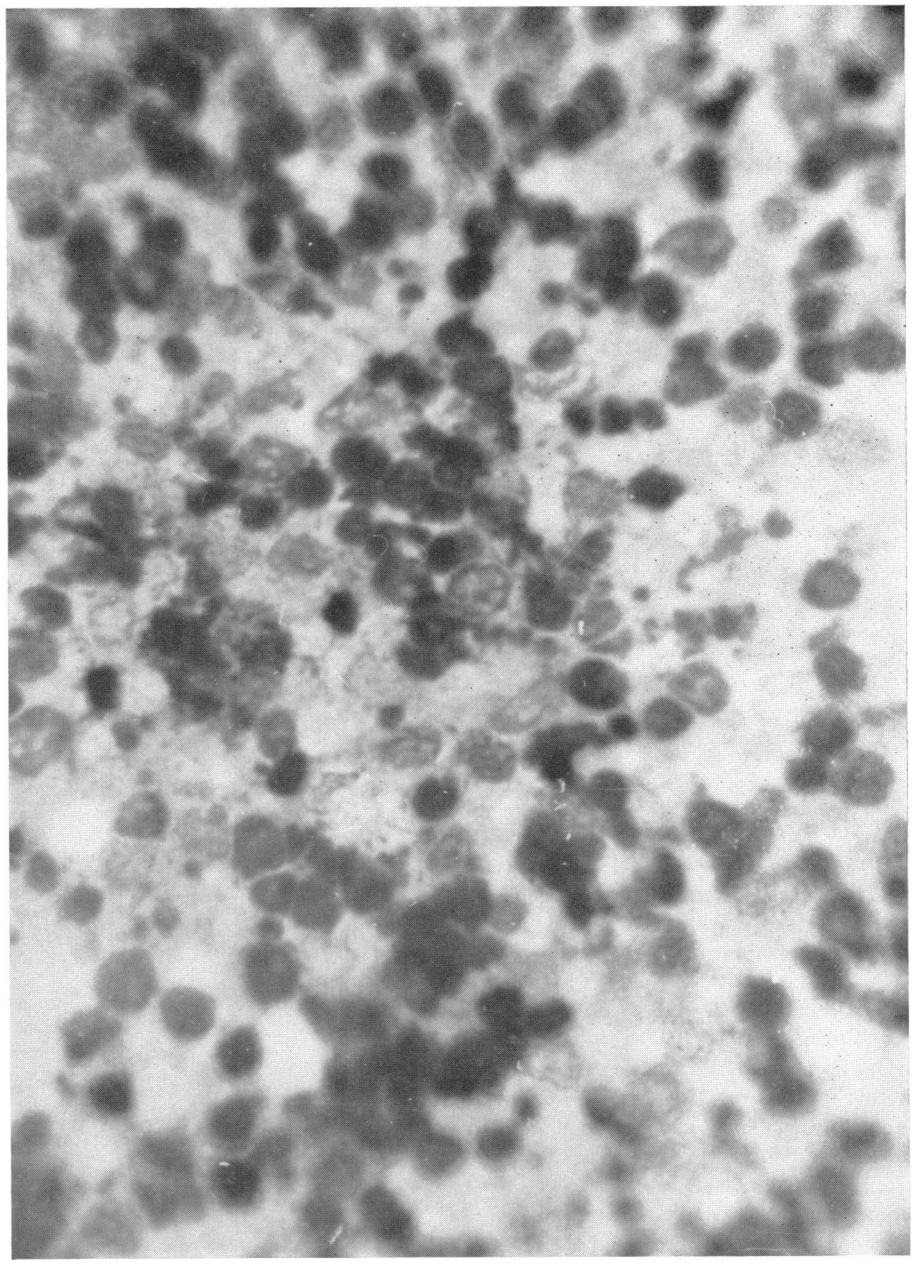

FIG. 2. *Large bodies derived from Proteus. The dense form and reticulated forms.*

opaque large bodies. Lysis was confined to the large reticulated or vacuolated forms. Water, however, caused dissolution of all types of large bodies. The long filamentous organisms were not affected by either water or saline.

Motility of large bodies and long forms. A drop of 20% sucrose solution was added to freshly prepared impression smears made from a culture of cephalothin-treated *V. comma*, and the preparations were examined in a hanging drop. The large bodies were noted to be nonmotile. The long forms, however, moved freely and rapidly, crossing a high-power field within 10 sec. The pattern of motility depended largely on the

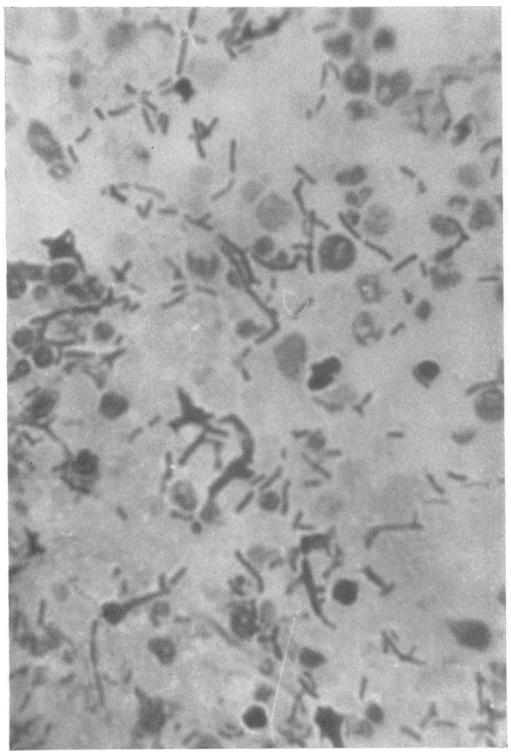

FIG. 3. *Reversion of large bodies to vibrio form: earliest changes.*

length of the organisms. The normal-sized vibrios moved up, down, forward, and backward, so that the orientation of the movement was difficult to determine. The longer forms (5 to 10 times the original size) moved only in one direction; the rods were rigid, and appeared to be propelled like a boat with a stern oar. The still longer ones (10 to 20 times the original size) also moved in one direction only, but exhibited a rhythmic undulation similar to, but slower than, that of spirochetes. The longest forms (greater than 30 times normal size) were nonmotile.

Formation of large bodies in organisms other than V. comma. Studies were carried out to determine formation of large bodies in organisms other than *V. comma*. Cephalothin discs, containing 2,500, 250, and 25 μg of drug, were applied to the surfaces of blood-agar plates which had been inoculated with suspensions of a variety of bacteria 1 hr earlier. Impression smears were prepared, fixed, stained, and mounted 18 to 20 hr after incubation at 37 C. Twenty-nine strains of eight different bacterial species were examined (Table 1). Of six cephalothin-sensitive strains of *Staphylococcus aureus*, none developed large bodies. Almost all of the gram-negative bacilli

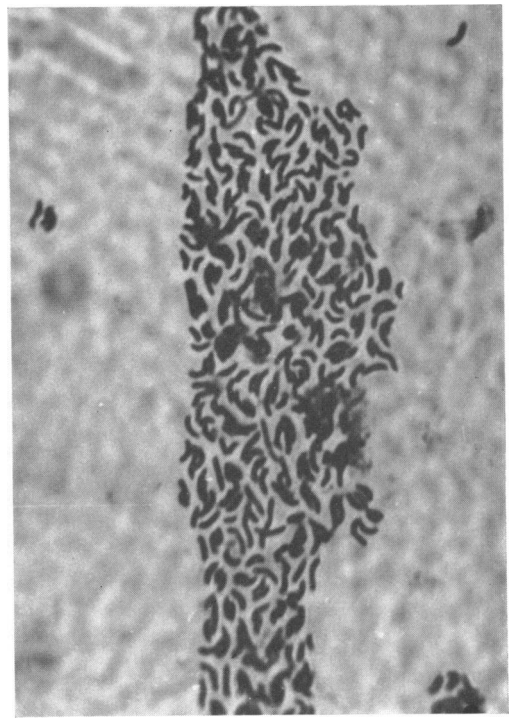

FIG. 4. *Reversion of large bodies to vibrio form: 3 hr.*

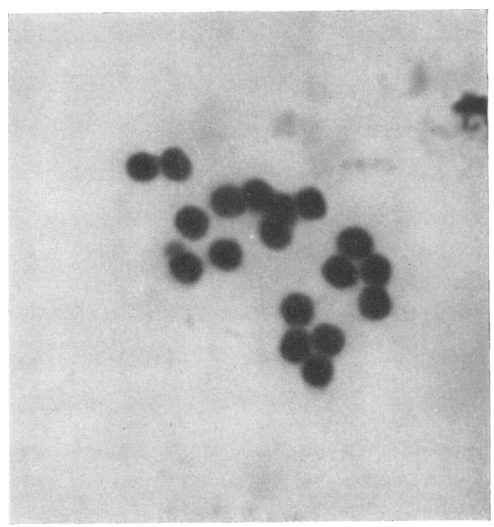

FIG. 5. *Large bodies in cephalothin-containing broth. The concentration of cephalothin was above minimal inhibitory concentration.*

TABLE 1. *Species differences in morphological transformations on exposure to cephalothin*

Organism	No. tested	No. showing large bodies	No. with filaments only	Changes not observed	Zone of inhibition
					cm
Salmonella	2	0	2	0	1.3–1.7
Shigella	2	0	2	0	1.0–1.4
Proteus	8	7	0	1*	0 0.6–2.3
Pseudomonas aeruginosa	1	0	0	1*	0
Vibrio comma	1	1	0	0	2.5
Aerobacter aerogenes	1	0	1	0	1.1
Escherichia coli	8	3	2	1*	0 0.1–1.0
Staphylococcus aureus	6	0	0	6	1.3–2.7

* Resistant to 2,500-μg cephalothin disc.

showed some degree of morphological change. The exceptions were one strain each of *E. coli*, *Proteus*, and *Pseudomonas;* these were not inhibited by the largest concentration of cephalothin. Seven out of eight strains of *Proteus*, and four out of eight strains of *E. coli* showed the series of transformations described above. Only filamentous forms, without large bodies, developed when the rest were exposed to 2,500 μg of the drug.

Correlation between changes in morphology and sensitivity of organisms after repeated exposures to cephalothin. Cephalothin-resistant organisms failed to develop any observable morphological changes when exposed to this drug; thus, studies were carried out to compare the reactions of initially susceptible strains of bacteria with those of the same strains after they were made resistant by repeated subculture in increasing concentrations of the antibiotic. Four strains each of *Proteus* and *E. coli* were transferred 24 times in cephalothin-containing medium; all became markedly resistant (Chang and Weinstein, 1963). Table 2 shows a comparison of the morphological alterations induced in the original strains, and in those which were made insensitive. Unlike the naturally resistant organisms, those which had been produced experimentally retained some of the characteristics of the originally sensitive ones, although the degree of morphological change was much less marked. Though the sensitive strains were completely converted into large bodies along the edge of the clear zone, fewer and fewer of the resistant ones underwent this change. This was particularly true of *E. coli.*

Bactericidal activity of cephalothin without the development of large bodies. Some gram-negative bacilli failed to develop large bodies in spite of exposure to large quantities of cephalothin (Table 3). Further examination of these organisms revealed that high concentrations of the drug were

TABLE 2. *Morphological alterations on exposure to cephalothin in Proteus and Escherichia coli before and after in vitro development of resistance to cephalothin*

Organism	Strain*	Sensitivity		Morphological changes†		
		Tube method (μg/ml)	Disc (30 μg)	Large bodies	Long form	Normal form
Proteus V	Or	5	1.9	+	+	0
	P	5,000	0	±	+	+
Proteus R_1	Or	5	1.3	+	+	0
	P	250	0.3	+	+	+
Proteus 1	Or	5	2.6	+	+	0
	P	250	0.6	+	+	+
Proteus 2	Or	5	1.6	+	+	0
	P	5,000	0	+	+	+
E. coli 3	Or	5	0.9	+	+	0
	P	500	0	0	±	+
E. coli 5	Or	2.5	1.6	+	+	0
	P	50	0.3	+	+	+
E. coli 8	Or	2.5	1.3	+	+	0
	P	500	0	0	+	+
E. coli 12	Or	5	0.7	+	+	0
	P	250	0	0	±	+

* Or = original strain; P = after 24 transfers in the presence of cephalothin.

† Changes recorded here were along the edge of inhibition zone: ± = present but rarely; + = present; 0 = absent.

TABLE 3. *Bactericidal activity of cephalothin without development of large bodies*

Organism	2,500 μg[a]			250 μg[a]			25 μg[a]		
	I.Z.[b]	M.C.[c]	G.4[d]	I.Z.	M.C.	G.4	I.Z.	M.C.	G.4
	cm			cm			cm		
Shigella 1	2.4	0	0	1.1	L[e]	+	0.7	L	+
Shigella 2	2.5	0	0	1.1	L	+	0.6	L	+
Salmonella choleraesuis	1.6	0	0	1.2	L	+	0.9	L	+
Salmonella paratyphi	2.6	0	0	2.1	L	+	1.6	L	+

[a] Measurement of disc.

[b] I.Z. = inhibition zone (cm).

[c] M.C. = morphological change inside the inhibition zone.

[d] G.4 = growth within inhibition zone after 4 days.

[e] L = long filamentous form.

bactericidal for them. Two strains each of *Shigella* and *Salmonella* were inoculated on the surface of blood-agar plates on which cephalothin discs, containing 2,500, 250, and 25 μg, were laid 1 hr later. The zone of inhibition of growth was measured, and impression smears were made after incubation at 37 C for 20 hr. The plates were then allowed to stand at room temperature for 4 days, and growth was again recorded. Studies at the end of 20 hr revealed long filamentous forms just beyond the edge of the zone of inhibition produced by 25 and 250 μg of cephalothin, but not in the area surrounding the disc which contained 2,500 μg of the drug. The filaments were present just outside the edge of the 25-μg disc, and increased in number toward the margin of the area of suppression of growth. There was a marked reduction in the number of long forms in the vicinity of the 250 μg of drug. When the same cultures were examined 4 days later, growth was present. The colonies which developed in the zone of inhibition produced by 25 and 250 μg of cephalothin had a serpiginous appearance.

DISCUSSION

The results of the present study are in many respects quite comparable to those which have been produced by exposing some bacteria to penicillin (Gardner, 1940; Hahn and Ciak, 1957; Lederberg, 1956; Lederberg and Clair, 1958). Some of the phenomena observed in this investigation have, however, not been noted previously. Two types of large bodies have been differentiated on the basis of their morphology and reaction to different osmotic pressures. This finding suggests that the osmotic fragility of the abnormal forms, induced by exposure to cephalothin, may be due to progressive deficiency in cell-wall material. Lysis was noted to occur even in isotonic solutions. Evidence that the mode of action of cephalothin is similar to that which has been demonstrated for penicillin (Park and Strominger, 1957; Lederberg, 1957; Kandler, Hurd, and Zehender, 1958; Ciak and Hahn, 1962; Chang and Weinstein, 1964) is the fact that the long forms and the dense large bodies reverted to bacilli when removed from contact with the drug; as the large bodies increased in size, viability was lost. These observations confirm the results of studies of the resistance of the morphologically altered organisms to hypotonic fluids. Though the long forms were unaffected by changes in osmolarity, the small round forms were less resistant; the reticulated ones exhibited marked fragility. This suggests that progression in the degree of cell-wall damage was associated with an increase in the intensity of the morphological changes.

The occurrence of continuous growth without formation of the septum, characteristic of cell division, may have been responsible for the development of the filamentous forms. A somewhat related septum defect has been reported by Murray, Framcombe, and Mayall (1959), who demonstrated the formation of imperfect septa in staphylococci exposed to penicillin. The quantity of cephalothin appears to be critical in determining the type of morphological transformation. This suggests that low concentrations of the antibiotic, produced by inhibiting the development of the cell septum, produced long forms; high concentrations, produced by causing more intense damage, led to the appearance of round forms. Lysis of *Staphylococcus*, *Salmonella*, and *Shigella* was observed in the absence of morphological changes when these organisms were exposed to high concentrations of cephalothin. The mechanism of this phenomenon is not apparent at present.

In contrast to the round forms of *Proteus* (Hughes, 1956; Fleming et al., 1950), those of *V. comma* were nonmotile. The filamentous rods were actively motile—all movement being unidirectional. This may be because the cholera vibrio has a single-pulse flagellum. The fact that

the motility rate of the elongated forms (20 times normal size) was approximately the same as that of organisms not exposed to the drug suggests the possibility that, as the rod enlarged, the size of the flagellum increased proportionately.

The sensitivity of an organism to cephalothin, and the degree of morphological disturbance developing after exposure to the drug, appeared to be directly related. Bacteria resistant to the antibiotic remained unaltered, but those susceptible to it exhibited striking changes. Those made insensitive in vitro underwent alterations of an intermediate degree. This suggests that most of the cells of cephalothin-sensitive organisms were transformed and lysed after contact with the drug; a small number were converted to drug-resistant variants.

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